Comparison of Increased Aromatase versus ERα in the Generation of Mammary Hyperplasia and Cancer

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Abstract

Factors associated with increased estrogen synthesis increase breast cancer risk. Increased aromatase and estrogen receptor α (ERα) in both normal epithelium and ductal carcinoma in situ lesions are found in conjunction with breast cancer, leading to the idea that altered estrogen signaling pathways predispose the mammary gland to cancer development. Here, we developed a transgenic mouse that conditionally expresses aromatase in the mammary gland, and used it along with a deregulated ERα expression model to investigate the molecular pathways involved in the development of mammary gland neoplasia and carcinoma. Both increased ERα and aromatase expression led to the development of neoplasia, but increased neoplasia, in addition to carcinoma, was found in aromatase overexpressing mice. Increased prevalence of mammary pathologic changes in mice expressing aromatase correlated with increased cyclin E and cyclin-dependent kinase 2 expression. Gain of both ERα and aromatase increased expression of ERα and progesterone receptor, but aromatase produced a higher increase than ERα, accompanied by higher levels of downstream target genes Ccnd1, Myc, and Tnfsf11. In summary, whereas gain of both ERα and aromatase activate abnormal growth pathways in the mammary gland, aromatase induced a wider range of abnormalities that was associated with a higher prevalence of mammary neoplasia and cancer progression. Cancer Res; 71(16):5477–87. ©2011 AACR.

Introduction

Breast cancer develops as genetic changes accumulate in the ductal epithelium giving rise to precursor lesions which may progress to ductal carcinoma in situ (DCIS) and eventually invasive breast cancer (1). Factors associated with increased estrogen synthesis, such as obesity and increased dietary fat, increase the risk for breast cancer. Estrogens have been considered one of the most important factors in driving this process (2). Estrogens are biosynthesized from androgens by aromatase, the product of the CYP19 gene and a member of the cytochrome P450 enzyme superfamily (3). In postmenopausal women, estrogens are synthesized in peripheral tissues through the aromatization of androgen precursors produced by the adrenal glands (4). The concentration of estradiol, the most potent endogenous estrogen, in breast tumors can be significantly higher than plasma concentrations in postmenopausal women (5) and higher in DCIS lesions and tumor tissues than normal breast areas (6, 7). Aromatase expression (7, 8) and activity (9) in DCIS lesions and tumors is greater than those in normal breast tissue.

Hormone receptor status is one of the main differentiating characteristics of human breast cancers and modifies response to therapy. Approximately 60% to 70% of human breast cancers are estrogen receptor α (ERα)-positive and estrogen dependent (10). In humans, estrogen receptors are expressed in the mammary gland epithelial cell compartment (11). Increased expression of ERα in normal breast epithelium has been found in conjunction with breast cancer supporting the concept that loss of normal regulatory mechanisms controlling ERα expression levels in normal breast epithelium increases breast cancer risk (12). Intratumoral estradiol levels showed a strong positive correlation with ERα expression in pre- and postmenopausal breast cancer patients (13).

Deregulated estrogen and progesterone signaling contributes to breast cancer pathophysiology. Estrogen and progesterone combination hormone replacement therapy increases the risk of development of breast cancer in postmenopausal women (14, 15). The effects of progesterone are mediated by 2 isoforms of the progesterone receptor, PR-A and PR-B. PR-B plays a role in alveologenesis, whereas PR-A is involved in ductal development and side branching (16). Ccnd1 and Myc are both ERα and PR downstream genes, whereas Areg, Tnfsf11 (RANKL), and Wnt4 are more specifically PR downstream genes (17–21). Different observations point to potential interactions between ERα and the STAT proteins. Both STAT3 and STAT5 are activated in a significant proportion of breast cancers, both ERα-positive and -negative (22–24).
Cross-talk between ERα and STAT5a has been shown in both normal and breast cancer cells (25). Changes in Stat5a expression levels can modulate progression of ERα-initiated mammary preneoplasia (26).

Normally, aromatase is not expressed in the mouse mammary gland. In the first mouse model of aromatase overexpression, MCF-7 cells transfected with the human placental aromatase gene (MCF-7Ca cells) were inoculated into nude mice (27). In the first transgenic mouse model of mammary-targeted aromatase, murine aromatase produced mammary gland hyperplasia (28).

Here we characterized a novel conditional transgenic mouse model of mammary-targeted human aromatase expression and then compared the impact of aromatase expression to ERα overexpression. Altered estrogen signaling pathways predispose the mammary gland to cancer development; however, few studies have systematically examined similarities and differences between substrate excess versus increased receptor. The objective of this study was to investigate whether development of mammary preneoplasia and carcinoma caused by ERα overexpression (26, 29, 30) results from the same or different aberrant molecular pathways than that induced by increased local estrogen production through mammary-targeted aromatase expression. Results showed that aromatase by itself mediated increased ERα and PR expression levels accompanied by increased p-AKT and increased cyclin E and CDK2 expression that were not found with overexpression of ERα alone.

Materials and Methods

Conditional aromatase transgenic mice
A 2.4-kb full-length human aromatase cDNA was cloned downstream of the tet-op promoter in pPF43 (31). Integrity was verified by nucleotide sequencing. The tet-op-Arom construct was restriction digested with NotI to remove bacterial DNA sequences. Transgenic mice were generated by standard procedures. Potential tet-op-Arom founders were identified by PCR of tail DNA (5'-CAGGATGCTGACGGTCG-3' (forward) and 5'-CAGGGATGGCTTCCAGGACGA-3' (reverse) and bred with MMTV-rtTA transgenic mice (32) to generate double transgenic "Arom" mice.

Mouse models, genotyping, and necropsy
Coohorts of experimental female nulliparous MMTV-rtTA/tet-op-Arom (Arom) and MMTV-rtTA/tet-op ERα (CERM; ref. 29) mice were maintained on a C57Bl/6 genetic background on diet containing 200 mg doxycycline (dox) per kilogram food (Bio-Serv) from weaning until euthanasia at age 12 months to test for development of preneoplasia and cancer (CERM, n = 34; Arom, n = 40). A cohort of Arom mice (n = 3) were exposed at age 10 months to letrozole (2.5 mg/60-day release subcutaneous letrozole pellet; Innovative Research of America) and euthanized at age 12 months to test the impact. Cohorts of CERM and Arom mice (n = 4–5) were exposed at the age of 5 to 6 months to mifepristone (Mife) and ICI 182,780 (ICI; 17.5 mg/60-day and 7.5 mg/60-day release subcutaneous pellets, respectively), and euthanized at age 6 to 7 months. Additional cohorts of Arom mice were fed dox diet (n = 3) or regular diet (n = 3) for 3 months and euthanized at age 4 months to test dox responsiveness. Wild-type (WT) C57Bl/6 mice served as controls (n = 42). To test the effect of exogenous estrogen, cohorts of WT and CERM mice (n = 5) were exposed at age 10 months to 17β-estradiol (0.72 mg/60-day release subcutaneous pellets) and euthanized at age 12 months. At the time of necropsy, mice were anesthetized by isoflurane inhalation and blood samples were obtained by cardiac puncture for measurement of serum 17β-estradiol levels by radioimmunoassay (Cayman Chemical Company). All mice were maintained and euthanized in accordance with institutional and federal guidelines approved by the Georgetown University Animal Care and Use Committee.

RNA expression analyses
Total RNA was isolated by using TRIzol reagent (Invitrogen) as described previously (30). Reverse transcriptase (RT)–PCR primers; CTP19: 5'-CCTTGCACCCAGATGAGACT-3' (forward), 5'-GACAGCAACAAACCGACACG-3' (reverse). Real-Time PCR Taqman Gene Expression Assays (ABI Prism 7700): COX-2 (Pig2; Mm01307329_m1), PR (Pgr; Mm00435625_m1), Pgr-b isoform (forward: TGGACTCAGGTCCCTTCCA; reverse: CCGTCCAGGAGATCGGTA- TAGG; reporter: ACCTGTCGCTGATGCTC), RANKL [TNF ligand superfamily member 11 (Tnfsf11); Mm01313941_g1], wingless-related mouse mammary tumor virus (MMTV) integration site 4 (Wt4; Mm01194003_m1), amphiregulin (Areg; Mm00437583_m1), c-myc (myelocytomatosis oncogene; Myc; Mm00487804_m1), cyclin D1 (Ccnd1; Mm00432360_m1), Erbb2 (Mm0065854_m1), and eukaryotic 18s rRNA (Hs99999901_s1). Relative mRNA gene expression normalized against WT control mice [2−ΔΔCt]; where ΔCt = Ct (target gene) – Ct (18s rRNA). Four independent samples were randomly selected from each group for RNA analysis.

Histologic analyses
Hyperplastic alveolar nodules (HAN; ref. 33) were detected by using whole mounts of inguinal mammary glands (30). Ductal morphology was analyzed by reflectance confocal microscopy (RCM) by using VivaCell 5000 (VivaCell-TiBa) with a 30× water immersion lens as described previously (34). Ductal hyperplasia (DH) and carcinoma was detected on hematoxylin and eosin (H&E)-stained formalin-fixed sections of the second inguinal gland. DH was defined as mammary gland ductal epithelium consisting of at least 4 epithelial cell layers. Immunohistochemistry (IHC) was carried out on unstained tissue sections by using the Vectastain ABC kit or the Mouse On Mouse (M.O.M) Peroxidase kit (Vector Laboratories, Inc.) as appropriate by standard procedures. Primary antibodies: ERα (sc-542), PR (sc-538), Her2/Neu (sc-284), E-cadherin (sc-7870), cyclin E (sc-81), CDK2 (sc-163), E2F-1 (sc-193), retinoblastoma (Rb; sc-50), obtained from Santa Cruz Biotechnology, Inc.; p-STAT5 (D3AT7), STAT3 (#9132), p-STAT5 (C11C5), STAT5 (9363), p-Rb (Ser807/811), from Cell Signaling Technology, Inc.; aromatase antibody Ab677, a gift from Dr. Dean B. Evans, Novartis, Basel, Switzer-

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land; KI-67 (RTU-Ki67-MM1), from Novoceastra; cyclin D1 (RM-9104-S), from Thermo Scientific; COX-2 (#160126), from Cayman Chemical. The proliferative index (PI) was calculated as the percentage of Ki-67–positive cells in a total of more than 1,000 cells per section. Five sections were randomly selected from each experimental and control group for evaluation. Negative control slides in which primary antibody was omitted, were analyzed in parallel. Digital photographs were taken by a Nikon Eclipse E800M microscope with Nikon DMX1200 software (Nikon Instruments, Inc.).

Aromatase activity
Aromatase enzyme activity was measured by using a tritium water-release assay. Dissected thoracic mammary gland was homogenized in 0.1 mol/L sodium phosphate buffer (pH = 7.4). Protein (1.5 mg) was used for each assay, and each sample was assayed in triplicate. Protein solution was preincubated at 37°C for 3 minutes followed by the substrate addition (androstenedione including [1β-3H]androst-4-ene-3,17-dione) and a NADPH regeneration system (containing glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP+) to initiate enzyme reaction in a 37°C water bath with constant shaking for 1 hour 45 minutes. Reaction was terminated with ether. After 3 cycles of this extraction step, 200 mL of dextran-treated charcoal solution was added and incubated at 42°C for 10 minutes. After centrifugation, a 600-mL aliquot was counted in 5 mL of liquid scintillation mixture. Results were expressed as picomoles of 3H2O formed per milligrams of protein per hour incubation time (pmol/mg protein/h).

Immunoblotting
Dissected thoracic mammary gland was homogenized in radioimmunoprecipitation assay buffer (Cell Signaling Technology) containing 1 mmol/L phenylmethylsulfonyl fluoride plus protease and phosphatase inhibitors (Roche Diagnostics). Primary antibodies: p-extracellular signal-regulated kinase plus protease and phosphatase inhibitors (Roche Diagnostics); COX-2 (160126), from Thermo Scientific; KI-67 (RTU-Ki67-MM1), from Novoceastra; cyclin D1 and E-cadherin were expressed in both with low levels of HER2 expression (Fig. 2D). To determine whether exogenous estrogen exposure could mimic the effect of mammary-targeted aromatase overexpression, WT and CERM mice were exposed to 17β-estradiol. Mammary glands from both genotypes showed lobuloalveolar development following estrogen exposure but the prevalence of HANs or DH was not changed (Fig. 3A and B). A normal 2-layered mammary epithelium was observed in the WT mice whereas the CERM mice showed DH. As reported previously (30), CERM mice showed significantly higher levels of nuclear-localized ERα and PR (10 ± 1% and 15 ± 1%, respectively) than WT mice (5 ± 1% and 8 ± 2%, respectively), and this was unchanged by exposure to exogenous 17β-estradiol (P < 0.05; Fig. 3C). Serum 17β-estradiol levels were increased more than 3-fold in the mice exposed to 17β-estradiol whereas no significant differences were found between nonexposed WT, CERM, and Arom mice (Fig. 3D).

Expression of ERα, PR, and downstream genes linked to mammary cancer progression were significantly increased in the mammary glands of Arom mice
Percentages of mammary epithelial cells expressing nuclear-localized ERα and PR were compared in Arom, CERM, and WT mice (Fig. 4A). The percentage of mammary epithelial

Results

MMTV-rtTA/tet-op-Aromatase transgenic mice express mammary targeted aromatase
Aromatase transgene RNA was identified in Arom mice exposed to dox (Fig. 1A and B). Significant levels of aromatase activity were measured in mammary tissue of Arom but not WT mice (Fig. 1C). HANs and multiple foci of abnormal ductal epithelial cell growth appeared along secondary and tertiary branches in Arom mice exposed to dox (Fig. 1D). Letrozole exposure for 2 months resolved these abnormalities (Fig. 1D). Aromatase and increased COX-2 protein expression were documented by IHC in Arom mice (Fig. 1E). Increased COX-2 was paralleled by a corresponding increase in RNA (Ptgs2) levels: Arom (1.7 ± 0.3) as compared with WT (1.00 ± 0.02) mice (P < 0.05 unpaired t test, data not shown).

Mammary-targeted aromatase led to more diffuse ductal disease and a higher prevalence of HANs, DH, and invasive adenocarcinomas as compared with mammary-targeted ERα overexpression
The impact of aromatase versus ERα overexpression was compared in nulliparous WT, CERM, and Arom mice at the age of 12 months. DH prevalence was significantly higher in aromatase and ERα overexpressing mice than control WT mice (48%, 41%, 2%, respectively, P < 0.0001; Fig. 2A–C). Diffuse disease was defined as multifocal HANs appearing along multiple secondary and tertiary branches. Aromatase expression resulted in a higher prevalence of diffuse ductal disease (47%) than ERα overexpression (6%, P < 0.0001; Fig. 2B). In the cohorts studied, invasive ductal adenocarcinomas appeared in 7.5% of the Arom mice but none of the CERM or WT mice (Fig. 2D). Whereas DH were ERα/PR-positive, carcinomas were ERα/PR-negative (Fig. 2D). Cyclin D1 and E-cadherin were expressed in both with low levels of HER2 expression (Fig. 2D). To determine whether exogenous estrogen exposure could mimic the effect of mammary-targeted aromatase overexpression, WT and CERM mice were exposed to 17β-estradiol. Mammary glands from both genotypes showed lobuloalveolar development following estrogen exposure but the prevalence of HANs or DH was not changed (Fig. 3A and B). A normal 2-layered mammary epithelium was observed in the WT mice whereas the CERM mice showed DH. As reported previously (30), CERM mice showed significantly higher levels of nuclear-localized ERα and PR (10 ± 1% and 15 ± 1%, respectively) than WT mice (5 ± 1% and 8 ± 2%, respectively), and this was unchanged by exposure to exogenous 17β-estradiol (P < 0.05; Fig. 3C). Serum 17β-estradiol levels were increased more than 3-fold in the mice exposed to 17β-estradiol whereas no significant differences were found between nonexposed WT, CERM, and Arom mice (Fig. 3D).

Expression of ERα, PR, and downstream genes linked to mammary cancer progression were significantly increased in the mammary glands of Arom mice
Percentages of mammary epithelial cells expressing nuclear-localized ERα and PR were compared in Arom, CERM, and WT mice (Fig. 4A). The percentage of mammary epithelial
cells showing nuclear-localized ERα was significantly increased in Arom (33 ± 2%) compared with both CERM (12.5 ± 0.9%) and WT (8.0 ± 0.6%) mice (P < 0.0001; Fig. 4B). Percentages of mammary epithelial cells expressing PR were evaluated as a measure of downstream ERα signaling. The percentage of mammary epithelial cells showing nuclear-localized PR was highest in Arom mice (30 ± 2%) followed by CERM (15.1 ± 0.8%) and WT (8.4 ± 0.5%) mice (P < 0.0001, P < 0.0005, respectively; Fig. 4B). Cyclin D1 lies downstream of both ERα and PR. Arom mice showed the highest percentage of mammary epithelial cells with nuclear-localized cyclin D1 (68 ± 2) compared with CERM (43 ± 6) and WT (27.3 ± 0.9, P < 0.0001) mice. To determine whether increases in protein expression of ERα downstream genes were correlated with increases in steady state RNA levels, real-time PCR was used to quantify expression levels of Pgr, Pgr(b), Ccnd1, Myc, Tnfsf11, Areg, and Wnt4 (Fig. 4C). Arom mice showed 2-fold higher levels of total Pgr (PR) expression than WT mice (P < 0.005). Both CERM and Arom mice expressed significantly higher levels of Pgr(b) (PR-B isoform) than WT mice (P < 0.005). Tnfsf11 (RANKL) showed the largest magnitude of difference in expression with an 8-fold increase in Arom and a 5-fold increase in CERM mice (P < 0.0005, compared with WT mice). Ccnd1 (cyclin D1) and Myc (c-Myc) were expressed at significantly higher levels in Arom but not CERM mice (P < 0.05). No significant changes in expression levels of Areg and Wnt4 were found in either Arom or CERM mice. Expression levels of Erbb2 (ErbB2/Her2) and Egfr (epidermal growth factor receptor) were examined because of their roles in the initiation and progression of both ERα-positive and -negative breast cancers (35). Erbb2 mRNA expression was significantly increased in the mammary gland of both CERM and Arom mice as compared with WT mice (P < 0.005) whereas only CERM mice showed a significant increase in Egfr expression levels (Fig. 4D).

**Aromatase expression increased expression of cell-cycle proteins cyclin E, CDK2, p-Rb, E2F-1, upregulated Bcl-2, downregulated Bax and p27, and increased relative levels of AKT phosphorylation**

The PI was highest in Arom (21 ± 1%) as compared with CERM (12.5 ± 0.9) and WT (22.3 ± 0.3) mice (Fig. 5A). Combined Western blot (Fig. 5B and C) and IHC (Fig. 5D) studies probed for significant differences in expression or activity of components of proteins that regulate the cell cycle (cyclin E, CDK2, CDK4, p27, E2F-1, RB), proliferation (ERK1/2, IGFR, STAT5, STAT3, STAT1), and survival (JNK, AKT, Bcl-2, Bax) of mammary epithelial cells (22, 24, 26, 36–38). The percentage of mammary epithelial cells with CDK2 expression was significantly higher in Arom (26 ± 1%) than CERM (2.1 ± 0.7%) and WT (1.7 ± 0.1%) mice. Association of cyclin E with CDK2 results in Rb phosphorylation. Arom mice also showed a statistically significant increase in nuclear localized p-Rb
protein (44 ± 5%) as compared with CERM (19.9 ± 0.9%) and WT (8 ± 2%) mice. Phosphorylation of Rb leads to release of E2F-1, which in turn facilitates the transcription of genes necessary for entry into the S-phase of the cell cycle. Arom mice showed a significant increase in nuclear-localized E2F-1 expression (12 ± 1%) as compared with CERM and WT mice (2.4 ± 0.3% and 2.6 ± 0.8%, respectively). Total protein levels of ERK1/2, AKT, JNK, IGF1R, STAT3, STAT5, and STAT1 were not altered in CERM and Arom mice as compared with WT mice. Relative levels of p-AKT were significantly increased in mammary tissue from Arom mice. In contrast, relative levels of p-ERK1/2, p-IGF1R, p-STAT3, and p-STAT5 were increased in both Arom and CERM mice. Only Arom mice showed significantly higher expression levels of cyclin E, CDK2, p-AKT, and Bcl-2, and decreased Bax and p27 expression. CERM, but not Arom, mice showed increased p-JNK. There was no evidence of STAT1 activation in any of the mice and CDK4 levels were equivalent in all models.

**Comparison of an ERα antagonist versus a PR antagonist on mammary ductal regression and expression of ERα and PR in CERM and Arom mice**

The ERα antagonist ICI resulted in a modest regression of the mammary gland ductal tree in both CERM and Arom mice.
with no changes in DH incidence (Fig. 6A and B). In contrast, the PR antagonist Mife resulted in more regression of the ductal tree in both CERM and Arom mice with a marked reduction in the prevalence of HANs and DH in Arom mice (Fig. 6A and B). The percentage of mammary epithelial cells showing nuclear-localized ERα was significantly decreased in Arom treated with both ICI (11 ± 2%) and Mife (10 ± 2%) as compared with nontreated mice (33 ± 2%; both \( P < 0.0001 \); Fig. 6C). Similar results were also obtained for PR expression (12 ± 1% and 12 ± 2% compared with 30 ± 2%; \( P = 0.0003 \) and \( P = 0.002 \), respectively; Fig. 6C). Tet-op-Arom and tet-op-ERα transgene expression was verified by RT-PCR following treatment and no significant changes were observed (Supplementary Fig. S1).

**Discussion**

This study compared the roles of increased local estrogen production versus ERα overexpression in mammary gland preneoplasia and cancer development in vivo. Although previous studies associated increased aromatase (28) and ERα expression (26, 29, 30) with mammary preneoplasia and cancer, this investigation compared the effects of each genetic lesion using the same conditional system to target and regulate transgene expression levels. Differences in activation of downstream signaling pathways were correlated with a higher prevalence of preneoplasia and cancer in mice with mammary-targeted aromatase expression than those with ERα overexpression.

CERM mice are an established tool to investigate the mechanisms involved in progression and regression of ERα-induced mammary hyperplasia, DCIS, and invasive cancer (26, 29, 30). Expression of ERα is increased 2-fold in the mammary epithelial cells of these mice and is considered deregulated because it is not downregulated by estrogen exposure. Here we introduce a companion model of mammary-targeted human aromatase expression with levels of aromatase activity comparable with those found in normal human breast samples and tumors (39, 40). In women, DH and DCIS lesions in the breast are associated with increased risk of invasive breast cancer development (1). HANs are considered a precancerous lesion in murine mammary gland (33). Our investigations identified local aromatase expression as a more potent inducer of DH, HANs, and invasive cancers than ERα overexpression. Control experiments were conducted to determine whether local aromatase overexpression could increase serum estradiol levels and to test whether exogenous estradiol would produce effects similar to those found with local aromatase overexpression. Results showed that aromatase overexpression in the Arom mice did not alter serum estradiol levels and that exogenous estradiol did not induce either pathologic changes in the mammary gland or increase expression levels of ERα or PR. Significantly, although increased aromatase activity stimulated the development of ERα/PR-negative invasive cancers, the DH lesions were ERα/PR-positive. In women, a higher percentage of ER-negative breast tumors among samples with high aromatase activity has been reported (41) and development of ERα/PR-negative disease from aberrant estrogen signaling is documented (42). While the exact mechanism is unclear, it is suggested that tumor heterogeneity, clonal selection of tumor cell subpopulations, or genetic instability of tumor cells may be some of the factors involved. Increased COX-2 expression has been detected in DCIS (43), and invasive breast carcinoma (44). Studies have shown a strong linear association between the sum of COX-1 and COX-2 expression and *CYP19* expression in breast cancer specimens compared with normal breast tissue (45). The aromatase mouse model presented here also shows an association between aromatase and COX-2 expression.

Increased PR expression in CERM mice from higher levels of ERα expression was anticipated (30). The significantly higher levels of ERα and PR expression following aromatase expression.
expression were not predicted but parallel findings in human breast disease were (13). Progesterone has been linked to breast carcinogenesis due to its mitogenic effects (46). PR-A is reportedly upregulated by estradiol and downregulated by progesterone, whereas PR-B is upregulated by progesterone (47). In a randomized controlled trial, the Women's Health Initiative linked the use of progestins to the onset and incidence of breast cancer (48). Overall, Arom mice showed

expression levels of ERα, PR, c-myc, cyclin D1, and RANKL were found in Arom as compared with CERM and WT mice. A, patterns of nuclear-localized ERα, PR, and cyclin D1 expression in WT, CERM, and Arom mice by IHC. Black arrows indicate representative cells showing nuclear-localized protein. Magnification, ×20; size bar, 20 μmol/L. B, bar graphs summarizing percentages of ERα and PR-positive mammary epithelial cells in WT, CERM, and Arom mice. Mean ± SEM shown. ** P < 0.005 versus WT; *** P < 0.0001 versus WT; ### P < 0.0005 versus CERM. C, bar graphs comparing relative RNA expression levels of total Pgr (PR), Pgr(b) (PR-B isoform), Ccnd1 (cyclin D1), Myc (c-Myc), Tnfsf11 (RANKL), Areg (Amphiregulin), and Wnt4 (WNT4) in WT, CERM, and Arom mice. *, P < 0.05 versus WT; **, P < 0.005 versus WT; *** P < 0.0005 versus WT; #, P < 0.05 versus CERM; ##, P < 0.01 versus CERM. D, bar graphs comparing relative RNA expression levels of Erbb2 and Egfr in mammary tissue from WT, CERM, and Arom mice. **, P < 0.005 versus WT; *** P < 0.0005 versus WT. C and D, mean ± SEM normalized to 18S RNA.
Figure 5. Rates of mammary epithelial cell proliferation were highest in Arom as compared with CERM and WT mice. Aromatase, but not ERα overexpression, increased cyclin E and CDK2 and Bcl-2 and downregulated Bax and p27 expression, and increased relative levels of AKT phosphorylation. A, bar graph comparing rates of mammary epithelial cell proliferation in WT, CERM, and Arom mice. Mean ± SEM shown. *** P < 0.0001 versus WT; ### P < 0.0005 versus CERM. B, Western blot analysis of relative levels of phosphorylated and total ERK1/2, AKT, JNK, IGFR, STAT3, STAT5, and STAT1. B and C, fold changes in expression levels of total and phosphorylated proteins as compared with WT mice indicated. β-Actin shown as loading control. *, P < 0.01 versus WT. D, IHC detection of KI67, cyclin E, Cdk2, p-RB, total RB, E2F-1, p-STAT3, total STAT3, p-STAT5, and total STAT5 in mammary epithelial cells from WT, CERM, and Arom mice. Black arrows indicate representative cells showing nuclear-localized protein. Magnification, ×40; size bar, 20 μm/L.
higher expression levels of PR downstream genes than CERM mice and this was associated with a higher prevalence of preneoplasia and cancer. For example, both CERM and Arom models expressed significantly increased levels of RANKL mRNA, but levels in Arom mice were significantly higher than CERM mice. RANKL and its receptor (RANK) are expressed in primary breast cancers in humans and breast cancer cell lines (49) and RANKL is reported to be a pivotal factor regulating incidence (20), development, and growth of murine mammary cancer (19). Arom mice also showed higher mRNA expression levels of cyclin D1 and c-Myc mRNA, consistent with the idea that ERα and PR downstream transcription was more highly activated in Arom as compared with CERM mice in correlation with the higher mammary disease prevalence.

Rates of mammary epithelial cell proliferation were higher in Arom mice as than in CERM mice. Expression and activation, as measured by phosphorylation, of key signaling pathways in mammary epithelial growth and survival (37) were measured to determine where similarities and differences could be found. Increased ERα and mammary-targeted aromatase resulted in similar levels of ERK1/2, IGFR, STAT3, and STAT5 activation but expression levels of cyclin D1 and phosphorylated Rb were

Figure 6. Impact of ICI and Mife on mammary ductal structure and prevalence of mammary preneoplasia in CERM and Arom mice. A, representative mammary gland WM, H&E-stained, and ERα and PR IHC images of ICI- and Mife-treated mice. Note HAN in ICI-treated Arom WM (inset). B, bar graph summarizing mean percentages of CERM and Arom mice with HANs and DH. C, bar graph summarizing percentages of ERα and PR-positive mammary epithelial cells. Mean ± SEM shown. Magnification, ×40 for WM and ×40 for H&E and IHC. Size bar WM, 100 μmol/L; size bar ×40 images, 20 μmol/L.
significantly higher in Arom mice than in CERM mice. Only Arom mice showed increased Akt activity and increased expression of cyclin E, CDK2, E2F-1 and Bcl-2 and reduced expression levels of Bax and p27, all changes favoring cell proliferation and survival. E2F-1 deregulation may be involved in the progression of breast cancer because its expression levels are higher in DCIS and invasive cancers then in the normal breast (36). Both models showed increased ErbB2 mRNA. CERM, but not Arom, mice showed significant increases in EGFR mRNA expression and phosphorylated JNK. Overall, however, Arom mice exhibited a higher number of alterations than CERM mice among the signaling pathways examined.

STAT proteins are involved in the normal physiology of the mammary gland and also can regulate growth of breast cancer cells (22–26). It is possible that the abnormally high levels of phosphorylated STAT3 and STAT5 found in both CERM and Arom mice contributes to development of mammary disease. Increased expression of cyclin D1 and c-Myc mRNA, known STAT3, and STAT5 target genes (50), were documented in the Arom, but not CERM, mice.

Introduction of aromatase expression in the mammary gland resulted in increased levels of the antiapoptotic protein Bcl-2 and decreased expression of the antiapoptotic protein Bax, changes that favor cell survival and are coincident with a previous report (38).

Mammary-targeted aromatase expression led to a different profile of molecular changes than ERT expression, suggesting that the pathophysiological mechanisms triggered by aromatase overexpression (that include an upregulation of ERT and PR expression levels) may be similar but not the same as those induced by primary ERT overexpression. This possibility is supported by the differential response of Arom and CERM mice to the PR antagonist Mife. In the Arom mice the PR antagonist resulted not only in regression of the ductal tree but also HANs and DH, whereas in CERM mice the DH persisted despite overall ductal regression. This is consistent with a model in which aromatase overexpression results in increased activity of the progesterone pathway that may then contribute to an increased risk of breast preneoplasia and cancer development. Because the impact of Mife was greater than ICI on regression of pathophysiological changes in the Arom mice, it raises the possibility that a PR antagonist could have a role in reversing disease related to increased levels of aromatase expression. The absence of a significant reduction in either ERT or PR levels following ICI or Mife treatment in the CERM mice may again reflect differences in molecular pathophysiology. This may be related to the fact that in the CERM model ERT expression is directly increased by a transgene whereas in the Arom model increased ERT expression is secondary to aromatase overexpression. The reduction of hormone receptor expression levels by both the ERT and PR antagonists in the Arom mice suggests that increased hormonal pathway activity contributed to the observed increase in ERT and PR expression levels.

All of the molecular changes identified following aromatase expression in the mammary gland correlate with alterations found in human disease. The deregulated signaling pathways identified here may represent candidate biomarkers for evaluating an interim response to aromatase inhibitors or tamoxifen. In the future, this conditional aromatase model can be used to determine how the time of mammary-targeted aromatase expression influences disease development, for example, duration of exposure to aromatase expression during a specific developmental stage such as puberty or menopause.

In conclusion, this study revealed that mammary-targeted aromatase expression correlated with higher ERT and PR expression levels, resulted in an increased number of aberrations in cell-cycle regulation and signaling, and produced more extensive mammary disease than more modest levels of ERT overexpression alone. This suggests that changes in aromatase levels and increased local estrogen production may be more pathogenic than alterations in ERT expression, by itself, for breast cancer progression.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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