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Mammary tumor suppression by transforming growth factor β1 transgene expression

(transgenic mice/mouse mammary tumor virus/breast cancer)

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ABSTRACT **In cell culture, type α transforming growth factor (TGF-α) stimulates epithelial cell growth, whereas TGF-β1 overrides this stimulatory effect and is growth inhibitory. Transgenic mice that overexpress TGF-α under control of the mouse mammary tumor virus (MMTV) promoter/enhancer exhibit mammary ductal hyperplasia and stochastic development of mammary carcinomas, a process that can be accelerated by administration of the chemical carcinogen 7,12-dimethylbenz[*a*]anthracene. MMTV-TGF-β1 transgenic mice display mammary ductal hypoplasia and do not develop mammary tumors. We report that in crossbreeding experiments involving the production of mice carrying both the MMTV-TGF-β1 and MMTV-TGF-α transgenes, there is marked suppression of mammary tumor formation and that MMTV-TGF-β1 transgenic mice are resistant to 7,12-dimethylbenz[*a*]anthracene-induced mammary tumor formation. These data demonstrate that overexpression of TGF-β1 *in vivo* can markedly suppress mammary tumor development.**

The type β transforming growth factors (TGF-β) are potent inhibitors of proliferation of most cell types in culture and *in vivo* (1). TGF-β1, TGF-β2, and TGF-β3 are all secreted in a latent form (2) and show different patterns of expression in the mouse mammary gland (3), suggesting that these peptides play an important role in mammary gland development. Direct delivery of exogenous TGF-β1, TGF-β2, or TGF-β3 into mammary glands inhibits proliferation of end bud cells and ductal elongation (3, 4); however, their administration does not inhibit proliferation of alveolar cells during pregnancy (5). Expression of a constitutively active mutant TGF-β1 protein in transgenic mice under the control of the mouse mammary tumor virus (MMTV) promoter/enhancer results in hypoplasia of mammary ductal epithelium but does not inhibit alveolar growth during pregnancy (6). Moreover, no mammary carcinomas have been observed in these MMTV-TGF-β1 transgenic mice.

In contrast to the TGF-βs, TGF-α is a mitogen for most cell types (7) and may also play a significant role in mammary gland development. TGF-α is localized *in vivo* in the epithelium of the advancing terminal buds and in stromal fibroblasts at the base of terminal buds (8). Furthermore, implantation of pellets containing TGF-α into regressed mammary glands of ovariectomized mice stimulates the reappearance of end buds (8). MMTV-TGF-α transgenic mice exhibit enhanced production of TGF-α in the smaller ducts of the mammary gland (9). These mice exhibit mammary epithelial hyperplasia with a marked increase in the rate of benign and malignant mammary tumor development (9, 10). In addition, administration of the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) results in a dramatic acceleration of mammary tumors in the TGF-α transgenic mice compared to their nontransgenic littermates (11).

Because TGF-β1 growth inhibitory effects are dominant over the growth stimulatory effects of TGF-α in cultured epithelial cells (12), we hypothesized that expression of the TGF-β1 transgene would suppress the phenotype of MMTV-TGF-α mice, including mammary tumor development. To test this hypothesis, crossbreeding experiments were performed. Animals carrying both the MMTV-TGF-α and MMTV-TGF-β1 transgenes developed no tumors, whereas mammary tumors were detected by 17 months of age in 53% of those expressing only the TGF-α transgene. Furthermore, MMTV-TGF-β1 transgenic mice were found to be resistant to DMBA-induced mammary tumor formation. These findings demonstrate that TGF-β1 can suppress mammary tumor formation at least in its early stages.

MATERIALS AND METHODS

Transgene Detection. Transgenic mice were identified by Southern blot analysis with a probe made from either mouse TGF-β1 or an *Eco*RI/*Xho* I fragment of the transgene construct containing 526 bp of rabbit β-globin exon 3 sequence as described (6). The MMTV-TGF-α transgene was detected by PCR as described (9).

Transgenic Mice. The MMTV-TGF-α and MMTV-TGF-β1 transgenic animals were generated in a (C57BL × DBA/2)F_1 ($B6D2_{F1}$) background, which has a low frequency of spontaneous mammary tumor formation (6, 9–11). Wild-type animals used in various experiments were from the same genetic background. For the crossbreeding experiments, the line 29 MMTV-TGF-α transgenic mouse line was selected (9, 10). The line 29 females cannot lactate, have a high rate of mammary epithelial cell proliferation, and have a high frequency of development of mammary gland tumors with onset earlier in multiparous than virgin animals (9, 10). Line 46.11 of the MMTV-TGF-β1 transgenic animals was used. Female offspring from this line can lactate and feed full litters of pups repeatedly (6). They do, however, show pronounced ductal hypoplasia and no mammary tumors have been observed. Male MMTV-TGF-α were mated to female MMTV-TGF-β1 mice, and the female offspring were assayed for the inheritance of both transgenes by Southern blot analysis and PCR amplification (6, 9); expression of both transgenes was confirmed by Northern blot analysis. Female mice expressing both transgenes (MMTV-TGF-α/β1 mice) were mated twice to wild-type males and produced two litters. Twenty-one MMTV-

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Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; MMTV, mouse mammary tumor virus; TGF, transforming growth factor; EGF, epidermal growth factor.

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TGF-α/β1 female mice that successfully passed both transgenes on to progeny were selected for the study. These 21 mice having both transgenes integrated along with 30 MMTV-TGF-α, 30 MMTV-TGF-β1, and 30 wild-type $B6D2_{F1}$ female mice that had each produced two litters of pups were used in the study.

For studies on DMBA induction of mammary tumors, 21 virgin wild-type $B6D2_{F1}$ and 19 virgin MMTV-TGF-β1 female mice were given DMBA by orogastric lavage as described (11). The dose regimen was 1 mg/kg per week for 6 weeks beginning at 8 weeks of age.

Mice for tumor formation experiments were housed in the animal care facility without males, kept on 12-hr light/dark cycles, and fed standard chow and water ad libitum. All mice were carefully examined by palpation for tumors twice weekly for 17 months. Mammary tumors were typically removed 3 weeks after detection; tumors were permitted to grow to a maximum diameter of 1 cm, then the mice were sacrificed by intraperitoneal administration of Ketaset (ketamine hydrochloride) and halothane inhalation, and complete autopsies were performed to determine pathological changes in all mammary glands and to characterize metastases, if present. Tissues were fixed in 4% formaldehyde and sections were stained with hematoxylin and eosin for histological diagnosis.

Transgene Expression. Northern blot analysis of total RNA from mammary glands was carried out as described for TGF-α (9) or TGF-β1 (6) transgene expression. RNase protection assays were carried out according to Melton *et al.* (13) with minor modifications. Briefly, 10 μg of total RNA was added to 5×10^6 cpm of ^{32}P-labeled RNA probes for the TGF-β1 and the TGF-α transgene as well as endogenous TGF-β1 gene and then hybridized overnight at 50°C. RNA hybrids were analyzed on 8% acrylamide/urea gels. For the transgenic TGF-β1 RNA probe, a 386-bp fragment from the 3′ end of the simian cDNA (14) was subcloned into the *Bam*HI and *Pst* I sites of pBluescript KSII+. Similarly, an endogenous TGF-β1 RNA probe was made by subcloning a 425-bp fragment from the 5′ end of the murine TGF-β1 cDNA (15) into the *Sma* I and *Eco*RI sites of pBluescript KSII+. No cross-reactivity with endogenous TGF-β1 was observed with the transgenic probe (6). A 560-bp fragment from the 3′ end of the human TGF-α cDNA was subcloned into pGEM7Z and was used to probe for the TGF-α transgene transcript. Glyceraldehyde-3-phosphate dehydrogenase and cyclophilin, putative constitutively expressed genes, were used as controls for loading and transfer. RIA on serum or mammary gland extracts was performed as described (10) using ^{125}I-labeled recombinant TGF-α.

Morphological Assessment of the Mammary Glands. The third thoracic mammary glands were resected and fixed en bloc and processed for whole mounts as described (4).

RESULTS

Transgene Effects on Mammary Gland Morphology. To determine the effect of expression of the different transgenes on mammary ductal structure, three multiparous female mice from each group (not included in the tumor formation study group) were sacrificed at 6 months of age and the third thoracic mammary gland was removed for whole mount analysis of ductal morphology (Fig. 1). The mammary ductal tree from the multiparous MMTV-TGF-β1 mice (Fig. 1*b*) had a less complex structure than that from wild-type mice (Fig. 1*a*), an observation similar to that made in 7- and 13-week-old virgin mice (6). The mammary glands from MMTV-TGF-α mice showed the expected hyperplastic changes (Fig. 1*c*) (9). The TGF-α transgene-induced hyperplasia was markedly suppressed in glands from MMTV-TGF-α/β1 mice, although widely scattered hyperplastic alveolar outgrowths remained, indicating that expression of the TGF-β1 transgene did not completely suppress the effects of the TGF-α transgene.

Transgene Effects on Mammary Tumor Formation. Solid mammary tumors appeared in 53% of the MMTV-TGF-α mice by 17 months of age (Fig. 2*a*). Consistent with previous studies of line 29 mice (10), 12 of 30 animals (40%) had mammary adenocarcinomas by this time (Fig. 2*b*). Necrotic tumors in which a histological diagnosis of carcinoma was not verified occurred in an additional three animals. Since these necrotic tumors were probably carcinomas, the estimated carcinoma incidence would increase to 50% in the MMTV-TGF-α mice. Mammary carcinomas appeared in more than one mammary gland in five animals, probably representing multiple primaries since metastasis from one mammary gland to another is unlikely. No distant metastases were observed in any MMTV-TGF-α mice with adenocarcinomas. Mammary tumors other than carcinomas appearing in the MMTV-TGF-α mice included eight solid adenomas, three cystic ade-

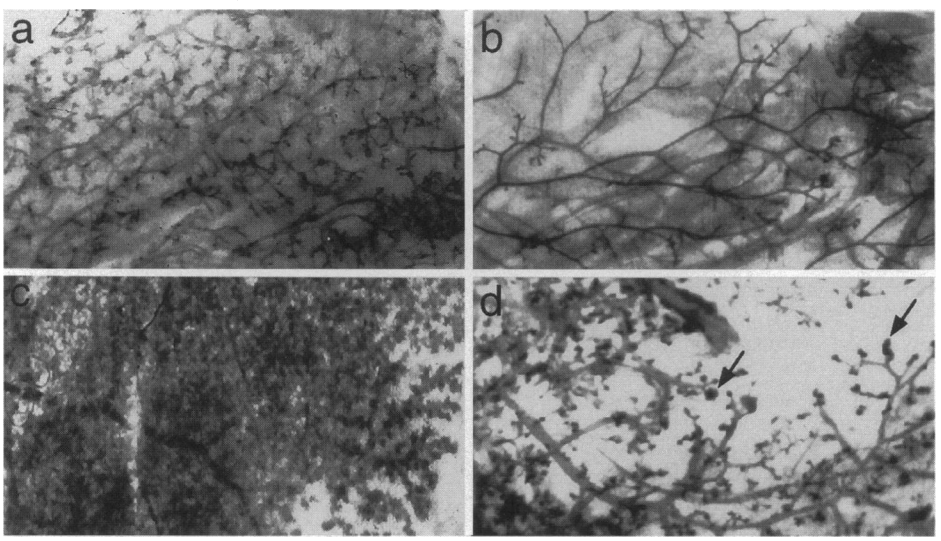

FIG. 1. Ductal morphology of the third thoracic gland from multiparous 6-month-old female mice that mothered two litters caged without pups or males for at least 1 month. (*a*) Wild-type mammary gland reveals a normal postinvolutional ductal tree. (*b*) MMTV-TGF-β1 glands appear hypoplastic compared with wild-type glands. (*c*) MMTV-TGF-α mammary gland exhibits lobuloalveolar hyperplasia. (*d*) TGF-α-induced hyperplasia is markedly suppressed in glands from MMTV-TGF-α/β1 mice; however, widely scattered hyperplastic outgrowths (arrows) were apparent, indicating that expression of the TGF-β1 transgene did not completely suppress the effects of the TGF-α transgene.

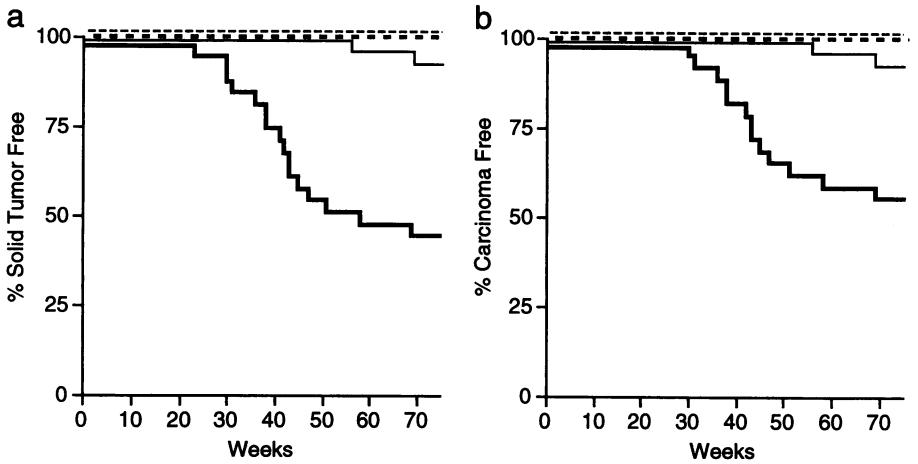

FIG. 2. Solid mammary tumor-free (*a*) and mammary carcinoma-free (*b*) survival of wild-type (thin solid line) (n = 30), MMTV-TGF-β1 (thin broken line) (n = 30), MMTV-TGF-α (thick solid line) (n = 30), and MMTV-TGF-α/β1 (thick broken line) (n = 21) multiparous mice.

nomas, one adenomyoepithelioma, and one lymphoma with liver involvement (Table 1). Some of these tumors occurred in mice that had carcinomas in other mammary glands.

TGF-β1 expression inhibited mammary tumor formation in mice carrying both transgenes; no carcinomas or other types of solid tumors developed in the MMTV-TGF-α/β1 mice (0/21). Benign cysts developed in 2/30 wild-type, 0/30 MMTV-TGF-β1, 19/30 MMTV-TGF-α, and 3/21 MMTV-TGF-α/β1 mice during the 17-month observation period. Two wild-type mice developed mammary carcinomas by 17 months of age, whereas the MMTV-TGF-β1 mice had no solid tumors (0/30). Thus, the rate of tumor development in wild-type mice was not sufficient to determine with statistical significance whether the TGF-β1 transgene suppresses spontaneous tumor formation, even though no tumors were observed in the MMTV-TGF-β1 mice (0/30).

Lack of MMTV-TGF-β1 Effects on TGF-α Expression and Epidermal Growth Factor (EGF) Receptor Expression. To determine whether the marked tumor suppressive effects of the TGF-β1 transgene in the MMTV-TGF-α/β1 mice were due to attenuated expression of the TGF-α transgene, Northern blot and RNase protection assays were performed. Fig. 3 illustrates Northern blot analysis of total RNA from mammary glands of four animals from each group. Quantitation with a PhosphorImager of the TGF-α transgene-specific transcript relative to cyclophilin, a constitutively expressed gene (6), showed no significant difference in TGF-α transgene expression in the two groups. The mean ± SD for expression of the TGF-α transgene relative to cyclophilin in MMTV-TGF-α mice was 11.2 ± 3.3, while that for MMTV-TGF-α/β1 mice was 15.9 ± 11. RNase protection assays gave similar results for the TGF-α transgene in the MMTV-TGF-α and MMTV-TGF-α/β1 groups and confirmed expression of the TGF-β1 transgene in the MMTV-TGF-β1 and MMTV-TGF-α/β1 mice (data not shown).

To estimate TGF-α protein in mammary glands of MMTV-TGF-α and MMTV-TGF-α/β1 mice, glands from four 16-week-old animals were obtained from each group and the tissue was processed for RIA. A wide variation was observed in the TGF-α content of glands from MMTV-TGF-α mice, consistent with a previous report (10), and also in the MMTV-TGF-α/β glands. The four MMTV-TGF-α mice showed a mean ± SD of 6050 ± 4390 pg of TGF-α per μg of DNA, while the four animals harboring both MMTV-TGF-α and MMTV-TGF-β1 transgenes gave 4075 ± 1860. Similar assays on 9- and 12-week-old female mice also showed no significant difference between the two groups (data not shown). Another possible mechanism for the TGF-β1 tumor suppressive effects would be inhibition of expression of the receptor for TGF-α, the EGF

Table 1. Histological diagnoses of mammary neoplasms arising in MMTV-TGF-α mice

Mouse	Age tumor noted, days	Diagnosis
α30	185	Lymphoma*
α38	207	Adenocarcinoma (medium grade)
α50	314	Adenocarcinoma (medium grade)
α60	263	Adenocarcinoma (medium grade)
α65	205	Adenoma
α79	213	Adenocarcinoma (medium grade)
α92	328	Adenocarcinoma (medium grade)
α93	161	Cystic adenosis
α94	401	Adenomyoepithelioma†
α104	266	Adenocarcinoma (high grade)
α110	298	Adenocarcinoma (low grade)
α116	388	Adenocarcinoma (low grade)
α121	477	Adenocarcinoma (low grade)
α131	249	Adenocarcinoma (medium grade)
α132	354	Adenocarcinoma (medium grade)
α134	301	Adenocarcinoma (low grade)
α137	287	Cystic adenoma

*Not included in Fig. 1, which plots occurrence of mammary gland epithelial tumors only.
†Judged to be a low-grade adenocarcinoma because of large size and high degree of cellularity.

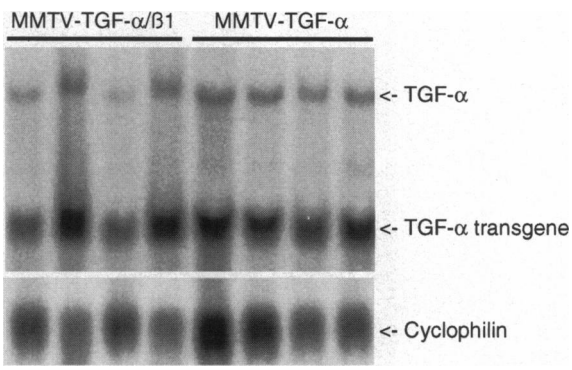

FIG. 3. Northern blot analysis of endogenous and transgene TGF-α expression in mammary glands of α and α/β mice. Total RNA was extracted from mammary glands and analyzed as described for TGF-α (9) or TGF-β1 (6) transgene expression. Transcripts from both the endogenous TGF-α gene (4.8 kb) and TGF-α transgene (1.4 kb) are visible. Lanes 1–4, RNA from four separate MMTV-TGF-α/β1 mice; lanes 5–8, RNA from four separate MMTV-TGF-α mice.

receptor. Northern blot analysis of EGF receptor mRNA was carried out on the same RNA samples as those used for TGF-α. Expression of EGF receptor mRNA relative to cyclophilin was 0.30 ± 0.08 for the four MMTV-TGF-α mice and 0.28 ± 0.06 for the four MMTV-TGF-α/β1 mice. Thus, the effects of the TGF-β1 transgene when coexpressed with the TGF-α transgene cannot be explained by suppression of TGF-α transgene or EGF receptor expression.

DMBA Induction of Mammary Tumor Formation in Wild-Type and MMTV-TGF-β1 Mice. To determine whether the MMTV-TGF-β1 transgene could affect the rate of mammary tumorigenesis induced by a chemical carcinogen, wild-type and MMTV-TGF-β1 female mice were given DMBA in a dosing regimen shown previously to induce a high rate of mammary tumors in wild-type and MMTV-TGF-α transgenic mice (11). DMBA-treated wild-type mice developed benign tumors by 5.5 months and carcinomas beginning at 7.5 months after the last DMBA treatment (Fig. 4). Table 2 presents the more specific details of tumor formation in DMBA-treated wild-type mice. By 12 months when the experiment was terminated, 11/21 (52%) of the wild-type mice had mammary tumors, nine of which (43%) were carcinomas (Table 2). The types of mammary tumors observed were similar to those previously reported in DMBA-treated mice (16). No carcinomas were observed in the DMBA-treated MMTV-TGF-β1 group; however, 1/19 (5%) developed a benign myoepithelioma. These results demonstrate that TGF-β1 transgene expression can suppress tumor formation in an experimental circumstance other than crossbreeding experiments with MMTV-TGF-α transgenic mice.

DISCUSSION

The MMTV-TGF-α phenotype is similar in many respects to that of transgenic mice expressing various oncogenes under control of the MMTV promoter/enhancer, including mammary epithelial hyperplasia and the stochastic development of mammary tumors, including carcinomas, with increased frequency (17–21). Coexpression of two MMTV-driven oncogenes in crossbred progeny even further enhances mammary tumor formation (22, 23). The MMTV-TGF-β1 phenotype is very different, with mammary ductal hypoplasia and no increase in mammary tumors (6). Data derived in the present study demonstrate that coexpression of MMTV-TGF-β1 with MMTV-TGF-α markedly suppresses mammary tumor development. We further show that MMTV-TGF-β1 transgenic mice are highly resistant to DMBA-induced mammary tumor formation.

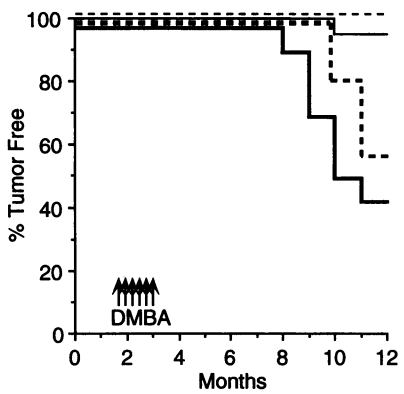

FIG. 4. DMBA induction of mammary tumors in wild-type and MMTV-TGF-β1 transgenic mice. Arrows represent DMBA administration. Carcinomas in DMBA-treated wild-type mice (thick broken line) include five adenocarcinomas, one adenosquamous carcinoma, and three adenomyoepitheliomas (judged to be the equivalent of low-grade adenocarcinomas based on size and cellularity). Total number of mammary tumors in DMBA-treated wild-type mice (thick solid line) include two cystadenomas and one adenoma in addition to the carcinomas. The DMBA-treated MMTV-TGF-β1 mice developed one benign myoepithelioma (thin solid line) and no malignant tumors (thin broken line).

Table 2. Histological diagnoses of mammary gland neoplasms arising in DMBA-treated wild-type mice

Mouse	Age tumor noted, days	Diagnosis
1	196	Adenosquamous carcinoma
2	201	Adenomyoepithelioma* and cystadenoma
3	208	Papillary cystadenoma
4	210	Adenocarcinoma (high grade), comedocarcinoma, adenoma, and cystadenoma
5	237	Adenocarcinoma (medium grade)
6	240	Adenocarcinoma (medium grade) and papillary cystadenoma
7	243	Adenomyoepithelioma* and nodular sclerosis
8	245	Adenoma
9	250	Adenomyoepithelioma* and adenosis
10	255	Lymphoma†
11	260	Adenocarcinoma (low grade)
12	264	Adenocarcinoma (low grade)
13	267	Lymphoma†

*Judged to be low-grade adenocarcinomas because of their large size and high degree of cellularity.
†Not included in Fig. 1, which plots occurrence of mammary epithelial tumors only.

The mechanism(s) of the marked tumor suppressive effect of TGF-β1 when overexpressed in the mammary gland is not known. We found no evidence for the TGF-β1 transgene having an effect on expression of TGF-α or its receptor, the EGF receptor. It has been shown that DMBA-induced mammary tumors are hormonally dependent in the rat (24), and it is possible that the mammary tumors that develop in the MMTV-TGF-α transgenic mice are also hormonally dependent. Thus, TGF-β1 transgene effects on the hormonal status of the animals or the hormonal responsiveness of mammary epithelial cells might account for the observed effects on tumor formation. However, the effects of TGF-α and TGF-β1 on mammary epithelial cell proliferation in culture are direct (25, 26). Furthermore, if expression of the TGF-β1 transgene alters the hormonal status of animals, it is not of sufficient severity to affect pregnancy and lactation; both MMTV-TGF-β1 and MMTV-TGF-α/β1 mice had fertility rates and lactation abilities similar to wild-type mice.

Another possible explanation for TGF-β1 suppression of mammary tumor formation is a decrease in the epithelial mass reducing the target size for mutational events leading to neoplastic transformation. The morphology of the MMTV-TGF-α/β1 mammary glands (see Fig. 1) suggests that TGF-β1 expression does suppress the stimulatory effects of TGF-α on end bud and terminal duct proliferation and the resulting hyperplasia. It has been reported that stimulation of mammary ductal growth early in the life of virgin SHN mice increases the incidence of mammary tumor development in older animals (27). By limiting the total ductal tree mass in both MMTV-TGF-β1 and MMTV-TGF-α/β1 mice, TGF-β1 expression may lower the risk of development of mammary carcinoma. However, TGF-β1 is known to have many diverse effects on cells (28), so other possible mechanisms of TGF-β1 suppression of tumor formation exist.

DMBA requires metabolism to reactive intermediates to have carcinogenic effects (16). It is possible that TGF-β1 inhibits DMBA metabolism; however, this would not account

for TGF-β1 transgene suppression of the TGF-α-induced mammary tumors. In this circumstance, TGF-β1 could suppress endogenous adduct formation. The most likely inhibitory mechanism is that suppression of proliferation either lowers the extent of adduct formation or prevents fixation of adducts into mutations. Alternatively, the major TGF-β1 effect may be suppression of outgrowth of initiated cells instead of inhibition of DNA adduct formation. Future studies will need to address both issues.

The data presented in this paper on the tumor suppressive effects of the MMTV-TGF-β1 transgene in the mammary gland are consistent with data on tumor formation in TGF-β1 null skin keratinocytes. Grafts of v-Ha-ras-initiated TGF-β1 null keratinocytes progressed much more rapidly to multifocal squamous cell carcinomas than similarly initiated keratinocytes from wild-type animals, suggesting that autocrine TGF-β1 suppresses the frequency and rate of malignant progression (29). Other studies have indicated that TGF-β1 protein staining in benign mouse skin tumors is prognostic for a low probability of malignant conversion (30, 31). Thus, the data derived from studies of skin keratinocytes indicate that decreased expression of endogenous TGF-β1 and TGF-β2 can lead to an increased development of squamous cell carcinomas. The data presented in the present paper demonstrate that overexpression of TGF-β1 can markedly suppress mammary carcinoma formation.

That TGF-β1 can suppress carcinoma development appears superficially to contradict many previous reports that TGF-β1 overexpression in cells that have already undergone malignant transformation enhances tumorigenicity, invasion, and metastases. Carcinoma cells frequently lose the growth inhibitory response to TGF-β (32, 33), and overexpression of TGF-β1 in such cells appears to enhance tumor growth and metastatic spread (34–38).

We propose the following model to account for the apparent bifunctional effects of TGF-β in the carcinogenic process. In normal epithelial cells, TGF-β not only inhibits proliferation but also suppresses the early stages of carcinoma development. Once carcinoma cells have progressed to a state in which TGF-β can no longer inhibit proliferation in an autocrine manner, increased expression of TGF-βs may then have paracrine effects on the host, including suppression of immune surveillance or alterations in tumor stroma formation and angiogenesis (38), that favor tumor growth and spread.

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