A Mouse Mammary Tumor Virus-Wnt-1 Transgene Induces Mammary Gland Hyperplasia and Tumorigenesis in Mice Lacking Estrogen Receptor-α


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ABSTRACT

Estrogens have important functions in mammary gland development and carcinogenesis. To better define these roles, we have used two previously characterized lines of genetically altered mice: estrogen receptor-α (ERα) knockout (ERKO) mice, which lack the gene encoding ERα, and mouse mammary virus tumor (MMTV)-Wnt-1 transgenic mice (Wnt-1 TG), which develop mammary hyperplasia and neoplasia due to ectopic production of the Wnt-1 secretory glycoprotein. We have crossed these lines to ascertain the effects of ERα deficiency on mammary gland development and carcinogenesis in mice expressing the Wnt-1 transgene. Introduction of the Wnt-1 transgene into the ERKO background stimulates proliferation of alveolar-like epithelium, indicating that Wnt-1 protein can promote mitogenesis in the absence of an ERα-mediated response. The hyperplastic glandular tissue remains confined to the nipple region, implying that the requirement for ERα in ductal expansion is not overcome by ectopic Wnt-1. Tumors were detected in virgin ERKO females expressing the Wnt-1 transgene at an average age (48 weeks) that is twice that seen in virgin Wnt-1 TG mice (24 weeks) competent to produce ERα. Prepubertal ovariectomy of Wnt-1 TG mice also extended tumor latency to 42 weeks. However, pregnancy did not appear to accelerate the appearance of tumors in Wnt-1 TG mice, and tumor growth rates were not measurably affected by late ovariectomy. Small hyperplastic mammary glands were observed in Wnt-1 TG males, regardless of ERα gene status; the glands were similar in appearance to those found in ERKO/Wnt-1 TG females. Mammary tumors also occurred in Wnt-1 TG males; latency tended to be longer in the heterozygous ERα and ERKO males (86 to 100 weeks) than in wild-type ERα mice (ca. 75 weeks). We conclude that ectopic expression of the Wnt-1 proto-oncogene can induce mammary hyperplasia and tumorigenesis in the absence of ERα in female and male mice. The delayed time of tumor appearance may depend on the number of cells at risk of secondary events in the hyperplastic glands, on the carcinogenesis-promoting effects of ERα signaling, or on both.

INTRODUCTION

Estrogens are important for reproductive tract growth and function, including proliferation of the uterine epithelium and ovarian folliculogenesis. Furthermore, estrogens function in the development of the mammary gland, external genitalia, appropriate reproductive behavior, and other female secondary sex characteristics. There is also a strong correlation between the action of ovarian hormones, particularly 17β-estradiol (estradiol), and carcinogenesis in the mammary gland and uterus (2, 3). The biological effects of estrogen hormones are mediated through ERα, a member of the superfamily of nuclear receptors that can function as ligand-inducible transcription factors (4). A second gene encoding another ER, termed ERβ, has been identified and may mediate estrogen signaling in some tissues (5–8).

During puberty, mammary epithelial cells divide and migrate into the stromal fat pad as TEBs in response to estradiol and growth hormone in a process called ductal morphogenesis (9–12). Mitosis occurs in the cap cell layer of the TEBs that appear to be devoid of ERα; ERα is present, however, in the ductal epithelium and mammary stromal cells (9). ERβ mRNA is detectable in human and rat mammary tissue (8, 13–15); however, its role in mammary development remains inconclusive.

The estrogen/ERα signaling pathway may stimulate proliferation of both ERα-positive and -negative mammary epithelium (16, 17). Estrogens can act directly on epithelial cells and/or stimulate stromal cells to secrete growth factors that induce mitogenesis in the epithelium (18, 19). Furthermore, estrogens can stimulate the secretion of pituitary prolactin that induces mitogenesis in mammary epithelium (20, 21). Therefore, estrogens may affect the growth of both ERα-positive and -negative mammary tumors (11, 22).

To assess the role of ERα in mammary gland carcinogenesis, we have introduced into ERKO mice an MMTV-Wnt-1 transgene known to induce mammary hyperplasia and carcinomas (23). Wnt genes encode secretory glycoproteins that normally act in autocrine and paracrine fashions to stimulate cell proliferation and differentiation (24). Several members of the Wnt gene family are expressed in the mouse mammary gland during various stages of development (24–26). Although Wnt-1 is not normally produced in the mammary gland, insertional activation of the Wnt-1 gene by MMTV proviruses initiate mammary carcinogenesis (27, 28), and a transgene that simulates an insertional activated locus produces mammary hyperplasia in both male and female animals (23). Virtually all female mice with the MMTV-Wnt-1 transgene develop mammary tumors within the first year of life, with half the animals displaying tumors at ~5–6 months of age. In addition, 15–30% of the male Wnt-1 TGs have mammary tumors by the age of 1 year (23).

In previous crosses of the Wnt-1 TG mice to genetically altered mice, we found that other oncogenes (e.g., FGFR-3) and tumor suppressor genes (e.g., p53) affect Wnt-1-induced tumorigenesis (29, 30). Here, we report that the Wnt-1 transgene can overcome a genetic deficiency of ERα to produce mammary hyperplasia and tumors, but the hyperplasia is limited by impaired ductal morphogenesis and the onset of tumors is significantly delayed. In addition, the effect on tumor latency can be mimicked by prepubertal ovariectomy. Thus, estrogen/ERα-mediated signaling is not required for Wnt-1-induced proliferation and oncogenic conversion of mammary epithelium. Estrogenic stimulation of the mammary gland can affect mammary carcinogenesis in this model system by determining the number of cells at risk of neoplastic conversion, directly promoting the growth of tumour cells, or both.

MATERIALS AND METHODS

Breeding. The mice were housed and treated in accordance with the NIH Guide to Humane Use of Animals in Research. All surgical procedures were approved by the National Institute of Environmental Health Sciences Animal Care and Use committee. Three males HET for the Wnt-1 transgene (derived from line 303; Ref. 23) were crossed with six females HET for the ERα gene (ERα+/−). First-generation HET/Wnt-1 TG males were then crossed with...
the HybSpeed RPA kit (Ambion) protocol. Protected probe fragments were ethanol overnight at 9°C. ERα gene primers (forward, 5'-CGGCTACCGCCAGTCCGGATC-3'; and reverse, 5'-GTGAAAGCAGGGAAGGGGTTGTC-3') anneal to sites that flank the neo gene insertion site used to disrupt the ERα gene in exon 2 (32). These primers generate a 239-bp PCR product from the intact ERα gene. The neo gene primers (forward, 5'-GTGTCGCGGTCTGACGGC-3'; and reverse, 5'-GTCTGTAGTCGCTGCGCCA-3') anneal to the neo gene used to disrupt the ERα gene and generate a PCR product of 555 bp. To detect the Wnt-1 transgene, the forward Wnt-1 primer (5'-GGACTTGGTCTTTCTTCTCATAGCC-3') anneals to the 3' end of the Wnt-1 gene and the reverse SV40 primer (5'-CCACACAGCATAGAGTGTCTGC-3') anneals to the SV40 polyadenylation sequence inserted immediately downstream of the Wnt-1 gene in the TG construct (23). This primer pair generates a 407-bp PCR product. All three primer sets (5 pmol of ERα primers, 10 pmol of Wnt-1 primers, and 50 pmol of neo primers) were used in a single-tube reaction. The PCR conditions were 95°C denaturing, 65°C annealing, and 72°C extension for 30 cycles using Taq polymerase and a DNA Thermal Cycler from Perkin-Elmer. The PCR products were resolved by electrophoresis in 2% Nusieve low melting agarose-0.7% agarose-1× T Tris-borate EDTA gels (FMC Bioproducts).

Ovariectomy and Pregnancy Studies. To determine whether tumors could continue to grow after estradiol deprivation, mice were ovariectomized under anesthesia by Veterinary Medicine (National Institute of Environmental Health Sciences, Research Triangle Park, NC) after a tumor of 0.5–1.0 cm diameter was observed. The mice were monitored weekly for tumor enlargement and then sacrificed when the tumors grew a further 3-fold in diameter. To determine whether estradiol at puberty is required for tumor development, mice were ovariectomized before puberty (15 days old) and then monitored for the development of tumors 0.5 cm in diameter. To determine whether pregnancy could accelerate tumor onset, Wnt-1 TG female mice were continuously mated beginning at 8 weeks of age and monitored for tumor development.

Kaplan-Meier Analysis. The compiled data of mouse age at the time of tumor appearance were subjected to Kaplan-Meier analysis and plotted as a function of the probability of a mouse being tumor free versus its age in weeks. This analysis also takes into account the time a mouse on study remains tumor free and then is removed from study before tumor appearance due to death by old age, sickness, and so on. The various plots were then compared to each other using a life table test (33) to determine whether the rate of tumor development was statistically different among the Wnt-1 TG mice with different ERα genotypes.

Mammary Whole-Mount and Histological Analyses. Mammary glands were stained and whole mounted according to a modified procedure from Russo et al. (34). Inguinal mammary fat pads were excised from euthanized mice and placed in a tissue histocassette containing a biopsy foam pad. The fat pads were placed in 10% formalin for at least 24 h and then defatted in acetone over 5–7 days. The fat pads were rehydrated in a graded series of alcohols to distilled water and then stained in a 0.035% toluidine blue dye solution for 30–60 min. The fat pads were dehydrated in methanol for 30–60 min, 70% ethanol for 30 min, and postfixed in 4% ammonium molybdate for 30 min. After remaining in distilled water overnight, the fat pads were dehydrated in a graded series of alcohols to xylene and then mounted using Permount (Fisher). Mammary glands and tumors excised for histological analyses were fixed in 10% formalin, embedded in paraffin, sectioned at 5-μm thickness, and stained with H&E.

RPA. Radiolabeled antisense RNA probes for mouse Wnt-1, keratin 18, and cyclinophilin were generated from linearized cDNA templates in pBluescript (Wnt-1 and keratin 18) and pTRI-cyclinophilin (Ambion) using T7 RNA polymerase and [32P]CTP (Amersham) according to the Maxiscript kit protocol (Ambion). The length of protected probe fragments were: Wnt-1, 404 nt; keratin 18, 547 nt; cyclinophilin, 103 nt.

RPA reactions consisting of 5 × 10^4 cpm of each riboprobe, mammary gland RNA (1 μg), and yeast tRNA (24 μg) were mixed and precipitated in ethanol overnight at −70°C. The pellets were further processed according to the HybSpeed RPA kit (Ambion) protocol. Protected probe fragments were resolved by electrophoresis using a 1.5-mm 6% bis-acrylamide, 8.3 M urea, and 1× Tris-borate EDTA gel (National Diagnostics). The gels were fixed, dried, and exposed to a Phosphorimager screen followed by exposure to X-ray film. RPA results were analyzed using the Phosphorimager Storm 860 and ImageQuant software (Molecular Dynamics).

RESULTS

Wnt-1 Induces Hyperplasia in Female ERKO Mammary Glands. To determine the effect of a disrupted ERα gene on Wnt-1-induced mammary neoplasia, the ERKO mouse line was crossed with the Wnt-1 TG line. Three Wnt-1 TG males were mated with six females heterozygous for the ERα gene (ERα/+; HET) to generate HET/Wnt-1 TG males, which were then crossed with HET females to generate wild-type, ERKO (ERα−/−), Wnt-1 TG, and ERKO/Wnt-1 TG mice. Mammary gland morphology, mammary tumor incidence, and the effect of ovarian hormones on tumor development were examined in these mice.

Inguinal mammary glands from mice bearing the Wnt-1 transgene in the presence or absence of the ERα gene were analyzed at 10 weeks of age. Virgin wild-type female mice progressed through mammary ductal morphogenesis as indicated by the presence of bulbous TEBs and an ordered ductal network (Fig. 1A). As reported previously (23), mammary glands from Wnt-1 TG females showed excessive hyperplasia, consisting of extensive ductal side-branching and the development of aberrant lobular-like structures (Fig. 1C). The ERKO female mammary gland was undeveloped, as illustrated by the rudimentary ductal network that remained confined to the nipple region and lacked both TEBs and alveolar structures (Fig. 1B). In contrast, the ERKO/Wnt-1 TG mammary gland was comprised of a hyperplastic rudimentary ductal structure without TEBs (Fig. 1D).

To determine whether ERα affected progression of Wnt-1-induced hyperplasia, mammary glands from 6-month-old virgin Wnt-1 TG and ERKO/Wnt-1 TG female mice were examined. At 6 months, the Wnt-1 TG female mammary gland was comprised of hyperplastic epithelium that occupied the entire fat pad (Fig. 1E). In contrast, mammary glands from 6 month ERKO/Wnt-1 TG mice exhibited hyperplasia (Fig. 1F) that was similar to that seen in glands from the 10 week old mice (Fig. 1D). Hyperplasia remained confined to the nipple region of ERKO/Wnt-1 TG glands, with no directed growth into the inguinal fat pad beyond the lymph node (Fig. 1F). This restricted mammary hyperplasia was maintained with age in ERKO/Wnt-1 females.

Mammary gland tissue sections were analyzed by H&E staining to determine whether cellular morphology was altered due to Wnt-1 expression. Wnt-1 TG female mammary glands consisted of closely spaced ducts and more periductal connective tissue, indicative of ductal and lobuloalveolar hyperplasia, compared to the normal ductal spacing and adipose stroma of wild-type mammary glands (compare Fig. 2, A and B). The ERKO/Wnt-1 TG mammary gland contained regions of lobuloalveolar hyperplasia at the periphery of the gland. Ductal hyperplasia and a dense connective tissue stroma were evident in the interior of the gland (Fig. 2C).

Wnt-1 TG Males Develop Hyperplastic Mammary Glands Regardless of ERα Gene Status. The mammary glands from wild-type and ERKO males were far less developed than those from the ERKO females. There was no demonstrable ductal rudiment and only minimal epithelial remnants present in the fat pad (Fig. 3, A and B). In contrast, mammary glands from both Wnt-1 TG and ERKO/Wnt-1 TG male mice exhibited epithelial hyperplasia (Fig. 3, C and D, respectively), which appeared similar to the ERKO/Wnt-1 TG female mammary glands (Fig. 1F). Cellular morphology of Wnt-1 TG and ERKO/Wnt-1 TG male mice was similar to that of Wnt-1 TG female mice. The mammary gland morphology in these male Wnt-1 TG males was similar to Wnt-1 TG female mice. The absence of ERα in these female mice did not appear to contribute to the differences in tumor development or morphology between the male and female Wnt-1 TG mice.

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Wnt-1 TG male mammary glands (data not shown) was similar to that seen in female ERKO/Wnt-1 TG mammary glands (Fig. 2C).

The Absence of ERα Delays Wnt-1-induced Mammary Tumor Development. To determine the effect of differing ERα gene levels on Wnt-1-induced mammary tumor incidence and latency, we recorded the ages at which mammary tumors (≥0.5-cm diameter) were discovered and subjected the data to Kaplan-Meier analysis. Wnt-1 TG and HET/Wnt-1 TG females developed mammary tumors at the same rate; tumors appeared in one-half of the mice in each group by 24 weeks of age (Fig. 4A). In contrast, the onset of tumors in ERKO/Wnt-1 TG females was delayed, with one-half of the mice developing tumors by 48 weeks (Fig. 4A). The onset of tumors in Wnt-1 TG males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia. One-half of the Wnt-1 males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia. One-half of the Wnt-1 males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia. One-half of the Wnt-1 males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia. One-half of the Wnt-1 males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia. One-half of the Wnt-1 males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia. One-half of the Wnt-1 males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia. One-half of the Wnt-1 males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia. One-half of the Wnt-1 males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia. One-half of the Wnt-1 males of all three ERα genotypes was more delayed than in the ERKO/Wnt-1 TG females, despite similar levels of hyperplasia.

Wnt-1-induced Mammary Tumors Develop in the Absence of Ovarian Estrogens. The development and growth of Wnt-1-induced mammary tumors were also observed under conditions of pregnancy and estradiol deprivation due to ovariectomy. To determine whether elevated levels of mammary hormones induced by pregnancy could accelerate tumor onset, 14 breeding Wnt-1 TG females that carried one to three pregnancies to term were monitored for tumors. Pregnancy did not measurably accelerate mammary tumor development in these females, as determined by Kaplan-Meier analysis. The average age of tumor onset for parous and nulliparous Wnt-1 TG females was 24 weeks (Fig. 5).

To determine whether tumors could continue to grow under estradiol-deprived conditions, 20 Wnt-1 TG females that developed tumors of 0.5–1.0 cm in diameter were then ovariectomized. In all 20 cases, tumors continued to grow after ovariectomy, and in three of these mice, a second tumor appeared in a different mammary gland. Ovariectomy was performed on Wnt-1 TG females at 15 days of age to determine whether estradiol production at puberty is required for mammary tumorigenesis. This experiment also permitted a comparison of systemic estradiol deprivation versus an ERα-negative environment on mammary hyperplasia and tumor development. Prepubertal ovariectomy of Wnt-1 TG females did not prevent Wnt-1-induced mammary hyperplasia, which was morphologically similar to that seen in surgically intact ERKO/Wnt-1 TG females (see Fig. 1, D and F). Ovariectomized Wnt-1 TG females developed tumors at an average age of 42 weeks, which is delayed compared to the intact Wnt-1 TG females (24 weeks) but similar to the intact ERKO/Wnt-1 TG group (48 weeks; Fig. 5). In addition, eight ERKO/Wnt-1 TG females were ovariectomized before puberty and exhibited mammary hyperplasia similar in morphology to their surgically intact counterparts (Fig. 1, D and F). Four of these mice developed mammary tumors at an age (mean ± SD) of 60 ± 11 weeks, whereas four mice...
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By introducing an oncogenic transgene, MMTV-Wnt-1, into an ERα-deficient background, we have begun to dissect the complex relationship that exists between the hormonal factors that normally govern organ development and the oncogenic lesions that produce tumors in that organ. Here, it is apparent that the proliferative and tumor-inducing effects of ectopic Wnt-1 expression in the mammary gland can occur in the absence of ERα. Mammary epithelial cells responsive to Wnt-1 are produced without estrogenic stimulation. Furthermore, the Wnt-1 signaling pathway, which has been shown to use several transcription factors (37–41), does not require any conjugate gene products that contribute to these processes (42). The rudimentary ductal network emanating from the nipple in the ERKO female clearly demonstrates the importance of estrogen/ERα action in mammary gland development. The ERKO/Wnt-1 TG female mammary gland was comprised of hyperplastic epithelium in the nipple region of the gland. Therefore, the Wnt-1 signal did not require an interaction that exists between the hormonal factors that normally govern organ development and the oncogenic lesions that produce tumors in that organ. Here, it is apparent that the proliferative and tumor-inducing effects of ectopic Wnt-1 expression in the mammary gland can occur in the absence of ERα. Mammary epithelial cells responsive to Wnt-1 are produced without estrogenic stimulation. Furthermore, the Wnt-1 signaling pathway, which has been shown to use several transcription factors (37–41), does not require any contribution from ERα. However, tumor latency is prolonged in ERKO/Wnt-1 TG mice and in mice ovariectomized before sexual maturity. This effect may be ascribed to the reduced size of the mammary glands, direct effects of estrogen on tumor promotion, or both. Estrogen/ERα signaling is essential for mammary ductal morphogenesis and lobuloalveolar development by inducing estrogen-responsive gene products that contribute to these processes (42). The rudimentary ductal network emanating from the nipple in the ERKO female clearly demonstrates the importance of estrogen/ERα action in mammary gland development. The ERKO/Wnt-1 TG female mammary gland was comprised of hyperplastic epithelium in the nipple region of the gland. Therefore, the Wnt-1 signal did not require a functional ERα to stimulate proliferation or hyperplasia. However, the hyperplasia did not progress into the inguinal mammary fat pad beyond the lymph node, indicating that ductal elongation did not occur as a result of Wnt-1 action in the ERKO female. Therefore, Wnt-1 cannot substitute for ERα-mediated ductal morphogenesis involving cap cell division and TEB formation. A previous report demonstrated that reconstituted mammary epithelium expressing Wnt-1 from a retrovirus vector did not develop TEBs, and the rate of hyperplastic growth was reduced after ovariectomy (43). However, another study showed that mammary epithelium transplanted from 3-month-old Wnt-1 TG females into ovariectomized, athymic nude mice developed hyperplasia that occupied the entire mammary fat pad (44). Because ovariectomy was performed during adulthood in those

died at the ages of 80, 81, 87, and 112 weeks without developing tumors.

ESTROGEN ACTION IS NOT MEDIATED BY ERβ AND WNT-1 GENE EXPRESSION IS NOT ALTERED IN THE ABSENCE OF ERα. As described above, ERKO/Wnt-1 TG females developed tumors, and tumor latency was increased in these mice by prepubertal ovariectomy. Possible tumor-promoting actions of estradiol and progesterone may be mediated by ERβ and PR, respectively, in the ERKO/Wnt-1 TG mammary gland. RPAs demonstrated that ERβ mRNA was not detected in wild-type or ERKO mammary glands, confirming a previous report (35). Furthermore, ERβ gene expression was apparently not induced by Wnt-1 because ERβ mRNA was not detected in hyperplastic mammary or tumor tissue from Wnt-1 TG and ERKO/Wnt-1 TG mice (data not shown). In contrast, PR mRNA was readily observable by RPA in hyperplastic mammary and tumor tissue from Wnt-1 TG and ERKO/Wnt-1 TG mice (data not shown).

The possibility remained that Wnt-1 transgene expression may be reduced in mammary epithelium lacking ERα, accounting for the increased latency of mammary tumors in ERKO/Wnt-1 TG mice. Normalizing the level of Wnt-1 mRNA in bulk mammary tissue to keratin 18 mRNA, an epithelial cell marker, can distinguish differences in Wnt-1 gene expression that are due to variations in epithelial cell abundance or to altered expression within the epithelial compartment (29, 36). As expected, keratin 18 mRNA was detected in the mammary glands of wild-type females but not in ERKO females, and Wnt-1 mRNA was not detected in wild-type or ERKO mammary glands from either sex (Fig. 6). When normalized to keratin 18 mRNA, Wnt-1 mRNA levels were essentially the same in Wnt-1 TG and ERKO/Wnt-1 TG mammary tissues from either sex (Fig. 6). Wnt-1 mRNA levels were increased in mammary tumors compared to hyperplastic glands; however, keratin 18 mRNA was also increased, indicating epithelial cell abundance (Fig. 6).

DISCUSSION

Fig. 2. Histological analysis of female mammary glands expressing TG Wnt-1 with or without ERα. Inguinal mammary glands from wild-type (A), Wnt-1 TG (B), and ERKO/Wnt-1 TG (C) females were sectioned and stained with H&E. Note the increased ductal structures in the wild-type fat pad (A). Scale bar, 200 μm (A–C) and 4.0 μm (insets).

By introducing an oncogenic transgene, MMTV-Wnt-1, into an ERα-deficient background, we have begun to dissect the complex relationship that exists between the hormonal factors that normally govern organ development and the oncogenic lesions that produce tumors in that organ. Here, it is apparent that the proliferative and tumor-inducing effects of ectopic Wnt-1 expression in the mammary gland can occur in the absence of ERα. Mammary epithelial cells responsive to Wnt-1 are produced without estrogenic stimulation. Furthermore, the Wnt-1 signaling pathway, which has been shown to use several transcription factors (37–41), does not require any contribution from ERα. However, tumor latency is prolonged in ERKO/Wnt-1 TG mice and in mice ovariectomized before sexual maturity. This effect may be ascribed to the reduced size of the mammary glands, direct effects of estrogen on tumor promotion, or both. Estrogen/ERα signaling is essential for mammary ductal morphogenesis and lobuloalveolar development by inducing estrogen-responsive gene products that contribute to these processes (42). The rudimentary ductal network emanating from the nipple in the ERKO female clearly demonstrates the importance of estrogen/ERα action in mammary gland development. The ERKO/Wnt-1 TG female mammary gland was comprised of hyperplastic epithelium in the nipple region of the gland. Therefore, the Wnt-1 signal did not require a functional ERα to stimulate proliferation or hyperplasia. However, the hyperplasia did not progress into the inguinal mammary fat pad beyond the lymph node, indicating that ductal elongation did not occur as a result of Wnt-1 action in the ERKO female. Therefore, Wnt-1 cannot substitute for ERα-mediated ductal morphogenesis involving cap cell division and TEB formation. A previous report demonstrated that reconstituted mammary epithelium expressing Wnt-1 from a retrovirus vector did not develop TEBs, and the rate of hyperplastic growth was reduced after ovariectomy (43). However, another study showed that mammary epithelium transplanted from 3-month-old Wnt-1 TG females into ovariectomized, athymic nude mice developed hyperplasia that occupied the entire mammary fat pad (44). Because ovariectomy was performed during adulthood in those
studies, the host mice were exposed to estradiol before transplant which may account for the expanded mammary hyperplasia. Our results demonstrate that prepubertal ovariectomy and/or the lack of ERα in Wnt-1 TG females prevents hyperplastic progression beyond the inguinal lymph node.

Wild-type male mammary ductal rudiments generally undergo regression in response to fetal testicular androgens on days 14–16 of gestation (45). The lack of ERα in male mammary cells did not appear to have an impact on this apoptotic process because ERKO male mice did not develop the mammary ductal rudiments seen in ERKO females. However, Wnt-1 TG males possessing or lacking the ERα gene retained a hyperplastic mammary rudiment indicating that male mammary regression was impaired and/or the rate of cellular proliferation was greater than apoptosis. Indeed, Wnt-1-induced hyperplasia was shown to be evident in mammary rudiments at day 18 of gestation (46).

ERKO/Wnt-1 TG females exhibit extensive mammary hyperplasia and likely possess the elevated concentration of serum estradiol associated with the female ERKO phenotype (47). Yet, the onset of tumors was delayed in ERKO/Wnt-1 TG mice compared to Wnt-1 TG females. Furthermore, ovariectomized Wnt-1 TG females possessed hyperplastic mammary glands and a tumor latency that was similar to intact ERKO/Wnt-1 TG mice. These data suggest that ERα may promote mammary tumor growth in a ligand (estradiol)-dependent manner, which is absent in ERKO/Wnt-1 TG females. The ERKO/Wnt-1 TG and ovariectomized Wnt-1 TG females possess a reduced number of mammary epithelial cells at risk of neoplastic transformation, which may also contribute to increased tumor latency compared to intact Wnt-1 TG females. In addition, ductal morphogenesis is absent in ERKO/Wnt-1 TG and ovariectomized Wnt-1 TG females. Dividing cap cells, which may be very susceptible to neoplastic conversion (11), are probably lacking in these mammary glands, as noted by the absence of TEBs. Therefore, defective ductal morphogenesis may also contribute to tumor latency.

Ovariectomized Wnt-1 TG mice developed tumors earlier than did ovariectomized ERKO/Wnt-1 TG mice. These results indicate that the presence of ERα in Wnt-1 TG mice may also promote tumorigenesis in the absence of estradiol, suggesting a role for ligand-independent activation of ERα. Growth factors acting through their tyrosine kinase receptors have been shown to indirectly activate ERα (48–52), and the mitogen-activated protein kinase pathway has been implicated in this process (53, 54). A Wnt-1-induced neoplastic environment may possess aberrant growth factor signaling that indirectly influences ERα signaling.

Prepubertal ovariectomy appeared to increase tumor latency in ERKO/Wnt-1 TG females, suggesting that ERα-negative mammary glands could still respond to estradiol. A recently identified second ER, termed ERβ (5), may mediate estrogen signaling in certain adult tissues. ERβ mRNA has been detected in rat mammary glands and human breast tissue, tumors, and immortalized mammary cancer cell lines using RT-PCR and RPA (8, 13–15). ERβ mRNA has also been detected in mouse mammary tissue by RT-PCR (55), but an RPA did...
not detect ERβ mRNA in mammary glands from wild-type or ERKO female mice (35). In our study, ERβ mRNA expression was not detected in Wnt-1-expressing mammary or tumor tissue of either sex. Therefore, ERβ is unlikely to mediate the apparent tumor-promoting effects of estradiol in Wnt-1 TG mammary glands. However, progesterone circulates at near normal levels in ERKO female mice (47) and could provide a growth stimulus to ERKO/Wnt-1 TG mammary glands that express the PR gene.

Unlike the female cohort, male mice expressing the Wnt-1 transgene possessed a similar degree of mammary hyperplasia regardless of Eα genotype, and there was little difference in the circulating sex steroid levels of wild-type and ERKO males (56, 57). Therefore, differences in mammary tumor latency among the Wnt-1 TG males could be attributed more directly to the promotional effects of Eα. An apparent Eα gene dosage effect on mammary tumor development was noted in TG Wnt-1 males, i.e., a decrease in Eα gene levels resulted in increased tumor latency. Wnt-1 TG males of each Eα genotype developed mammary tumors later than ERKO/Wnt-1 TG females despite similar levels of mammary hyperplasia. This difference in tumor onset may be due to the tumor-promoting effect of ovarian hormones and to other sex-specific factors that contribute to the susceptibility of female mammary epithelium to carcinogenesis.

Fig. 4. Tumor incidence in Wnt-1 TG mice with or without Eα. When mammary tumors of 0.5-cm diameter were found, mouse age was recorded and subjected to Kaplan-Meier analysis. The data were plotted as the probability of a mouse being tumor free versus its age in weeks. A, Wnt-1 TG females with the following Eα genotypes are indicated as follows: ●, wild-type, n = 40; ○, HET, n = 38; ■, ERKO, n = 26. Statistical comparisons were made of the following plots (significance in parentheses): wild-type versus HET (P = 0.957), wild-type versus ERKO (P = 0.001), and HET versus ERKO (P = 0.001). B, Wnt-1 TG males with the following Eα genotypes are indicated as follows: ●, wild-type, n = 45; ○, HET, n = 58; ■, ERKO, n = 25; ◊, female ERKO/Wnt-1 TG, n = 26. Statistical comparisons were made of the following plots (significance in parentheses): wild-type versus HET (P = 0.237), wild-type versus ERKO (P = 0.057), and HET versus ERKO (P = 0.352). Statistical comparisons of the female ERKO/Wnt-1 TG plot versus the following male Wnt-1 plots were made (significance in parentheses): wild-type (P = 0.354), HET (P = 0.002), and ERKO (P = 0.003).

Fig. 5. The effect of pregnancy and prepubertal ovariectomy on tumor onset in Wnt-1 TG females. Wnt-1 TG mice ovariectomized (Ovxed) on day 15 of age and parous Wnt-1 TG mice were monitored for tumors. The age at tumor onset was recorded, and the data were subjected to Kaplan-Meier analysis. The female mice are indicated as follows: ●, nulliparous Wnt-1 TG, n = 40; ○, parous Wnt-1 TG, n = 14; ■, ovariectomized Wnt-1 TG, n = 20; ◊, intact ERKO/Wnt-1 TG, n = 26. Statistical comparisons were made of the following plots (significance in parentheses): nulliparous wild-type versus parous wild-type (P = 0.759), nulliparous wild-type versus ovariectomized wild-type (P = 0.002), intact ERKO versus parous wild-type (P = 0.001), and intact ERKO versus ovariectomized wild-type (P = 0.441).

Fig. 6. Detection of Wnt-1 and keratin 18 mRNA transcripts in mammary tissue by RPA. The relative levels of Wnt-1 and keratin 18 mRNAs in mammary glands and tumors from female (A) and male (B) mice were analyzed. Each lane represents the analysis of 1 µg of total RNA isolated from inguinal mammary gland (Lanes M) and tumor (Lanes T) tissue from the same mouse, where applicable. Individual samples from two mice from each indicated genotype were analyzed. Full-length riboprobes are on the left, and the protected riboprobe fragments for Wnt-1, keratin 18 (K-18), and cyclophilin (Cyc) are indicated to the right (arrowheads). The protected cyclophilin probe fragments were used to normalize loading between lanes. Visible size markers (Lanes m) are 500 and 400 nt.
Differences in tumor latency between ERα-positive and negative mammary tissue were not due to altered levels of Wnt-1 transgene expression. Wnt-1 mRNA levels were dependent on mammary epithelial cell abundance, and neither ERα gene levels nor the sex of the mouse influenced Wnt-1 expression. Expression of the Wnt-1 transgene is dependent on the Wnt-1 promoter and the MMTV LTR enhancer, which provides epithelial cell-specific expression (23, 58). Our results are consistent with the fact that the MMTV LTR is not inducible by estrogen (59). Although the MMTV LTR is progestosterone inducible (59, 60), pregnancy did not appear to accelerate tumor onset in Wnt-1 TG females in this study.

The action of ovarian hormones, particularly estradiol, is strongly associated with the development of breast cancer (2, 61). Approximately 70% of primary breast tumors in women are ERα positive and exhibit estrogen-dependent growth (62). The most malignant mammary tumors, which are ERα negative and exhibit estrogen-indepen-
dent aggressive growth, are thought to progress from an ERα-positive state (11, 22). However, only 10–25% of human and mouse mammary epithelial cells possess ERα, raising the possibility that tumors could initiate directly from either ERα-positive or -negative epithelium (16, 17). ERα is also present in mammary stromal cells, which are thought to secrete growth factors that stimulate mitogenesis in the epithelial compartment (18, 19). Therefore, the ERKO/Wnt-1 TG mouse model has demonstrated that hyperplasia and tumorigenesis can be induced in mammary glands deficient in estrogen signaling. Furthermore, ERα-negative mammary tumors do not necessarily have to progress from an ERα-positive state but can be initiated directly in ERα-negative epithelium.

Finally, many other TG mice expressing different oncogenes de-
velop mammary tumors (36). On the basis of our mouse model, it may be useful to determine whether the absence of ERα has any impact on the development and growth of mammary tumors in these TG lines.

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cine at National Institute of Environmental Health Sciences, especially James Clark, Page Meyers, and Dr. Michele Hawk, is greatly appreciated.

REFERENCES


2. Pike, M. C., Spencer, D. V., Dahmoul, L., and Press, M. F. Estrogens, proges


11. Russo, I. J., and Russo, J. Mammalian mammary tissue were not due to altered levels of Wnt-1 17). ERα exhibit estrogen-dependent growth (62). The most malignant mam-
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