Deregulated Estrogen Receptor α Expression in Mammary Epithelial Cells of Transgenic Mice Results in the Development of Ductal Carcinoma In situ

M. Silvina Frech, Ewa D. Halama, Maddalena T. Tilli, Baljit Singh, Edward J. Gunther, Lewis A. Chodosh, Jodi A. Flaws, and Priscilla A. Furth

1Lombardi Comprehensive Cancer Center, Departments of Oncology and Pathology, Georgetown University, Washington, District of Columbia; 2Department of Cancer Biology and Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; and 3Department of Epidemiology and Preventive Medicine, School of Medicine, University of Maryland, Baltimore, Maryland

Abstract

A conditional tetracycline-responsive transgenic mouse model with deregulated estrogen receptor α expression in mammary epithelial cells developed ductal hyperplasia (DH), lobular hyperplasia, and ductal carcinoma in situ (DCIS) by 4 months of age. Higher proliferative rates were found in both normal and abnormal ductal and lobular structures. DH and DCIS but not normal ductal structures showed an increased percentage of cells with nuclear-localized cyclin D1. No differences in either the prevalence or extent of these phenotypes following exogenous 17β-estradiol treatment were found suggesting that alteration of ERα expression was the rate-limiting factor in initiation of DH, lobular hyperplasia, and DCIS. (Cancer Res 2005; 65(3): 681-5)

Introduction

The mandatory role of estrogen receptor α (ERα) during normal mammary gland development is illustrated using mice in which the ERα gene is disrupted (1). Loss of functional ERα is associated with a reduction in mammary tumorigenesis in transgenic mice expressing different oncogenes in mammary epithelial cells (1, 2) and overexpression of ERα with an oncogene in transgenic mice is associated with an increased rate of mammary adenocarcinoma development (3). In humans, deregulated expression of ERα in normal breast epithelium has been found in conjunction with breast cancer, leading to the concept that loss of the normal regulatory mechanisms that control expression levels of ERα in normal breast epithelium may confer an increased risk for the development of breast cancer (4). ERα expression in mammary epithelial cells is normally down-regulated following estrogen exposure (5, 6). It has been suggested that development of at least some human breast cancers is associated either with increasing failure to down-regulate ERα as cells enter the cell cycle or to suppress division of ERα-positive cells (7). Higher ERα expression levels are found in normal breast tissue of women from a population at high risk for breast cancer as compared with a population at relatively low risk for the disease (8). The percentage of ERα-expressing cells increases at early stages of ductal hyperplasia (DH) and rises further with increasing atypia. A high percentage of contiguous cells in atypical DHs and in ductal carcinoma in situ (DCIS) express ERα in contrast to normal tissue in which ERα-positive cells are surrounded by ERα-negative cells (9, 10). Expression levels of known ERα-responsive genes are augmented in DHs and DCIS including progesterone receptor (11) and cyclin D1 (12–14). In DCIS, expression of p27 is correlated with cyclin D1 immunopositivity and ERα expression (14). The presence of proliferative breast epithelial lesions such as DH and lobular hyperplasia (LH) is correlated with an increased risk of breast cancer development (15, 16). Women with a history of atypical hyperplasias and in situ carcinomas have ~5- and 10-fold increased relative risks, respectively, of eventually developing invasive breast cancer (17). In this study, we show that deregulated expression of ERα in mammary epithelial cells of mice is sufficient by itself to increase rates of mammary epithelial cell proliferation and to induce the development of a multilayered ductal epithelium, ductal abnormalities, DH, LH, and DCIS by 4 months of age.

Materials and Methods

MMTV-rtTA, tet-op-ERα Double Transgenic Mice, Doxycycline Administration, 173-Estradiol Exposure, Euthanasia, and Necropsy.

Mice carrying a transgene composed of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) linked to sequences encoding the tetracycline responsive reverse transactivator (rtTA: tet-on gene regulation; ref. 18) and a transgene composed of the tetracycline responsive promoter (tet-op) linked to sequences encoding murine ERα [ref. 19; MMTV-rtTA/tet-op-ERα double transgenic or conditional estrogen receptor α in mammary tissue (CERM) mice] and single transgenic (MMTV-rtTA or tet-op-ERα) were identified using the PCR on DNA extracted from 3-week-old mice. Doxycycline (200 µg/ml) was given in the drinking water. Both nontransgenic and single transgenic mice were used as controls. Cohorts of female C57Bl/6 mice were exposed to either 60-day release 17β-estradiol 0.72 mg/pellet (Innovative Research of America, Sarasota, FL) or placebo pellets at 7 weeks of age: 7 CERM 17β-estradiol-treated, 6 CERM placebo-treated, 2 single transgenic 17β-estradiol-treated, 2 single transgenic placebo-treated, 3 nontransgenic 17β-estradiol-treated, and 7 nontransgenic placebo-treated mice. All cohorts of mice described above were composed of nulliparous mice that received doxycycline throughout their lifetime until they were sacrificed at 4 months of age. Cohorts of CERM mice were sacrificed either at 2 months (n = 6) or 12 months (n = 5) of age to study the relation between age and development of lesions. Four 2-month-old nontransgenic mice were used as controls. Eight CERM mice were ovarioctomized at 3 weeks of age and mammary gland phenotype was analyzed at 2 months of age. Specific cohorts of female C57Bl/6 mice were used to test if deregulated expression of ERα during embryonic and early postnatal life was sufficient to induce the development of ductal abnormalities and DCIS (n = 3) as well as to investigate the reversibility of phenotypic changes when ERα expression was discontinued for 2 weeks at 4 months of age (n = 4). All procedures involving animals were done in accordance with the institutional animal care guidelines.
acqured with current federal (NIH Guide for the Care and Use of Laboratory Animals) and university guidelines and were approved by the Georgetown University Institutional Use and Care Committee.

Mammary Gland Whole Mounts. One number four mammary gland from each animal was dissected and processed as previously published (3). Whole mounts were examined under 0.5×, 0.1×, and 4× magnification to evaluate the presence or absence of abnormal branching, lateral budding, dilated ducts, terminal end buds, and hyperplastic alveolar nodules. Digital photographs were taken using the Nikon Eclipse E800M microscope setup with Nikon DMX1200 software (Nikon Instruments, Inc., Melville, NY).

Histologic Analyses and Immunohistochemistry. Mammary gland specimens were fixed in 10% buffered formalin overnight at 4°C and embedded in paraffin using standard techniques. Sections (5 µm) were stained with H&E. H&E sections were evaluated by a board certified human pathologist (B.S.) for the presence or absence of DH, LH, and DCIS. Detection of ERα, progestere receptor, cyclin D1, Ki67, and p27 protein expression and cellular localization in mammary gland tissue was accomplished using the mouse on mouse (MOM) peroxidase kit (Vector Laboratories, Inc., Burlingame, CA). Tissue sections were deparaffinized, rehydrated, antigens exposed with BORG solution (Biocare, Walnut Creek, CA) in a decloaking chamber (Biocare), quenched with 3% hydrogen peroxide, blocked with mouse IgG-blocking reagent, incubated with MOM diluent, exposed to the primary antibody diluted in MOM diluent for 1 hour and to MOM biotinylated anti-mouse IgG reagent for 10 minutes at room temperature. The tissues were exposed to MOM elite stain for 30 minutes, stained with diaminobenzidine peroxidase substrate kit (Vector Laboratories) for 5 minutes and counterstained with hematoxylin. Primary antibodies included 1:25 dilution of mouse monoclonal ERα antibody clone 1D5 (Beckman Coulter Immunotech, Miami, FL); 1:100 dilution of mouse monoclonal progestere receptor antibody clone 16 and SAN27 (Novocasta, United Kingdom); 1:50 dilution of mouse monoclonal cyclin D1 antibody clone A-12 (Santa Cruz Biotechnology Inc., Santa Cruz, CA); 1:50 dilution of mouse monoclonal p27 antibody (Santa Cruz Biotechnology); 1:100 dilution of mouse monoclonal Ki67 antibody clone MM1 (Novocastra). The percentage of cells demonstrating proliferating cell nuclear antigen (PCNA) as detected by immunohistochemistry was used as a relative measure of the rates of cell proliferation. Mammary tissue sections were exposed to two drops of the EPOS PCNA immunostaining system (DAKO Cytomation California Inc., Carpinteria, CA) for 1 hour at room temperature and stained with the diaminobenzidine kit for 5 minutes. Ki67 immunohistochemistry was done in a subset of the samples as a second technique to confirm the findings obtained from PCNA immunohistochemistry. Determination of the percentage of cells demonstrating positively stained nuclei was obtained by counting a minimum of 1,000 cells per section. One section from each mouse was selected randomly and consecutive fields examined under 40× magnification until a total of 1,000 cells were counted. Slides from control and CERM 4-month-old female nullparous mice were analyzed: ERα (n = 19), cyclin D1 (n = 15), PCNA (n = 14), progestere receptor (n = 16), Ki67 (n = 18), and p27 (n = 10). Negative control slides in which the primary antibody was omitted were analyzed in parallel. In the absence of primary antibody no nuclear-specific staining was observed.

RNA Isolation and Analysis of Gene Expression by Real-time PCR. Total RNA was isolated by Trizol extraction (Invitrogen, Carlsbad, CA) from mammary gland tissue snap frozen at the time of necropsy, quantified on a spectrophotometer, and cDNA synthesis done. Taqman Assays on Demand (Mm 00433149 m1, Applied Biosystems, Foster City, CA) were used to simultaneously detect ERα cDNA from both the endogenous gene and the ERα transgene. Reactions were carried out with the ABI Prism 7700 sequence detector and analysis was done using ABI Software (Applied Biosystems).

Statistical Analyses. Statistical differences among groups were analyzed with either t tests or ANOVA as appropriate using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Post hoc test for the ANOVA included Bonferroni’s multiple comparison test. Significance was assigned at P ≤ 0.05.

Results and Discussion

Expression of ERα Was Deregulated in CERM Mice. Real-time PCR showed a statistically significant 2-fold increase in total ERα mRNA expression levels in CERM as compared with control mice (2.07 ± 0.24 to 1.0 ± 0.05, P < 0.041; t test). In parallel, the mean percentage of mammary epithelial cells demonstrating nuclear-localized ERα was 2-fold higher in CERM mice than that observed in control mice (35 ± 8% to 17 ± 4%, P < 0.037; t test) when both 17β-estradiol- and placebo-treated groups were combined. Control mice showed a statistically significant decrease in the mean percentage of mammary epithelial cells with nuclear-localized ERα following exposure to 17β-estradiol (25 ± 1 to 10 ± 6%, P < 0.032; t test; Fig. 1). The mean percentage of mammary epithelial cells demonstrating nuclear-localized ERα expression was decreased following
17β-estradiol exposure in CERM mice (46 ± 10% to 19 ± 12%), but expression levels were still higher than those found in control mice. Maintenance of higher levels of ERα expression following exposure to exogenous 17β-estradiol in the CERM mice was likely due to the fact that unlike the endogenous ERα, gene transcription from the tetracycline responsive conditional transgene system was not down-regulated by 17β-estradiol exposure.

Figure 2. Mammary gland whole mounts with representative normal mammary ductal structure in control mice treated with 17β-estradiol (B) and the presence of abnormal secondary and tertiary branching (black arrows, A, and C), dilated ducts (dashed arrows, C, and D), and lateral budding (arrowhead, D) in CERM mice. Mammary gland whole mounts from 2-month-old ovariectomized mice (I) and 12-month-old CERM mice (thin black arrow, hyperplastic alveolar nodule, J). H&E-stained sections of mammary tissue from CERM mice show DCIS (E, F, G, and H). There were no structural differences found between placebo treated and 17β-estradiol exposed CERM mice. White arrows, mitotic figures (E and G). Bar, 200 μm (A, B, C, D, I, and J). Bar, 20 μm (E, F, G, and H).

Figure 3. Immunohistochemical detection of PCNA (A, C, E, and G) and cyclin D1 (B, D, F, and H) in mammary epithelial cells of control (A-B) and CERM mice (C-H). There was an increase in the mean percentage of mammary ductal epithelial cells that showed nuclear-localized PCNA in CERM (C, E, and G) compared with control (A) mice (P < 0.05; ANOVA, Bonferroni's test). The mean percentage of cells demonstrating nuclear-localized PCNA (arrows) was not significantly different between the normal-appearing ducts (C), LH (E), and DH and DCIS (G). The mean percentage of mammary epithelial cells demonstrating nuclear-localized cyclin D1 in normal appearing ducts was not increased in CERM (D) as compared with control (B) mice. In contrast, the mean percentage of mammary epithelial cells demonstrating nuclear-localized cyclin D1 in DH and DCIS (H, arrows) was significantly increased as compared with normal appearing ducts of CERM (P < 0.05) and normal ducts of control mice (P < 0.01; ANOVA, Bonferroni's test). Bar, 20 μm.
Deregulated ER\(_{\alpha}\) Expression in Mammary Epithelial Cells Led to the Development of Ductal Abnormalities, Ductal Hyperplasia, and DCIS. At 4 months of age, 95% of CERM mice displayed abnormal ductal structures, including lateral budding, dilated ducts, and abnormal branching (Fig. 2A, C, and D). These changes were uniform throughout the ductal tree. LH was found in 52% (Fig. 3E-F), DH in 36% (Fig. 3G-H), and DCIS in 21% (Fig. 3E-H) of 4-month-old CERM mice. Thirty-three percent and 17% of CERM mice developed DH and DCIS, respectively, by 2 months of age. Occasional mitotic figures were found in the DCIS lesions (Fig. 2E and G). None of the control mice showed any of these phenotypes. Exposure to exogenous 17\(\beta\)-estradiol did not increase or change the prevalence of phenotypic ductal abnormalities, proliferative disease or DCIS. When deregulated ER\(_{\alpha}\) expression was limited to in utero and postnatal exposure through 3 weeks of age, mammary gland development was normal without evidence of either proliferative disease or DCIS (data not shown). Neither hyperplasia nor DCIS regressed within a 2-week period following discontinuation of transgenic ER\(_{\alpha}\) expression in 4-month-old mice (data not shown). None of the eight ovariectomized mice showed either DH, DCIS, or other ductal abnormalities consistent with the hypothesis that lesion development was promoted by normal ovarian hormone function (Fig. 2F). Hyperplastic alveolar nodules and an increased frequency of mitotic figures within the hyperplasias were found in 12-month-old CERM mice (Fig. 2F). No externally palpable tumors developed by that age. These data show that deregulated ER\(_{\alpha}\) expression in mammary epithelial cells alters development and differentiation and is sufficient for the initiation of proliferative disease with progression to DCIS.

Deregulated ER\(_{\alpha}\) Expression in Mammary Epithelial Cells Increased the Rates of Mammary Epithelial Ductal Cell Proliferation. Rates of mammary epithelial cell proliferation were increased significantly in the normal appearing ducts (44 ± 7%, \(P \leq 0.05\)), LH (80 ± 4%, \(P \leq 0.001\)), and DH and DCIS (72 ± 6%, \(P \leq 0.001\); ANOVA, Bonferroni’s test) of CERM mice as compared with normal mammary epithelial cells in control mice (20 ± 2%; Fig. 3). Whereas there were statistically significant differences between CERM and control mice in the percentage of cells exhibiting proliferation markers, exposure to 17\(\beta\)-estradiol did not increase the percentage of cells demonstrating nuclear-localized PCNA in either CERM or control mice (17\(\beta\)-estradiol-treated CERM, 60 ± 7%; placebo-treated CERM, 80 ± 5%; 17\(\beta\)-estradiol-treated control, 19 ± 6%; placebo-treated control, 20 ± 2%). Similar to human disease, the DCIS lesions that developed in the CERM mice showed a high percentage of cells with nuclear-localized ER\(_{\alpha}\), cyclin D1, PCNA, Ki67, and p27 (Fig. 4).

The Percentage of Mammary Epithelial Cells Demonstrating Nuclear Localized Cyclin D1 Was Increased Selectively in Ductal Hyperplasia and DCIS. DH and DCIS but not normal ducts in CERM mice showed a statistically significant increase in the percentage of cells demonstrating nuclear-localized cyclin D1 expression (59 ± 4%) as compared with normal appearing ducts of CERM mice (42 ± 5%, \(P \leq 0.05\); ANOVA, Bonferroni’s test) and normal ducts of control mice (37 ± 4, \(P \leq 0.01\); ANOVA, Bonferroni’s test; Figs. 3 and 4). The percentage of cells demonstrating nuclear-localized cyclin D1 in the normal appearing ducts of CERM mice was not significantly different than the percentage found in the ducts of control mice. These findings parallel those reported for human atypical DH and DCIS where the percentage of cells demonstrating nuclear-localized cyclin D1 is higher than it is in normal mammary epithelial cells (12, 13).

In summary, this study showed that increasing expression levels of ER\(_{\alpha}\) in mammary epithelial cells combined with an inability to fully down-regulate ER\(_{\alpha}\) expression in response to estrogen led to the development of proliferative disease and DCIS. CERM mice mirror the pathophysiology of human-proliferative breast disease where DCIS is associated with the presence of hyperplasia in other areas of the mammary gland and the mammary epithelial cells that compose the DCIS lesions show expression of ER\(_{\alpha}\) in association with proliferative markers and increased levels of cyclin D1. This model offers the opportunity to study the biological and molecular events that act with ER\(_{\alpha}\) to promote the development of proliferative disease in the breast and dictate its progression to DCIS, investigate how physiologic and pharmacologic interventions might be used to promote regression, and identify factors that further progression of DCIS to invasive breast cancer. Combining the CERM model with mouse models of ER-negative breast cancer would allow a comparative analysis of the development of breast cancer with and without ER\(_{\alpha}\) signaling. Progression of genetic instability in the form of aneuploidy and loss of heterozygosity in DCIS could be compared in both the presence and absence of persistent ER\(_{\alpha}\) signaling (12). Because this is a conditional system, ER\(_{\alpha}\) expression can be deliberately

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**Figure 4.** Immunohistochemical detection of ER\(_{\alpha}\) (A), cyclin D1 (B), PR (C), Ki67 (D), PCNA (E), and p27 (F) in a single DCIS lesion from a 4-month-old nulliparous placebo-treated CERM mouse. Arrows, representative cells demonstrating nuclear-localized protein. Bar, 20 \(\mu\)m.
manipulated to investigate its role at different time points during cancer development from preneoplasia to adenocarcinoma to metastasis. For example, the ERKO/Wnt-1 transgenic mouse model shows that Wnt-1 initiates carcinogenesis directly in ERα-negative epithelium (20). Combining this type of model with the CERM mice could be used to determine whether or not introduction of ERα signaling at specific time points during Wnt-1-induced carcinogenesis is redundant, additive, or synergistic. Finally, ligand-independent and ligand-dependent activity of ERα during carcinogenesis could be compared in combination models in which ligand exposure is regulated through ovariectomy and administration of pharmacologic agents that restore ligand activity.

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References
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