Lack of Ductal Development in the Absence of Functional Estrogen Receptor α Delays Mammary Tumor Formation Induced by Transgenic Expression of ErbB2/neu

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ABSTRACT

Expression of the mouse mammary tumor virus (MMTV) neu/neuB2 transgene in mice induces mammary tumors. To examine the effect of removing estrogen receptor α (ERα) signaling on the ability of an MMTV-neu/neuB2 transgene to induce mammary tumors, the neu transgene was expressed in the ERα knockout (oERKO) mouse, which lacks functional ERαs. MMTV-neu females that lacked ERα still developed mammary tumors; however, tumor onset was significantly delayed. This study indicates that ERα is not required for mammary tumor induction by overexpression of neu/neuB2, but plays a role in the rate of tumor onset. The removal of ovarian steroid by ovarioectomy in adults did not alter the onset rate. In contrast, prepubertal ovarioectomy, which arrested mammary epithelial development, significantly delayed onset. In addition, manipulations that increase progesterone also accelerate the tumor onset, indicating the slower onset in the oERKO is primarily attributable to the anovulatory phenotype resulting in lack of progesterone stimulation and a decreased abundance of target cells in the oERKO mammary gland.

INTRODUCTION

Estrogens are known to be important for the development, growth and function of the mammalian reproductive tract and mammary gland. The majority of estrogen effects are mediated via ERα2 and ERβ, which are hormone-inducible nuclear receptors. The ERs bind specific estrogen-responsive DNA sequences in target genes and recruit transcriptional coregulator molecules, which transduce the hormone signal to the transcriptional machinery. Hormonal manipulation and gene disruption studies have indicated the importance of estrogens in the proliferation of the developing mammary epithelium in response to ovarian hormone levels that rise during puberty and pregnancy and have, furthermore, shown that these processes are dependent on functional ERα. There is also a strong correlation between exposure to estrogenic compounds and carcinogenesis and tumor growth in the mammary gland (1, 2). Thus, ERα has been a target of both chemotherapeutic and chemopreventative therapies in breast cancer.

The oERKO mouse, which lacks functional ERαs as a result of targeted-gene disruption, displays a phenotype that definitively illustrates the importance of ERαs in mammary gland development (3–5). At birth, the mouse mammary gland contains a rudimentary ductal structure, which undergoes dramatic elongation and branching development in response to the rising levels of ovarian steroids at puberty. This mammary ductal morphogenesis does not occur in the oERKO despite the elevated levels of circulating E2 (5). Because mammary epithelial development is minimal in the oERKO, the question arises as to whether the expression of mammary tumor-inducing oncogenes could promote carcinogenesis in an estrogen-insensitive mammary structure. We have previously shown that expression of the MMTV-Wnt-1 transgene resulted in mammary tumor induction in the oERKO (6); however, there was a significant delay in tumor onset in comparison to WT females expressing the MMTV-Wnt-1 transgene. Therefore, functional ERα was not obligatory to the induction of MMTV-Wnt-1-induced mammary tumors, but contributed to the rate of tumor progression.

In this study, we have further investigated the role of ERα in mammary carcinogenesis using transgenic expression of the ErbB2 oncogene, also called neu, an EGFR-like protein (7) reported to be overexpressed in 20–30% of human breast tumors (8). There is no identified ligand required for erbB2 activation, but, rather, dimerization with other ligand-activated erbB family members stimulates erbB2 activity. Several transgenic mouse lines with overexpression of WT erbB2 or a constitutively active mutant neu targeted to the mammary epithelium have been generated, all of which exhibit an increased incidence of mammary tumors (9–34), compared with nontransgenic littersmates.

Our earlier study used MMTV-Wnt-1 transgenic mice, expressing Wnt-1, which encodes a diffusible factor that signals through the frizzled membrane receptor and enables β-catenin-modulated gene expression (35). In contrast, neu/neB2 initiates signaling via the EGF RTK pathway, which is strongly implicated in the development and growth of mammary cancers. In addition, RTK pathway activation has been shown, in some instances, to cross-talk with and activate ERα signaling (36). Because ERα is involved in mammary development, and its expression correlates with breast cancer growth, and the receptor is a potential target of neu-activated RTK through cross-talk, we were interested in studying the effect of removing ERαs from mice overexpressing the MMTV-neu transgene in the mammary gland. MMTV-Tg.NK mice were engineered to express a constitutively active neu, containing a mutation (9) in the transmembrane region of the receptor (37). These mice were bred with the oERKO mouse line to obtain mice that express the neu transgene in the absence of functional ERα. The rates of mammary tumor onset were compared with WT animals expressing the neu transgene to determine the effect of removing ERα in this mammary carcinogenesis model.

MATERIALS AND METHODS

Animals. All of the studies were carried out according to NIH guidelines for humane use of research animals and were approved by the NIEHS Animal Care and Use Committee. A colony of Transgenic MMTV-Tg.NK mice was established at NIEHS with breeders received from Dr. Philip Leder (Howard Hughes Medical Institute, Harvard Medical School, Boston, MA). Females heterozygous for the ERα gene (38) were bred to male MMTV-Tg.NK transgenic MMTV-neu animals. Tail biopsies were collected at wean-
ing (21 days of age), and offspring were screened for ERα genotype, as described previously (38, 39), and for the presence of the neu transgene using primers in the SV40 polyadenylation signal sequence of the transgene. Offspring carrying the neu transgene were grouped according to ERα genotype and housed until palpable tumors (~0.5–1.0 cm in diameter) appeared. At that time, mice were euthanized, and mammary tissue and tumors were collected for whole-mount or RNA analysis. Other tissues and blood samples were collected as needed for further analysis.

Mammary Gland Whole Mount. Inguinal mammary glands (numbers 4 and 5) were excised and laid flat on a clean glass slide. The tissue was then processed and stained as described in the “Biology of the Mammary Gland Biology.” Briefly, tissue was fixed for 1–3 h in a solution of 75% ethanol and 25% acetic acid and then transferred to 70% ethanol for 15 min, followed by a 5-min rinse in water. The tissue was then stained overnight in Carminic red alun solution, dehydrated in 70% ethanol (two times for 10 min), 95% ethanol (one time for 10 min, then for 1–4 h), 100% Ethanol (two times for 10 min), and cleared in xylene for 30–60 min. Tissue was then flattened and cover-slipped with Permount (Fisher Chemicals).

Serum Analysis. Whole blood was collected and allowed to clot on ice, then centrifuged at 8000 RPM for 15 min to separate serum. Analysis for progesterone or E2 was carried out using RIA kits (Ultraseensitive Estradiol and Active Progesterone; DSL, Webster, TX).

RPAs. Tissues were collected and snap-frozen in liquid nitrogen and stored at −70°C until extraction. Frozen tissues were first pulverized into powder, and RNA was isolated using Trizol Reagent according to the manufacturer’s protocol (Life Technologies, Inc., Rockville, MD). Antisense riboprobes were generated and radiolabeled using the MAXIscript kit (Ambion, Austin, TX) and [35S]CTP (Amersham, Arlington Heights, IL). RPAs were then carried out on 10 μg of mammary gland RNA or 5 μg of tumor RNA using the RPAIII kit (Ambion). Protected fragments were separated on 6% Tris-borate EDTA-urea acrylamide gels (Invitrogen, Carlsbad, CA), fixed in a solution of 10% acetic acid with 0.5% glycerol, and dried, using the Easy Freeze gel drier ( Hoefer Scientific). Gels were then analyzed by autoradiography and phosphorimaging and accompanying image analysis software (Storm 860 Phosphorimager and Image Quant software; Molecular Dynamics, Sunnyvale, CA). The template for the neu transgene antisense riboprobe was constructed by cloning the 324-bp amplifier generated from the SV40 polyadenylation signal genotyping primers (5′ primer: 5′-ggacaacacaaactagctgctc-3′; 324-bp amplifier) into PCRII TOPO plasmid (Invitrogen) using the TOPO TA kit (Invitrogen). Linearized template was prepared by digestion with XhoI, and antisense probes were synthesized using the Ambion Maxicsript kit (Ambion) with SP6 polymerase. This generated a 440-nucleotide (nt) riboprobe with a protected fragment of 324 nt. The riboprobe for CK18, an epithelial cell marker, was used to normalize for amount of epithelium as previously described (40), and is 186 nt with a protected probe for CK18, an epithelial cell marker, was used to normalize for amount of epithelium as previously described (40).

Ovariectomy, Pituitary Grafts, and Progesterone Pellet Implants. Ovaries were surgically removed from either prepubertal (18–20 days of age) or adult WT/neu (WT/neu) mice (4–5 months of age), which were then housed until palpable (0.5–1.0 mg) mammary tumors were present.

For pituitary grafts, pituitary glands from age-matched 10–12-week-old WT females lacking the neu transgene were grafted under the kidney capsule of host WT/neu or eERKO/neu females. These animals were then housed until palpable (0.5–1.0 mg) mammary tumors were present.

For progesterone treatments, four pellets (35 mg each, 90-day progesterone releasing), designed to release a total of 200 ng/ml progesterone in the serum, (Innovative Research, Sarasota FL) were implanted s.c. into 10–12-week-old WT/neu or eERKO/neu females. These animals were then housed until palpable (0.5–1.0 mg) mammary tumors were present.

Kaplan-Meier Analysis. The compiled data of time of tumor appearance were subjected to Kaplan-Meier analysis and plotted as a function of the probability of a mouse remaining tumor free versus its age in weeks. This analysis also takes into account the time at which a tumor-free mouse is removed from the study because of death from other causes. The various plots were compared with each other as described previously (6) to determine whether the rate of tumor formation was statistically different among the different groups analyzed.

RESULTS

MMTV-neu Induces Mammary Tumors in eERKO Females. Female mice of all three ERα genotypes carrying the MMTV-neu transgene (WT/neu, ERα+/−/neu, eERKO/neu) were housed until palpable tumors were detected. The proportion of tumor-free animals over the course of the study was analyzed using a Kaplan-Meier plot (Fig. 1). All of the MMTV-neu mice developed palpable tumors at an incidence that approached 100%; however, the time-to-tumor onset was significantly delayed in eERKO/neu mice expressing the MMTV-neu gene compared with WT/neu and ERα+/−/− mice. Whereas WT/neu (n = 41) and ERα+/−/− mice mice (n = 53) exhibited a 50% incidence of tumors at 52 weeks of age, the population of eERKO/neu mice (n = 26) required 105 weeks of age to exhibit 50% incidence. These data, therefore, indicated that ERα is not required for the induction of erbB2/neu related mammary tumors, but plays a role in the rate of onset of neu-induced tumors. The rate of tumor onset in the WT/neu mice was identical to that in the ERα+/−/− mice, indicating that the loss of one ERα allele does not alter the tumor onset. For this reason, for the remaining studies, data from WT/neu and ERα+/−/− mice were pooled and treated as a single experimental WT/neu group.

Interestingly, the 50%-tumor-incidence at 52 weeks of age exhibited by the cohort of WT/neu mice generated in this study represents a dramatic delay compared with the incidence in the original parent colony (MMTV-Tg,NK), reported as reaching 50% incidence by 25 weeks of age (41). This disparity between the two WT/neu colonies may be attributable to differences in background strains, because the original Tg,NK line was carried on a FVBN strain, whereas the eERKO colony is on a C57BL/6 strain, a strain reported to be more resistant to multiple types of tumors (42–44).

Representative whole mounts of mammary glands from all three ERα genotypes carrying the neu transgene are shown in Fig. 2. As described previously (5, 45), the eERKO mammary gland duct failed to develop beyond the prepubertal stage and consisted of only a rudimentary ductal structure at the nipple. Before tumor onset, the appearance of the mammary glands from WT/neu and eERKO/neu transgenic mice shown in Fig. 2 was comparable with those previously described for WT and eERKO animals, indicating expression of

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MAMMTV-neu transgene did not alter the overall structure of the ducts, as reported previously (9). After tumor formation, however, hypoplastic nodes foci could be found in some glands (Fig. 2, arrow in the ERα+/−/neu panel). Transgene Expression in WT/neu and αERKO/neu Mammary Glands and Tumors. To determine whether ERα disruption altered neu transgene levels, RPAs were carried out to compare expression of the neu transgene in mammary glands or tumors, in each of the experimental groups. To account for differences in ductal development, all of the assays for the neu transgene were normalized to the expression of CK18, an epithelial cell marker. As reported previously (9), the transgene expression increases with age, with little or no expression detected in WT/neu animals at 2 months of age, but becoming detectable in two of the four samples by 3 months (Fig. 3). A limited number of samples from tumor-free WT/neu and αERKO/neu mammary glands of mice at ages ranging from 5 to 10 months were analyzed for neu transgene expression. No pattern of transgene induction was revealed in these samples (not shown), but much variation was apparent that was not correlated to age or genotype. This lack of a uniform pattern of neu transgene expression was previously observed in the parent Tg.NK line (9); however, expression was correlated with the appearance of dysplasia and tumors (9). Likewise, the neu transgene level was found to be elevated in samples from WT/neu mammary glands that were collected after tumor onset (Fig. 3). In addition, transgene expression in RNA prepared from tumors was higher than that of mammary tissue of the same animals (Fig. 3). Overall, transgene expression in the αERKO/neu was reduced compared with WT/neu, but a high degree of variation among individual αERKO/neu mice negated any significant differences (Fig. 3B). However, neu transgene mRNA was significantly decreased in the αERKO compared with the WT/neu when the level was normalized to cyc (not shown), which reflects the expression in the whole mammary gland, rather than the epithelial-specific expression reflected by normalization to CK18.

Effect of Hormonal Milieu on MMTV-neu-induced Tumor Incidence. There are several factors not directly related to functional ERα expression that may contribute to the delayed onset in neu-induced mammary tumors observed in the αERKO mice. First, the expression of the MMTV/neu transgene is positively regulated by progesterone (46). Because αERKO females are anovulatory and lack the cyclical postovulatory progesterone surge (5, 38, 47), it is conceivable that expression of the transgene in the αERKO female may be reduced. Secondly, the MMTV/neu transgene expression is targeted to the mammary epithelium, which is significantly reduced in volume in the underdeveloped gland of the αERKO. Therefore, these differences in hormonal milieu and mammary development between the WT/neu and αERKO/neu females make direct comparisons difficult. To overcome these differences, we undertook a series of experiments aimed at: (a) producing an underdeveloped mammary gland in the WT/neu mice that is comparable with that of the αERKO; and (b) manipulating the progesterone levels in WT/neu mice to mimic those of the αERKO.

The first of these approaches involved ovariectomy of WT/neu mice at 18–20 days of age, thereby stunting proper pubertal development of the mammary duct. Therefore, the WT/neu gland remains underdeveloped in an environment lacking progesterone, which is similar to that of the αERKO.

As expected, the mammary gland of the ovariectomized WT/neu mice remained immature and consisted of only a rudimentary ductal structure, appearing very similar to the αERKO (Fig. 4A). Prepubertal mammary whole mounts of MMTV-neu+/− mice. Mammary glands were collected after tumor onset and stained as described in the “Materials and Methods” section. The arrow in the ERα+/−/neu panel indicates an epithelial lesion. The arrow in the αERKO/neu panel indicates the ductal rudiment. Ovex, ovariectomized.

Fig. 2. Mammary whole mounts of MMTV-neu+/− mice. Mammary glands were collected after tumor onset and stained as described in the “Materials and Methods” section. The arrow in the ERα+/−/neu panel indicates an epithelial lesion. The arrow in the αERKO/neu panel indicates the ductal rudiment. Ovex, ovariectomized.

Fig. 3. RNase protection analysis for MMTV-neu transgene expression. RNA isolated from WT/neu, ERα+/−/neu, or αERKO/neu mammary glands (MG) or tumors was analyzed by RPA for expression of MMTV-neu transgene. Signal was normalized to CK18, an epithelial cell marker, to control for the amount of epithelial tissue. A, representative RPA of mammary gland tissue from tumor-free WT/neu females ages 2 (2 mo) or 3 (3 mo) months, or mammary gland (top) or tumor (bottom) tissues collected after tumor onset from WT/neu (+/+), ERα+/−/−/− (−/−), or αERKO/neu (−/−). WT/neu mice that were ovariectomized as adults (ov), or multiparous breeders (mp). The protected riboprobe for the MMTV-neu transgene and CK18, to normalize for epithelial tissue, are indicated. The undigested riboprobes (RP) are indicated. B, RPAs were quantified, and the neu signal was normalized to CK18 from mammary gland (MG) or tumors. Bars, data from four to six samples.
ovariectomy did result in an observable delay in mammary tumor onset in the WT/neu mice (50% incidence by 62 weeks for WT/neu with prepubertal ovariectomy (n = 24) compared with 52 weeks for WT/neu unmanipulated). However, this delay was not to the same extent as in the αERKO (50% incidence by 105 weeks; compare Figs. 1 and 5). Furthermore, treating prepubertally ovariectomized WT/neu females with progesterone, in the form of a slow-release pellet, returned the time-to-tumor onset to that of the intact WT/neu female rate [40% by 52 weeks (n = 6); Fig. 5]. These results indicated that the volume of mammary epithelium in the precancerous gland did not alter tumor onset to an extent that might fully explain the delay in tumor onset observed in the αERKO/neu females. Thus, the lack of ERα is, at least in part, responsible for the slower onset in the αERKO/neu.

In a second set of experiments, progesterone levels were manipulated in WT/neu mice to mimic those of the αERKO. This involved ovariectomy in mice of 16–20 weeks of age; thereby removing production of ovarian progesterone from WT/neu females in which the mammary glands were fully developed. Although whole tissue mounts showed the mammary ductal structure was sparser in WT/neu mice after ovariectomy (Fig. 2), there was no significant effect on the time-to-tumor appearance for this population [50% incidence by 48 weeks (n = 19)]. These data indicate that the removal of ovarian steroids in adult WT/neu animals had no effect on the rate of tumor formation or growth. Because the mice were ovariectomized at 16–20 weeks of age, the neu transgene may have initiated tumor progression before the removal of ovarian steroids. Indeed, analysis of neu transgene expression before the appearance of tumors indicated that the transgene mRNA level was beginning to rise in some animals by 12 weeks of age (Fig. 3). When measured after tumor onset, transgene expression in mammary gland or tumor RNA samples was not significantly affected by ovariectomy (Fig. 3).

**Increasing Progesterone Levels Accelerates Tumor Onset in WT/neu and αERKO/neu Mice.** To directly examine the promotional effect of progesterone exposure on mammary tumor incidence, three experimental approaches were used: (a) time-to-tumor onset was determined in multiparous WT/neu female breeders; (b) WT/neu and αERKO/neu mice were implanted with 90-day release progesterone pellets; and (c) WT/neu and αERKO/neu mice received a pituitary xenograft, thereby increasing prolactin-induced luteal functions in the ovary.

As shown in Fig. 6, the time-to-tumor formation in multiparous WT/neu breeders was significantly decreased [50% incidence by 32 weeks for WT/neu multiparous WT/neu (n = 15) versus 52 weeks for virgin WT/neu (n = 94)], which suggested that the hormonal milieu unique to pregnancy has a promotional effect on the neu-induced tumors. This is likely caused by the elevated progesterone and associated mammary epithelial proliferation that occurs during each pregnancy. Transgene expression was elevated in a mammary gland RNA sample taken from a tumor-free multiparous WT/neu female (neu signal 45% CK18 signal, not shown) compared with an age-matched virgin WT/neu female (neu signal, 6.7% CK18, not shown), which indicated that pregnancy can accelerate transgene expression. When measured after tumor onset, transgene expression in mammary gland and tumor RNA samples was slightly increased in multiparous animals (Figs. 3).

A second set of experiments was aimed at determining whether increasing the progesterone level and/or the amount of mammary...
epithelium would accelerate tumor onset. The first of two experimental approaches consisted of implanting 90-day progesterone-releasing pellets in 10–12-week-old WT/neu or αERKO/neu mice. This treatment resulted in a significant elevation in circulating progesterone (~200 ng/ml) for the first 90 days after implantation. A comparison of the tumor-onset rates for unmanipulated WT/neu or αERKO/neu and those receiving a progesterone pellet is shown in Fig. 7. Progesterone treatment dramatically accelerated the tumor onset in WT/neu animals [50% incidence by 37 weeks for WT/neu with progesterone (n = 6) versus 52 weeks for WT/neu unmanipulated]. The onset rate in this progesterone-treated group was not altered by ovariectomy at the time of pellet implant [50% incidence by 40 weeks for WT/neu with progesterone and ovariectomy (n = 5)], which indicated that progesterone is the principle ovarian hormone necessary for the decreased tumor-onset time in adult WT/neu females. Interestingly, tumor onset was also accelerated in the αERKO/neu mice receiving progesterone treatment when compared with untreated αERKO/neu adults [50% incidence by 70 weeks for αERKO/neu with progesterone (n = 8) compared with 105 weeks for αERKO/neu unmanipulated] but was still delayed compared with progesterone-treated WT/neu mice. Transgene expression was not increased in mammary gland RNA samples from tumor-free WT/neu or αERKO/neu females collected 20 days after progesterone pellet implantation, which indicated that the treatment did not directly induce MMTV-neu (not shown). Mammary gland RNA samples collected after tumor onset did indicate transgene induction, but the expression was not altered compared with untreated animals with tumors (not shown).

A second approach to increase progesterone exposure was to transplant an exogenous pituitary under the kidney capsule of mice in each of the experimental groups studied. Such manipulation has been used to increase serum prolactin, thereby inducing the luteal functions of the ovary and ultimately resulting in elevated progesterone levels in the recipient mouse (4, 48). We have previously used this approach to demonstrate that a pituitary xenograft combined with the elevated estradiol in the αERKO was able to stimulate mammary duct development (4). Therefore, it was also expected that this procedure would induce a more developed ductal structure in the αERKO mammary gland, which would be comparable with a WT/neu mammary gland and result in an increased volume of epithelial cells capable of transgene expression.

Pituitaries from WT donors were grafted into WT/neu or αERKO/neu recipients. Tumor onset was significantly accelerated in both the WT/neu and the αERKO/neu populations receiving a pituitary graft (Fig. 8). Tumors occurred in both WT/neu and αERKO/neu females with pituitary xenografts with 50% incidence in about 40 weeks (WT/neu n = 16; αERKO/neu n = 22), compared with 52 weeks for unmanipulated WT/neu and 105 weeks for unmanipulated αERKO (Fig. 1). Surprisingly, mammary gland whole mounts (Fig. 9, A and B) showed that pituitary grafts did not result in the expected ductal elongation in the αERKO, although the density and complexity of the epithelial tissue did increase because of the increased side branching and alveolar development. This was not attributable to neu expression, as reported in another model (49), because αERKO littermates from this colony that did not carry MMTV-neu transgene also did not respond with ductal growth (Fig. 9C). The pituitaries were implanted at 10–12 weeks of age and were in place until tumor onset (50% at 40 weeks, 28–30 weeks after implantation), which is longer than the 45 days in the previous study (4). The success of the transplant was confirmed by observing: (a) the increased mammary ductal density (Fig. 9); (b) increased serum progesterone levels in pituitary graft recipients (Table 1); (c) luteinized ovarian follicles in pituitary graft recipients (not shown); (d) increased expression of CK18 epithelial
cell marker mRNA (not shown), which indicated an increased population of epithelial cells. Because the pituitary graft did not result in a dramatic increase in the volume of ductal tissue in the αERKO/neu, it is likely that the increase in serum progesterone resulting from the transplant is an important component involved in the more rapid tumor onset. Transgene expression was not increased in RNA samples from mammary glands of a tumor-free WT/neu female and a tumor-free αERKO/neu female that received pituitary transplants (not shown). Mammary gland RNA samples that were collected after tumor onset did indicate transgene induction, but the expression was not altered compared with nontransplanted animals with tumors (not shown).

**DISCUSSION**

This study examined the effect of abrogated ERα signaling on the ability of a MMTV-neu/erbB2 transgene to induce mammary tumors. The mammary tumor onset was significantly delayed in the αERKO/neu mice as compared with the WT/neu mice (see Fig. 1), which indicated that, although ERα is not required for the induction of these tumors, ERα disruption alters the rate of onset and/or promotion.

Several experiments were undertaken to determine whether increasing the progesterone level and/or the amount of mammary epithelium would accelerate tumor onset. This is of interest in light of the association between increased mammary duct tissue density and increased breast cancer risk in women (50, 51). When the progesterone level in the αERKO/neu mice was increased, either by treatment with progesterone-release pellets or by pituitary graft, the mammary tumor-onset rate equaled or exceeded that of unmanipulated WT/neu mice (Figs. 7 and 8), despite the low content of epithelial tissue in αERKO/neu relative to WT/neu. Similarly, when WT/neu mice were exposed to elevated progesterone during pregnancy, by direct treatment with progesterone-releasing pellets or by stimulation of the ovaries with pituitary grafts, the onset rate was accelerated (Figs. 6, 7, and 8). When the transgene level in the animals that were treated with progesterone or that received pituitary transplants were measured after tumor onset, they were not significantly elevated compared with untreated animals (Fig. 3C).

Additional studies on a small number of samples from tumor-free females of various ages seemed to indicate that these treatments do not result in more rapid transgene induction (not shown). However, the ability of progesterone to accelerate tumor onset in WT/neu and αERKO/neu mice indicates that the underlying phenotypes of the αERKO (i.e., the lack of postovulatory progesterone and low volume of mammary epithelial cells in which to express transgene) contribute significantly to delayed tumor onset in the αERKO/neu. The more rapid onset after progesterone elevation may reflect progesterone’s role in pregnancy-associated ductal proliferation and lobuloalveolar development (52), as a possible component of tumor progression, rather than direct transgene induction. In addition, although progesterone signaling does not alter neu transgene expression, progesterone may have a role in neu/erbB2 signaling and activity.

The initial aim of this study was to explore the role of estrogen action in MMTV-neu-induced mammary tumors by removing ERα signaling. One role of estrogen in breast tumor growth is induction of growth factors in the stroma that stimulate growth factor RTK signaling in the epithelial cells. Although there was a delay in tumor onset in the αERKO/neu mice, there is a 100% tumor incidence, even in the absence of ERα. Thus, it is clear that ERα is not necessary for tumor induction in the neu/erbB2 model used here. Similarly, ovariectomized WT/neu mice also develop tumors, which indicates that neither E2 nor other ovarian hormones are necessary for mammary tumorigenesis in this transgenic mouse. Overexpression of the neu transgene is sufficient to activate growth factor-receptor signaling necessary for tumor induction in this model, independent of ERα activation or induction of ERα-regulated genes.

Interestingly, studies using MMTV-Wnt-1 transgenic animals (6) had a similar outcome in that tumor onset was also delayed in αERKO/Wnt-1 females, and the lack of ERα did not prevent tumorigenesis. Unlike the studies with the neu/erbB2 transgenics, multi-parous Wnt-1 females did not display accelerated tumor onset, although prepubertal ovariectomy did cause delay in onset. The different modes of activity and regulation of expression of the transgenes in these models may be responsible for some of the differences observed.
in these studies. Whereas, the MMTV-neu transgene encodes a membrane-bound receptor, the MMTV-Wnt-1 transgene produces a diffusible signaling molecule with activity that is not limited to cells in which it is expressed. Although expression of both of these transgenes is limited to the mammary epithelium, the neu transgene effects are limited to these same cells, whereas those of the Wnt-1 transgene can be communicated to other cells expressing the frizzled receptor.

The effect of removing ERα signaling on mammary tumor induction has also been studied in transgenic mice that express large T antigens in developing mammary duct cells. However, when this transgene was introduced into the oERKO females, it was not expressed, presumably because of the lack of ductal development in the oERKO; hence, tumors did not occur (53).

A recent study has shown that cyclin D1-ablated (CycD1−/−) mice that also express the MMTV-Wnt-1 transgene develop mammary tumors, whereas MMTV-neu Cyclin D1−/− mice do not (54), illustrating a distinction between these two transgenic lines in the pathways required for tumorigenesis. In both MMTV-Wnt-1 and MMTV-neu tumors, Cyclin D1 was up-regulated (31, 54); however, in the case of MMTV-neu mice that also express the MMTV-neu transgene, no increase in ductal density is associated with an increase in breast cancer susceptibility to tumor induction. If ERα does play such a role in mammary breast cancer in humans by increasing the population of cells that are susceptible to tumor induction (i.e., actively dividing cap cells that may be decreased in number in the oERKO) or that express molecules responsible for tumor induction. If ERα does play such a role in human breast cancer, one might predict that ERα antagonists may delay tumor onset; however, the identification of drug compounds that abrogate the function of signaling molecules directly involved in breast tumorigenesis may prevent, rather than merely delay, tumorigenesis effectively.

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REFERENCES


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