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This project sets out to establish *in vivo*, the basis for a dramatic cooperation between the *c-myc* oncogene and transforming growth factor α (TGFα). These two genes are commonly amplified or overexpressed respectively in human breast cancer. We will use a novel bitransgenic model to overexpress the two genes in the mouse mammary gland. In addition, we will use mammary epithelial transplant procedure to establish whether this interaction can occur when TGFα and Myc interact in a paracrine fashion. Finally, the expression of several human breast cancer associated genes will be studied in tumors which arise. In the first year of study, we successfully established the timecourse, sex dependence, and organ preference for TGFα - Myc interactions in our system. In addition, we characterized the expression of both transgenes and the epidermal growth factor receptor in mammary and salivary tumors which arose.

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TABLE OF CONTENTS

INTRODUCTION

These studies were carried out to evaluate the potential tumorigenic interaction of *c-myc* (a nuclear oncogene) and transforming growth factor α (TGFα, a growth factor). Previous work in human breast cancer has identified *c-myc* as a gene which is frequently amplified and over expressed. In addition, the growth factor TGFα is commonly overexpressed in breast cancer, along with other members of its family, including amphiregulin (1). While neither TGFα nor *c-myc* overexpression has been shown alone to be a strong prognostic indicator in the disease, our previous work had established that the two gene products strongly synergised *in vitro* to promote transformed growth of human mammary epithelial cells in soft agar colony assays (1,2). The purpose of the present work was to utilize transgenic mouse methodology to establish the malignancy - promoting interactions of TGFα and Myc in breast tumorigenesis. The tumors will be studied for expression of the transgenes, markers of differentiation, and of several other malignancy- associated genes. Finally, we will use xenograft transplantation of different genotype mammary cells combined in variable proportions and implanted in different genotype recipient mammary fatpads *in vivo* to asses TGFα - Myc paracrine interactions.

BODY

The goals of the research in the first year were two- fold. First, we wished to establish the time course of hyperplasia and tumor development in bitransgenic Myc/TGFα mice. Second, we wished to establish the range of paracrine models whereby TGFα is produced by one mammary cell type (stromal or epithelial) and allowed to interact with Myc - expressing mammary epithelial cells to promote their malignant function.

We used bitransgenic mating methodology (3) to co-overexpress the growth factor TGFα and nuclear oncogene *myc* in mouse mammary glands. Tables 1 and 2 summarize the striking results. A strong synergy was observed between the two gene products, leading to 100% incidence of mammary tumors with a mean onset time of 66 ± 12 days in both male and females. The tumors were classified as adenocarcinomas types A and B [see also histopathology in figure 1 in Ammundadottir et al (3), in the Appendix].

Table 1 Number of offspring in each of four genotype groups

Mean mammary gland tumor onset times and tumor frequency in each group is presented.

Genotype	No. of animals (%)[a]	Mean tumor onset time (days)	Tumor incidence
TGFα/c-*myc*	23 (15)	66 ± 12	100%[b]
TGFα	24 (15)	NA	0%
c-*myc*	39 (25)	298 ± 55	50%
Wild type	71 (45)	NA	0%

[a] Total offspring number is 157.
[b] One hundred % equals 20/20 animals; 3 animals died at very young ages.
NA, not available (tumors were not observed for more than 1 year).

Table 2 Summary of histopathological findings in mammary glands of transgenic mice at 3 and 7 months of age

Genotype	3 mos.	7 mos.
TGFα/c-*myc*	Multiple adenocarcinomas (types A and B) in females and males[a]	NA
TGFα	Normal	Cystic ducts
c-*myc*	Normal	Atypical hyperplasia to adenocarcinoma
Wild type	Normal	Cystic ducts

[a] Histopathology from both virgin females and males. All other diagnoses are from virgin females only. NA, not available (all animals of this genotype are deceased at this time point).

We next established the time course of tumor onset in males and females. We designed these experiments with the knowledge that upon necropsy (usually about 3 months of age), all bitransgenic, tumor bearing animals exhibited non-palpable malignancies in all other mammary glands. In addition, no normal tissue was observed in any mammary gland of any animal at this time (3). We carried out whole mount preparations to study the morphology of control and bitransgenic glands weekly from approximately 3-6 weeks of age. This is the time period during which the epithelial rudiment elongates and penetrates the mouse mammary fatpads. Surprisingly, at just 24 days of age, we could reproducibly observe tumorous appearing nodules, disrupted morphology, and enlarged ducts, [this can be observed in figure 2 of the Appendix, Amundadottir et al (3)]. Excision and reimplantation of these early tumors subcutaneously in nude mice yielded rapidly growing, invasive cancer (3).

We examined tumors and found, as expected, that they expressed high levels of TGFα and myc mRNA's by northern blot [see Figure 3 in Appendix, Amundadottir et al (3)] and by *in situ* hybridization [see Figure 4 in Appendix, Amundadottir et al (3)]. The epidermal growth factor receptor (EGFR) was also expressed in the tumors [see Figure 6a, b, in Appendix, by Ammundadottir et al (3)].

A second goal of our first year of study was to explore possible paracrine actions of TGFα to drive Myc- dependent tumorigenesis, since our bitransgenic model cannot readily discriminate autocrine paracrine, and endocrine modes of growth factor action. We initially exised mammary epithelial cells from single transgenic Myc mice and implanted them into the

mammary fatpads of single transgenic TGFα mice from the same litter (derived from a heterozygote TGFα/Myc cross). This is a well known technique initially described by deOme (4). In this experiment we did not observe rapid onset mammary tumors (data not shown). We reasoned that one possible explanation was that TGFα expressed in high levels from the transgene in the mammary stroma of single transgenic TGFα mice was not actually secreted in high levels. As a first step in testing this hypothesis, we exploited single transgenic TGFα and bitransgenic TGFα/Myc mammary tumor cells of *in vitro* culture. We reverified that the cultures were expressing high levels of TGFα mRNA by northern blot and immunohistochemistry. However, in contrast to the TGFα cells, we were unable to detect secretion of significant levels of TGFα from bitransgenic cells in using a standard cloning bioassay for TGFα that we had previously described (2). These results were consistent with autocrine, but not paracrine action of TGFα in our system to allow interaction with Myc to promote tumors. The reasons for non-secretion of TGFα were not yet determined. Current studies are focused on repeating attempts to demonstrate paracrine effects of TGFα *in vivo* and on evaluation of the possibility of any immunologic barrier to transplantation.

CONCLUSIONS

From these studies we have concluded the following relative to our year 1 specific aims:

1. TGFα and c-Myc interact in an extremely rapid fashion to induce the nearly simultaneous appearance of palpable tumors in males and females with an average latency of 66 days for each sex.
2. The time course for conversion of individual epithelial cells to full malignancy is extremely rapid, because disordered growth is apparent as early as the epithelium grows into the fatpad (3-4 weeks of age).
3. Testing of these early growing rudiments by transplantation subcutaneously into nude mice yielded invasive rapidly growing tumors. Thus, the TGFα/Myc interaction is profound, rapid, and seemly occurring too quickly for many further genetic changes to occur.
4. Surprisingly, although mammary tumors from single transgenic TGFα mice produced high levels of TGFα as determined by immunohistochemistry and northern blot, the protein did not appear to be secreted by the tumor cells in culture. This suggest that our proposed study to implant Myc - expressing epithelial cells into mice expressing a TGFα transgene in their stroma may fail. Specifically, stromal TGFα produced from the transgene may be cell- associated and unavailable for diffusion across the basement membrane to stimulate Myc - expressing epithelial cells. Indeed, in a pilot experiment where - Myc expressing mammary epithelial cells were implanted in TGFα - expressing stroma, mammary tumors failed to arise. Although additional attempts will be carried out for this experiment, it may be wise to concentrate further paracrine studies using our

second experimental design whereby single transgenic TGFα and Myc - expressing mammary epithelial cells are actually mixed prior to fatpad implantation. In this experimental design, juxtacrine contact could occur between TGFα and Myc cells to potenitally allow tumorigenic synergy to occur.

From our studies, we were also able to make some unexpectedly rapid progress toward later year specific aims:

1. We were able to confirm expression of the *TGFα*, *EGFR* and *Myc* transgenes in bitransgenic tumors by northern blot and *in situ* hybridization. (See reference 3, included in the Appendix)
2. An unexpectedly clear secondary interaction of TGFα and Myc was also observed in salivary glands of male and female bitransgenic animals. Because these lesions were focal, rather than involving the entire gland (as with mammary lesions) we were able to utilize staining proliferating cell nuclear antigen (PCNA) to confirm, on a cellular level, the concordance of coexpression of TGFα and Myc with hyperproliferation and hyperplastic glandular morphology. (See reference 3 in Appendix)
3. Finally, because we were able to stockpile transgenic tumors from our study, with no added cost to the project, we were able to address expression of the TGFα - related growth factor amphiregulin. Surprisingly, this factor was strongly unregulated in all transgenic mouse mammary tumors, further supporting the role of the TGFα family of growth factors in mammary tumorigenesis (5).

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Appendix

Amundadottir, L.T., Johnson, M.D., Merlino, G.T., Smith, G.H., and Dickson, R.B.: Synergistic interaction of transforming growth factor α and c-myc in mouse mammary and salavary gland tumorigenesis. Cell Growth and Differentiation. 6:737-748, 1995.

Synergistic Interaction of Transforming Growth Factor α and c-*myc* in Mouse Mammary and Salivary Gland Tumorigenesis[1]

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Abstract

The c-*myc* oncogene is commonly amplified in breast cancer and is known to interact synergistically with transforming growth factor α (TGFα) *in vitro* to promote phenotypic transformation of mammary epithelial cells. In addition, both genes are under sex steroid hormone regulation in breast cancer. We have used a bitransgenic mouse approach to test the relevance of Myc-TGFα interaction in mammary gland tumorigenesis of virgin animals *in vivo*. We mated single transgenic TGFα and c-*myc* mouse strains to yield double transgenic offspring for TGFα and c-*myc*. All (20 of 20) double transgenic TGFα/c-*myc* animals developed synchronous mammary tumors at a mean age of 66 days. An unexpected finding was that tumor latency and frequency in males and virgin females were identical. Thus, two gene products that are known to be coinduced in breast cancer by the sex hormones estrogen and progesterone strongly synergize to induce synchronous mammary tumors, independent of sex. The tumors, despite being estrogen receptor positive, were readily transplanted as highly malignant s.c. cancers in ovariectomized nude mice. Although approximately one-half of single transgenic c-*myc* virgin females also eventually developed mammary gland tumors, these were stochastic and arose after a long latency period of 9–12 months. Single transgenic virgin TGFα females and males, c-*myc* males, and transgene-negative littermates did not develop tumors (ages up to 15 months).

The salivary glands of double transgenic animals also coexpress the two transgenes and show pathological abnormalities ranging from hyperplasias to frank adenocarcinomas. In contrast, the salivary glands of single transgenic and wild-type animals showed only mild hyperplasias or metaplasias, but tumors were not observed.

***In situ* hybridization analysis of mammary and salivary glands revealed that hyperplastic and tumorous areas colocalize with regions that overexpress both the TGFα and c-*myc* transgenes. This indicates that there is a requirement for the presence of both proteins for transformation of these glands. In summary, TGFα and c-Myc synergize in an extremely powerful way to cause breast and salivary gland tumorigenesis in males and virgin females without a requirement for pregnancies.**

Introduction

Gene amplification and/or deregulated expression of a number of genes are frequent findings in human breast cancer. Among these are the genes for c-*myc* and TGFα.[3] The protein product of the c-*myc* gene is a nuclear phosphoprotein involved in transcriptional regulation, and TGFα is a member of the EGF family of mitogens, which bind to and activate the EGF receptor (1, 2). The c-*myc* proto-oncogene is amplified in 25 to 30% of breast cancer cases and is overexpressed (without gene amplification) in many more (3–6). Furthermore, amplification of the c-*myc* gene has been shown to correlate with poor prognosis of the disease (3, 7, 8). Although the TGFα gene is not found amplified in human breast cancer, its expression (and that of other EGF family members) is frequently increased compared to the normal gland (9–12). The EGFR is also found expressed in about 30–50% of human breast cancers with high expression associated with poor prognosis and high a degree of invasiveness (13).

Expression of both genes is induced during estrogen and progesterone treatment of hormone-responsive breast cancer cells *in vitro* (10, 14–18). In addition, treatment with antisense oligonucleotides to either TGFα or c-*myc* inhibits estrogen-induced expression of these genes and estrogen-stimulated growth *in vitro*, indicating that they are important mediators of estrogenic effects on cell growth (19, 20).

Transgenic mouse models have provided insight into the roles of both genes in mammary gland development and malignant progression *in vivo*. Overexpression of TGFα in the mammary gland from the mouse metallothionein promoter or the MMTV promoter/enhancer caused the appearance of mammary carcinomas after a relatively long latency period of 7–12 months. Tumors were stochastic and arose predominantly in female mice that had undergone multiple pregnancies (21–23). Transgenic mice with MMTV-*myc* constructs directing expression to the mammary gland also develop clonal tumors after a long latency period of 7–14 months, again with a requirement for multiple pregnancies (24).

Long latency times in transgenic mice are consistent with the hypothesis that oncogenesis is a multistage process composed of a series of genetic events (25, 26). Thus, although one proto-oncogene is overexpressed in a given

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[3] The abbreviations used are: TGFα, transforming growth factor α; EGF, epidermal growth factor; EGFR, EGF receptor; MMTV, mouse mammary tumor virus; MT, metallothionein; LTR, long terminal repeat.

organ of a transgenic mouse, the occurrence of additional events is necessary before cancer can arise. We chose to study the interaction of TGFα with c-*myc*, since *in vitro* studies had suggested possible cooperative interactions. In various cell types *in vitro*, overexpression of c-*myc* resulted in an increased responsiveness to the effects of mitogenic growth factors. For example, rodent fibroblasts and human and mouse mammary epithelial cell lines transfected with *myc* constructs showed transformed behavior, in many cases, only in the presence of TGFα or fibroblast growth factor family members (27–30). High levels of *myc* expression may, therefore, permit a tumorigenic transformation by a TGFα/EGFR autocrine growth mechanism, or it may sensitize cells to such a mechanism.

The interaction of TGFα and c-Myc has not been studied in mammary gland transformation *in vivo*, but two recent studies investigated their interaction in the liver and pancreas with bitransgenic mouse models. Animals expressing both transgenes from liver- and pancreatic-specific promoters formed tumors in these organs at an elevated rate compared to single transgenic animals, suggesting a synergistic interaction (31, 32).

We show here that TGFα and c-Myc cooperate in an extremely powerful, synergistic manner in mouse mammary and salivary gland tumorigenesis. Double transgenic male and virgin female mice develop synchronous mammary tumors in all glands at about 2 months of age, whereas single transgenic animals develop clonal tumors at about 12 months of age or not at all. In addition, epithelial rudiments from 3-week-old TGFα/c-*myc* double transgenic animals could be established in nude mice, indicating that the mammary gland is transformed right at the start of its development. Tumors were also found in salivary glands of double transgenic animals at 3 months of age, whereas single transgenic and wild-type mice have not been observed to develop tumors. Expression of the TGFα and c-*myc* transgenes was associated with hyperplastic and tumorous areas in mammary and salivary glands, indicating a requirement for the presence of both gene products for malignant transformation.

Results

Generation of TGFα/c-*myc* Double Transgenic Mice. Heterozygous mice transgenic for TGFα (MT-TGFα MT100 strain) (21) and c-*myc* (MMTV-c-*myc* M strain) (24) were mated to yield offspring of four possible groups: double transgenic TGFα/c-*myc* mice; single transgenic TGFα mice; single transgenic c-*myc* mice; and mice negative for transgenes (wild type). Since the parental mice are of FVB/N (TGFα) and CD-1 × C57BL/6J (c-*myc*) backgrounds, offspring mice of all groups are of the following genetic background: FVBN/CD-1 × C57BL6J. Mice of the two single transgenic groups and of the wild-type group serve as controls in a similar genetic background as the double transgenic TGFα/c-*myc* mice.

At 3 weeks of age, offspring were weaned, and DNA was extracted from tail biopsies. Screening for transgenes was performed by Southern blot analysis and/or PCR (data not shown). According to Mendelian rules, when mating animals heterozygous for two traits, 25% of offspring should fall into each of four possible genotype groups. However, of 157 offspring, 45% were wild type; 15 and 25% were single transgenic for TGFα and c-*myc*, respectively; and 15% were double transgenic TGFα/c-*myc* (Table 1). Thus, there appeared to be a selection bias against mice positive for the TGFα transgene. A reduced body weight at weaning was not associated with the lower frequency genotypes (data not shown), in contrast to observations of Luetteke *et al.* (33) for a different strain of MT-TGFα transgenic mice. However, we have noted that TGFα-positive mice consistently die at younger ages than c-*myc* single transgenic and wild-type mice and show signs of malnutrition. This is probably due to the effects of the TGFα transgene product on the stomach, as described previously (34). Offspring of each genotype group were approximately equally divided between females and males.

Table 1 Number of offspring in each of four genotype groups

Mean mammary gland tumor onset times and tumor frequency in each group is presented.

Genotype	No. of animals (%)[a]	Mean tumor onset time (days)	Tumor incidence
TGFα/c-*myc*	23 (15)	66 ± 12	100%[b]
TGFα	24 (15)	NA	0%
c-*myc*	39 (25)	298 ± 55	50%
Wild type	71 (45)	NA	0%

[a] Total offspring number is 157.
[b] One hundred % equals 20/20 animals; 3 animals died at very young ages.
NA, not available (tumors were not observed for more than 1 year).

Table 2 Summary of histopathological findings in mammary glands of transgenic mice at 3 and 7 months of age

Genotype	3 mos.	7 mos.
TGFα/c-*myc*	Multiple adenocarcinomas (types A and B) in females and males[a]	NA
TGFα	Normal	Cystic ducts
c-*myc*	Normal	Atypical hyperplasia to adenocarcinoma
Wild type	Normal	Cystic ducts

[a] Histopathology from both virgin females and males. All other diagnoses are from virgin females only. NA, not available (all animals of this genotype are deceased at this time point).

Synergistic Induction of Mammary Gland Tumors in TGFα/c-*myc* Double Transgenic Mice. Of 23 double transgenic TGFα/c-*myc* animals, 20 developed multiple mammary tumors at a mean age of 66 ± 12 days, and three mice died from other causes at very young ages. We can, therefore, conclude that all mice of the TGFα/c-*myc* genotype that reached an age of about 2 months developed mammary gland cancers. An additional striking finding was that tumors arose in both virgin female and male animals with the same latency and frequency. Frank tumors (*i.e.*, palpable) arose first in axillary mammary glands (glands nos. 1, 2, and 3) and then subsequently in inguinal glands (nos. 4 and 5). The average number of palpable tumors at time of necropsy were 2.5/mouse. In addition, pathological diagnoses of hematoxylin/eosin-stained sections revealed the presence of adenocarcinomas in glands without frank palpable tumors, thus showing that every mammary gland from double transgenic animals was cancerous. Surprisingly, no normal tissue was found adjacent to mammary gland tumors in double transgenic animals; therefore, the whole gland could be characterized as malignant. Even a very young (5 weeks old) TGFα/c-*myc*-positive female was diagnosed as having

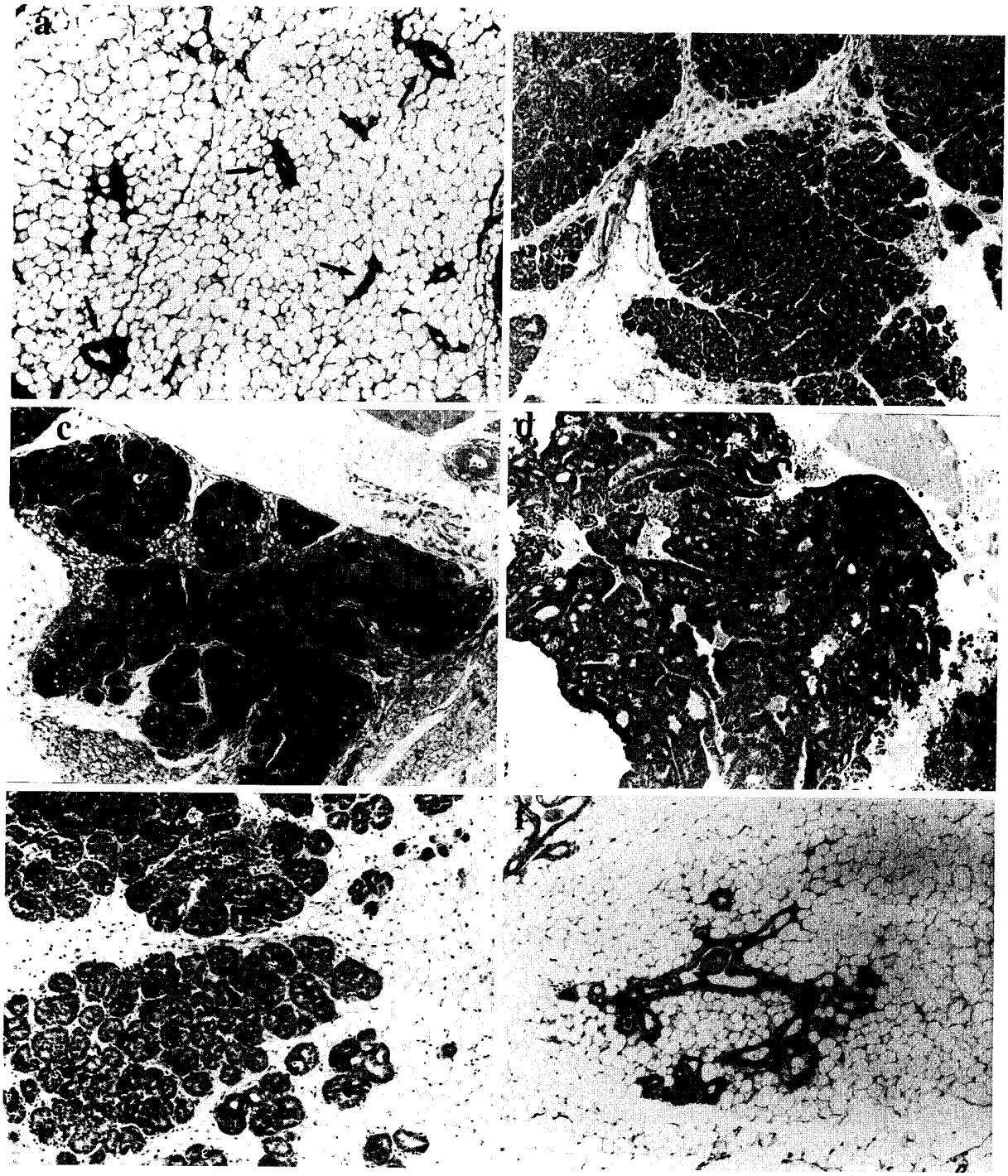

Fig. 1. Hematoxylin & eosin-stained sections of mammary glands. *a*, a normal virgin gland at 3 months of age. It is representative of TGFα and c-*myc* single transgenic animals and of nontransgenic littermates at this age. *Arrows*, epithelial ducts. Panels *b–d*, mammary gland tumors from double transgenic TGFα/c-*myc* animals at 3 months of age. *e*, a section of an inguinal mammary gland from a 5-week-old TGFα/c-*myc* female. *f*, an atypical hyperplastic gland from a c-*myc* single transgenic virgin female at 9 months of age. ×100.

mammary gland adenocarcinoma based on histopathology. This is striking since, at this age, the gland is not fully developed. Our results suggest that, in this model, overexpression of TGFα and c-*myc* is sufficient to cause a complete tumorigenic transformation of the mouse mammary gland.

Mean tumor onset times and frequency are shown in Table 1. Pathological diagnosis of mammary glands from

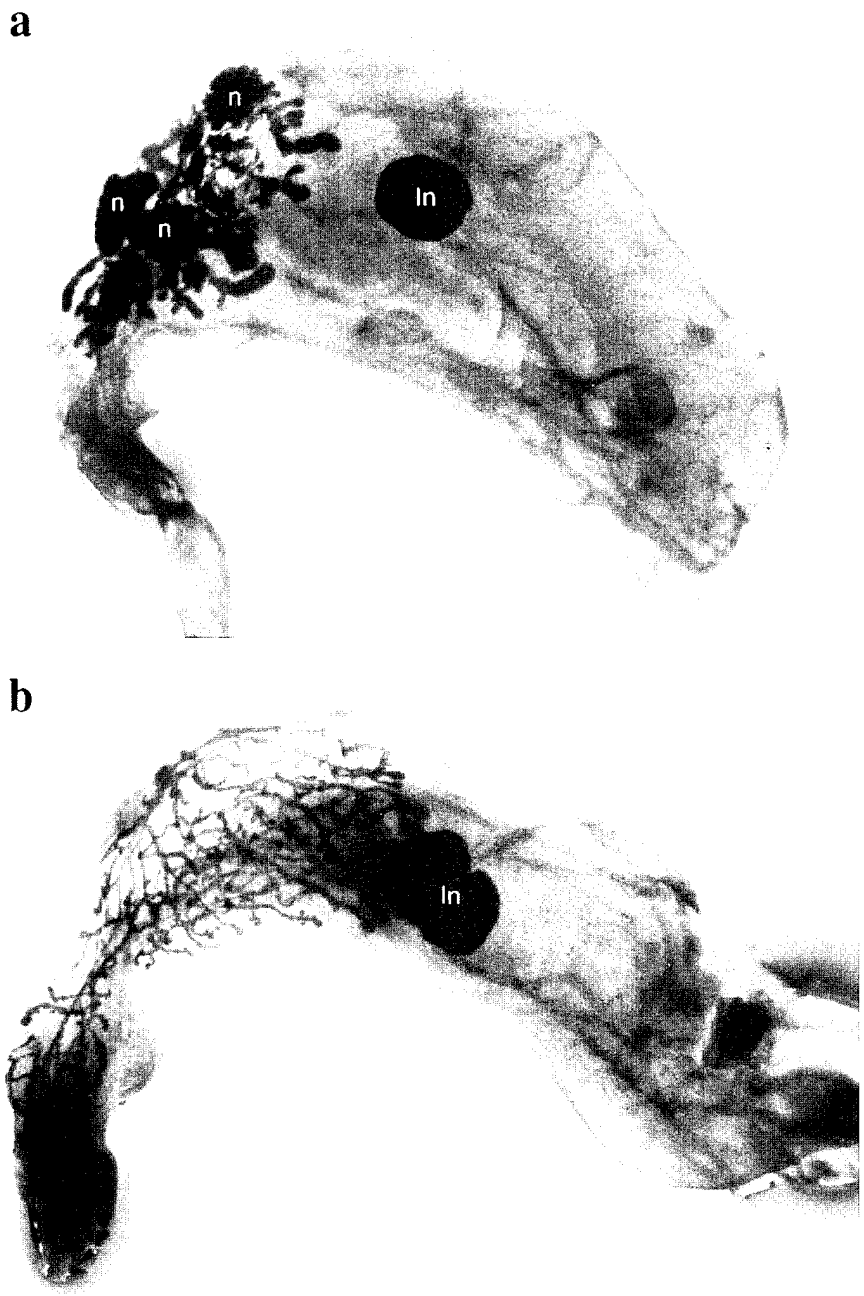

Fig. 2. Whole-mount staining of mammary glands from a TGFα/c-*myc* animal (*a*) and from a wild-type animal (*b*). The carmine alum stain reveales the epithelial network of the gland and the lymph node (*ln*). The animal in *a* was 24 days old, and the one in *b* was 28 days old. Note tumorous nodules (*n*) in the mammary gland from the TGFα/c-*myc* animal (*a*) and normal ductal pattern in the wild-type animal (*b*).

transgenic mice of each genotype group, at 3 and 7 months of age, is shown in Table 2. Representative hematoxylin/eosin-stained sections are shown in Fig. 1. The normal virgin gland at 3 months of age is mostly composed of adipose tissue, with scattered epithelial ducts consisting of two layers of cells (Fig. 1*a*). It is representative of pathology from the two single transgenic groups and the wild-type group at 3 months of age. In contrast, every mammary gland from double transgenic animals (virgin females and males) is tumorous at the same age (Fig. 1, *b–d*). Tumors were classified as adenocarcinomas of types A and B. A type tumors are fairly well differentiated, with the acinar structure of the gland prominent and two layers of epithelial cells seen surrounding lumens (Fig. 1*b*). Type B is less organized and locally invasive (Fig. 1*d*). When tumor sections were stained with periodic acid-Schiff stain, the basement membrane was seen intact in type A tumors but was often disrupted in type B tumors (data not shown). No distant metastases have been found to date. Fig. 1*e* shows adenocarcinoma from a 5-week-old double transgenic virgin female animal. At this age, the epithelial tree has not fully penetrated the mammary fat pad.

About 50% single transgenic c-*myc* virgin females also developed mammary gland tumors, but these were stochas-

tic and arose only after a very long latency period of 298 ± 55 days. The remainder had mild atypical hyperplasias and cystic ducts (Fig. 1*f*). Single transgenic virgin TGFα mice and transgene-negative littermates have not developed tumors to this date (ages up to 15 months). In the case of single transgenic males, we observed atypical hyperplastic areas in mammary glands of a 14-month-old single transgenic c-*myc* male (data not shown). Mammary glands from single transgenic TGFα males and wild-type males at the same ages were normal.

A whole organ staining (termed whole-mount staining) of mammary glands from virgin double transgenic animals at 24 days of age revealed multiple nodules in each gland that appeared tumorous (Fig. 2*a*). These were successfully established in nude mice, indicating that the gland is transformed from the start of its development. In comparison, whole-mount staining of mammary glands from wild-type virgin animals at 28 days of age revealed only the normal ductal pattern (Fig. 2*b*).

The observation that mammary gland tumors arose in double transgenic TGFα/c-*myc* males as well as in virgin females suggested that they might be estrogen independent. Estrogen receptor ligand binding assays revealed that tumors from males and females contained from 13–30 fmol/mg protein of the receptor, and are, therefore, considered estrogen receptor positive (data not shown). Control tumors (MCF-7 or MKL-4 cells grown as tumors in nude mice) contained about 3-fold higher levels of receptor. The ovariectomizing of TGFα/c-*myc* females ($n = 2$) at the time of weaning did not result in a significantly delayed tumor onset (69 *versus* 66 days). In addition, both axillary and inguinal mammary gland tumors could be successfully transplanted into ovariectomized nude mice (data not shown). Together, these data indicate that, although relatively low levels of the estrogen receptor are present in mammary gland tumors as measured by binding to ligand, they are not dependent on estrogen for growth.

Expression of Transgenes and the Epidermal Growth Factor Receptor in Mammary Gland Tumors. We have used Northern analysis, RNase protection assays, *in situ* hybridization analysis, and immunohistochemistry to examine the expression of transgenes and that of the EGFR gene in mammary gland tumors from double transgenic TGFα/c-*myc* animals. RNA expression was compared between axillary mammary gland tumors (frank tumors or lumps) and inguinal gland tumors (carcinoma revealed by histopathology) of TGFα/c-*myc* animals.

Transgenes were expressed in all mammary gland tumors from double transgenic animals but were not detectable in normal glands from single transgenic animals at 3 months of age (Fig. 3). There was about a 5-fold difference in the expression of the c-*myc* transgene, and about 7-fold for the TGFα transgene between the lowest- and highest-expressing tumor. An association of transgene expression at the RNA level and pathological diagnosis (adenocarcinoma type A *versus* B), location (axillary *versus* inguinal glands), tumor size, or sex was not observed. Expression of TGFα and c-*myc* was not detected in normal glands by this method. Transgene expression was also examined by *in situ* hybridization analysis to establish the pattern of transgene expression in the tumors (Fig. 4, *a*, *c*, and *e*). Sequential tumor sections from double transgenic TGFα/c-*myc* animals were hybridized to ^{35}S-labeled TGFα and c-*myc* probes. We observed a very strong and uniform expression of c-*myc* mRNA in mammary gland tumors from double

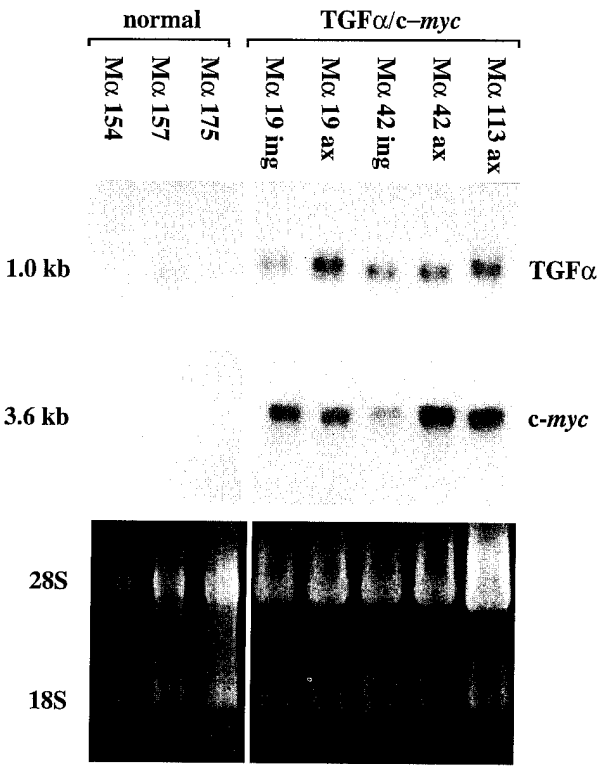

Fig. 3. Northern analysis showing expression of the TGFα and c-*myc* transgenes in mammary gland tumors from double transgenic animals and in normal glands from single transgenic and wild-type animals. The TGFα transcript is 1.0 kb, and the c-*myc* transcript is 3.6 kb. Note that the endogenous 2.3-kb c-*myc* transcript is not seen here. Loading controls are the 18S and 28S ribosomal RNA bands. Mα 19 through 175 denotes the number of each animal used here. *Ax*, axillary gland tumor; *ing*, inguinal gland tumor.

transgenic animals at 3 weeks of age and higher. A scattered expression of TGFα mRNA was noted in most areas of the mammary glands by this method. An immunohistochemical evaluation of TGFα protein levels in bitransgenic tumors with a TGFα-specific antibody also revealed a scattered pattern of strong expression but no association of enhanced staining with a more aggressive phenotype (Fig. 5, *a* and *b*). We also measured endogenous EGFR mRNA levels in tumors from double transgenic animals by RNase protection assays. As seen in Fig. 6, EGFR mRNA levels were comparable in all but one mammary gland tumor (axillary tumor from TGFα/c-*myc* animal no. 113). An association of EGFR mRNA levels with pathological diagnosis, location, tumor size, or sex was not seen.

Synergistic Induction of Salivary Gland Tumors by TGFα and c-*myc*. The MT promoter is active in most epithelial tissues, whereas the MMTV promoter is restricted to only a few tissues. Therefore, the MMTV promoter limits coexpression of the transgenes to mammary glands, salivary glands, and some reproductive organs. An interaction between TGFα and c-*myc* was not observed in reproductive organs, but a positive interaction was noted in the salivary glands. Ductule hyperplasia (sometimes with atypia) was seen in all salivary glands of double transgenic TGFα/c-*myc* virgin female and male animals at 3 months of age. In some cases, squamous metaplasia was observed in the sublingual gland,

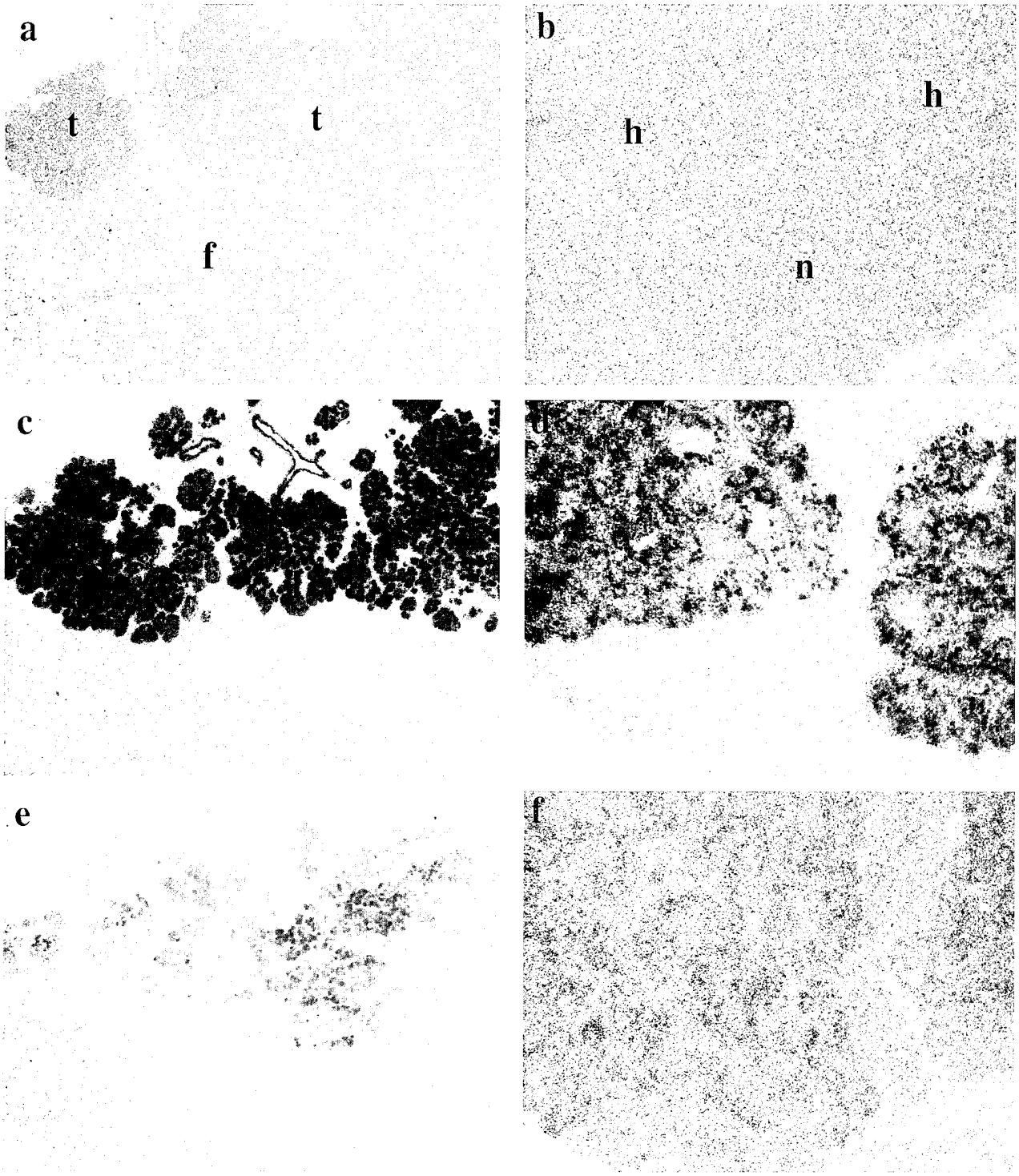

Fig. 4. *In situ* hybridization analysis of mammary and salivary glands from TGFα/c-*myc* animals. Sequential paraffin-embedded tissue sections were hybridized to ^{35}S-labeled riboprobes generated against the TGFα and c-*myc* transgenes. *a* and *b*, control sections hybridized to sense riboprobes. *c* and *d*, sections hybridized to c-*myc* antisense riboprobes. *e* and *f*, sections hybridized to TGFα antisense riboprobes. *a*, *c*, and *e*, from mammary glands; *b*, *d*, and *f*, from salivary glands. Note a near uniform expression of the transgenes in a mammary gland tumor from this 3-week-old double transgenic animal. Observe a patchy expression of c-*myc* and scattered distribution of TGFα in this 6-week-old double transgenic animal. Also note that where both transgenes are expressed, the glands appear hyperplastic and tumorous. *t*, tumor; *f*, fatty tissue; *h*, hyperplastic; *n*, normal. All panels are brightfield photographs. *a*, *c*, and *e*, ×50; *b*, *d*, and *f*, ×200.

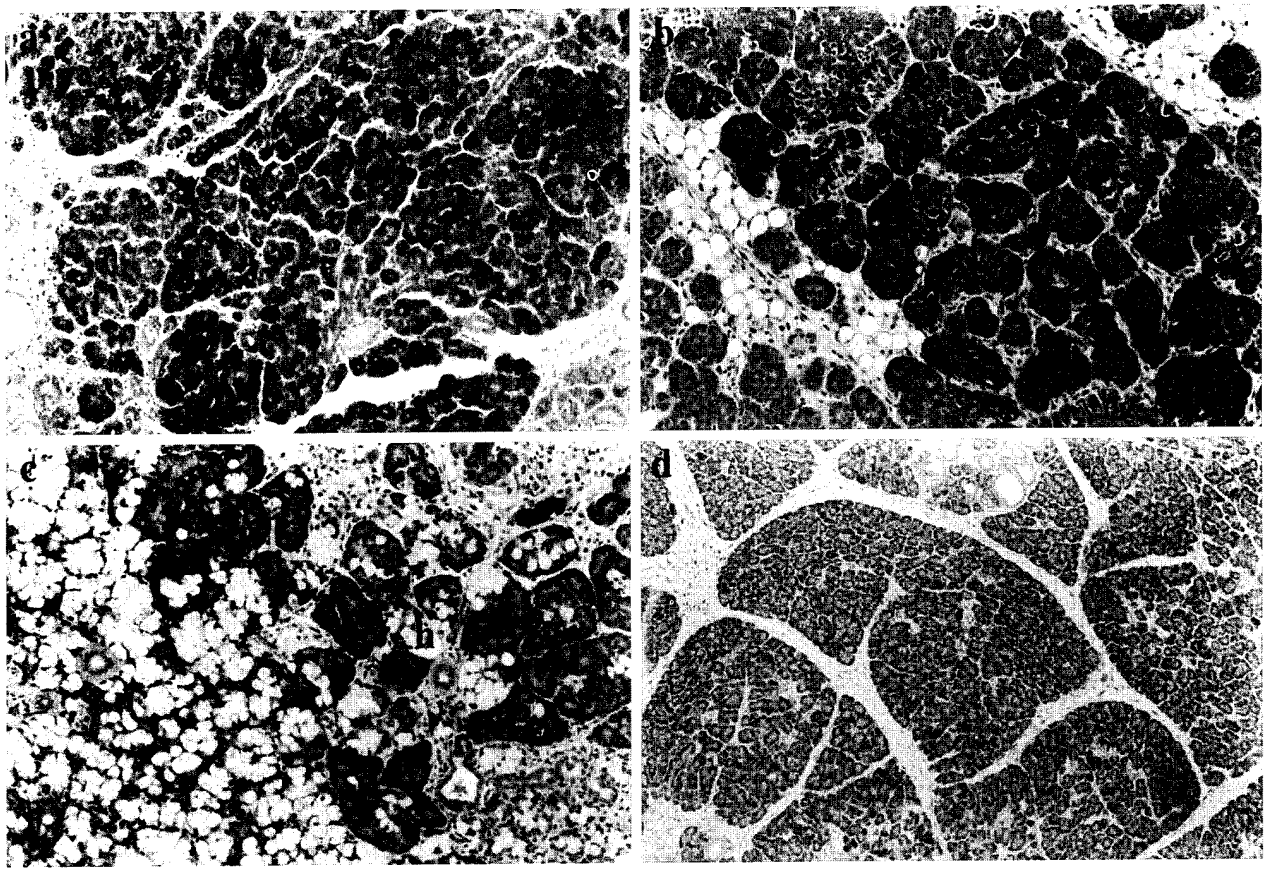

Fig. 5. Immunohistochemical staining of mammary and salivary glands from TGFα/c-*myc* double transgenic animals with an anti-TGFα polyclonal antiserum. *a*, a mammary gland tumor from a 3-month-old animal and *b*, from a 5-week-old animal. Both have a scattered pattern of strong TGFα staining. *c*, an immunohistochemical staining of a bitransgenic salivary gland (sublingual) with a premalignant atypical hyperplastic lesion (*h*) that stains strongly for TGFα, while the surrounding normal areas (*n*) appear negative. *d*, a negative control without a primary antibody.

and adenoma and adenocarcinoma in the parotid gland at the same age. Salivary glands of single transgenic TGFα animals showed minimal ductule hyperplasia, but single transgenic c-*myc* mice and transgene-negative littermates were free of pathological abnormalities at 3 months of age (Table 3; Fig 7).

At 7 months of age, histopathology of salivary glands from single transgenic animals revealed minimal serous metaplasia in the sublingual glands of c-*myc* mice, and mild duct(ule) squamous metaplasia and hyperplasia in the sublingual and submandibular glands of TGFα animals. Wild-type mice at 7 months of age had no apparent abnormalities. No tumors were ever observed in the salivary glands of single transgenic or wild-type mice (up to 10 months for TGFα mice and 15 months for c-*myc* mice).

To obtain information about the localization of expression of both transgenes within the gland, *in situ* hybridization analysis was performed on sequential sections of salivary glands from TGFα/c-*myc* animals (Fig. 4, *b*, *d*, and *f*). It revealed a very patchy pattern of expression of the c-*myc* transgene and scattered expression of the TGFα transgene. Expression was quite different from what we observed in the mammary glands in that only about 5% of salivary gland tissue was positive for both transgenes. Interestingly, areas where expression of both transgenes was detected appeared hyperplastic and atypical, whereas areas with only one transgene expressed looked quite normal. These areas might represent premalignant areas within the salivary glands, indicating that only when both transgenes are expressed does malignant conversion occur. Immunohistochemical staining of salivary glands from TGFα/c-*myc* animals was performed with a polyclonal antiserum that recognizes both the endogenous mouse TGFα and the transgene-derived human TGFα. In agreement with *in situ* hybridization data, we observed a scattered distribution of TGFα expression and an association of an intense staining with premalignant hyperplastic atypical nodules (as seen in the sublingual gland in Fig. 5*c*), whereas surrounding areas of normal or hyperplastic salivary gland had little or no TGFα staining. Finally, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) on salivary gland sections revealed a strong staining in areas that coexpressed the transgenes, indicating that DNA synthesis was occurring (Fig. 8).

Discussion

In this study, matings of MT-TGFα and MMTV-c-*myc* transgenic strains were carried out to investigate the *in vivo*

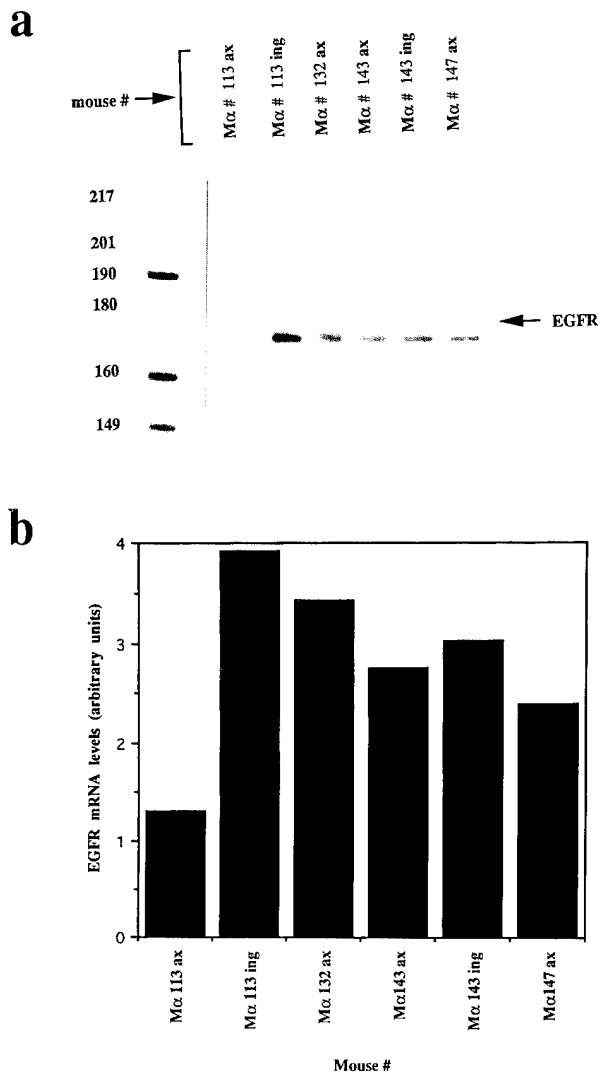

Fig. 6. Expression of the endogenous EGFR mRNA measured by RNase protection assay. RNA levels were compared between mammary gland tumors from double transgenic animals. *b*, a scanned version of the data in *a*. Mα113 through Mα147 denotes the number of each double transgenic mouse used in the assay. *ax*, axillary gland tumor; *ing*, inguinal gland tumor.

Table 3 Summary of histopathological findings in salivary glands of transgenic animals at 3 and 7 months of age

All data represent both male and female animals.

Genotype	3 mos.	7 mos.
TGFα/c-*myc*	Hyperplasia with atypia, squamous metaplasia and adenoma, and adenocarcinoma	NA[a]
TGFα	Ductule hyperplasia	Ductule hyperplasia and squamous metaplasia
c-*myc*	Normal	Serous metaplasia
Wild type	Normal	Normal

[a] NA, not available (all animals of this genotype are deceased at this time point).

interaction of TGFα and c-Myc in mouse mammary gland transformation and confirm our previous *in vitro* studies that showed cooperation between the two. We found that tumorigenesis in mammary glands of double transgenic TGFα/c-*myc* virgin females and males is strikingly different from single transgenic and wild-type animals and also from multiparous single transgenic TGFα and c-*myc* mice. In previous studies, both MT-TGFα and MMTV-c-*myc* single transgenic mice developed polyclonal mammary gland tumors only after a long latency period and multiple pregnancies (21, 24, 35). In contrast, our studies show that both virgin females and males harboring both transgenes develop multiple mammary gland tumors after a mean latency period of only 66 days. All of 20 double transgenic virgin females and males developed rapidly growing mammary gland tumors that could be established in nude mice in the absence of estrogens. Single transgenic virgin TGFα and wild-type animals of both sexes did not develop any tumors, whereas about one-half single transgenic c-*myc* virgin females developed stochastic mammary gland tumors after a long latency period of about 8–12 months. The early onset and multiple tumor formation in double transgenic TGFα/c-*myc* animals suggests that very few, if any, additional genetic events are necessary for tumorigenesis in our model. In fact, at 3 weeks of age, when the glandular tissue has just started to penetrate the fat pad, the mammary gland is already tumorous.

It is also quite interesting that tumors form in a synchronous manner in our model, so that normal mammary gland tissue is not found at all. Two previous studies have described transgenic models with synchronous tumorigenesis of mammary glands. In the first one, an activated rat *neu* oncogene was expressed from the MMTV-LTR promoter/enhancer, and in the second, the polyoma middle T oncogene was expressed from the same promoter (36, 37). An extremely high level of transgene expression observed in the former strain might have contributed to the phenotype, since MMTV-*neu* transgenic mice made by another group developed only stochastic mammary gland tumors (38). However, the study has been repeated using the same transgene construct with similar results (39). Our model is comparable to the effects of a mutated growth factor receptor, Neu, or the powerful viral protein product of the polyomavirus middle T oncogene that mediates cellular transformation by targeting a number of intracellular signaling pathways (40–43). The fact that overexpression of two normal proteins in the mammary gland of transgenic mice has a similar effect on tumorigenesis in this organ as a mutated, highly active growth factor receptor and a strong viral oncoprotein further emphasizes the cooperative effect of TGFα and c-Myc.

An intriguing finding from our studies was that double transgenic TGFα/c-*myc* males developed mammary cancer in a manner indistinguishable from virgin females. Mammary gland cancer has been described previously in transgenic male mice of MMTV-*neu*, MMTV-v-Ha-*ras*, MMTV-*int*-1, MMTV-*int*-3, and MMTV-polyomavirus middle T strains. However, tumor onset is typically delayed compared to female mice (35–37, 44, 45). Both TGFα and c-*myc* are estrogen-inducible genes, and each has been shown to be responsible, at least in part, for estrogen-mediated growth *in vitro* (19, 20). It is, therefore, possible that when both genes are overexpressed *in vivo*, they induce growth of the male mammary gland in the absence of

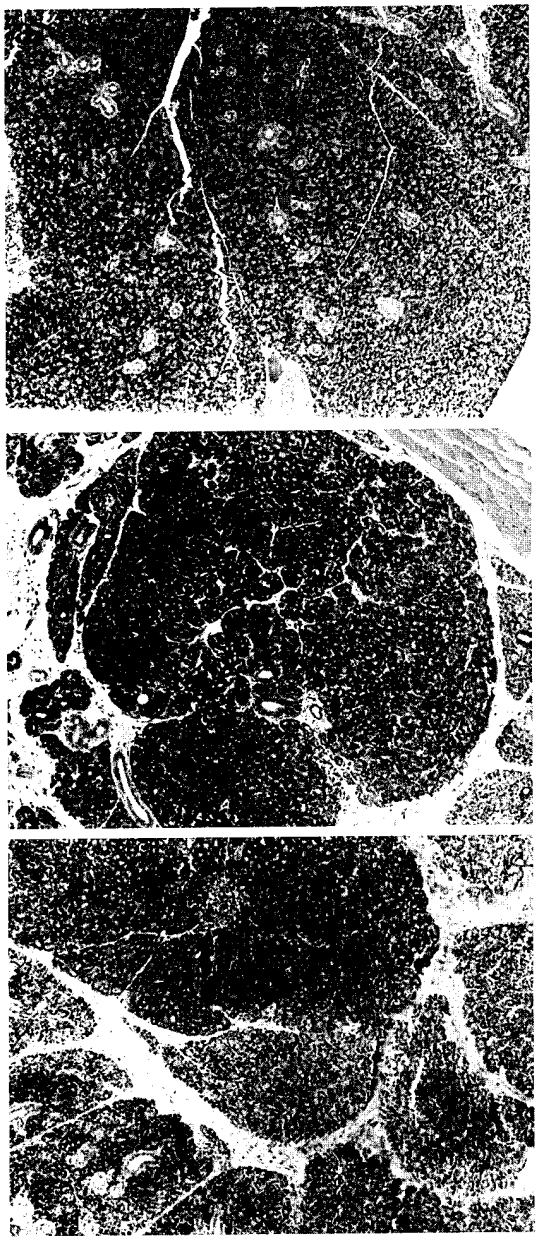

Fig. 7. Hematoxylin & eosin-stained sections of salivary glands. *a*, a normal parotid gland from a wild-type mouse. *b*, a parotid adenoma and *c*, an adenocarcinoma from TGFα/c-*myc* double transgenic animals. All animals are about 3 months old. ×100.

estrogens. Breast cancer in human males is extremely rare, but in the presence of exogenously applied estrogens, males can develop mammary gland hyperplasias (gynecomastia), thus showing that estrogens can cause proliferation in the male mammary gland in the presence of androgens. It is remarkable that overexpression of two estrogen-induced genes can stimulate the growth and malignant transformation of the male mammary gland as we have seen here. It encourages further studies to dissect the role of, and interaction between, mediators responsible for hormone action on the normal and malignant development of the mammary gland.

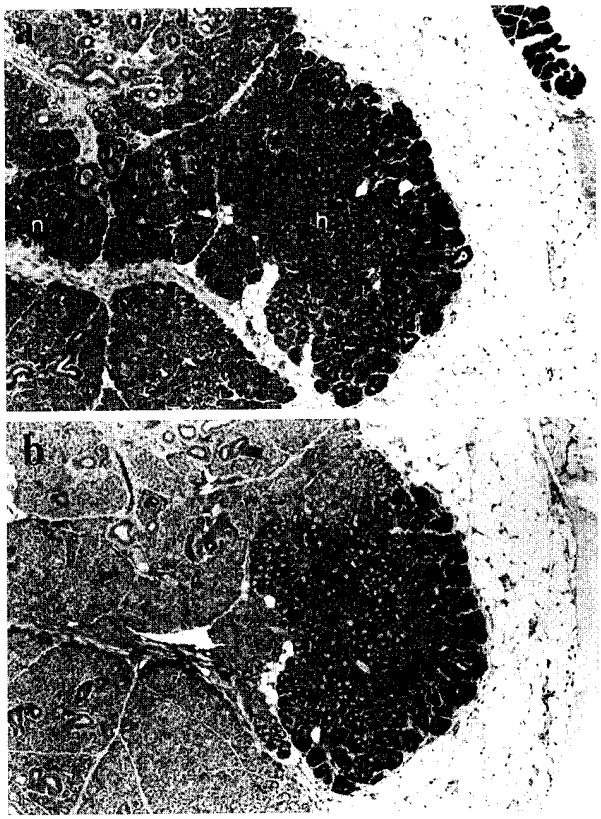

Fig. 8. Immunohistochemical staining of proliferating cell nuclear antigen in salivary glands from a 6-week-old double transgenic TGFα/c-*myc* animal. *a*, a hematoxylin & eosin-stained section of the salivary gland and *b*, a sequential section stained for proliferating cell nuclear antigen. Note intense proliferating cell nuclear antigen staining in hyperplastic areas of this field. This area in the parotid gland coexpressed the TGFα and c-*myc* transgenes. *h*, hyperplastic; *n*, normal. ×100.

A cooperative interaction between TGFα and c-Myc also exists in the salivary glands, although malignancies were somewhat less prominent than in mammary glands. In contrast to mammary glands of bitransgenic mice where normal tissue was not found, the salivary glands of double transgenic animals contained normal tissue juxtaposed with hyperplastic areas and frank tumors. However, single transgenic and wild-type animals did not develop salivary gland tumors, whereas they developed mammary gland tumors after a long latency (MMTV-c-*myc* female mice). Therefore, we conclude that a strong cooperative interaction also exists in the salivary glands.

In situ hybridization analysis revealed that expression of transgenes was more uniform in mammary glands than salivary glands. In fact, only about 5–10% of salivary gland tissue expressed detectable levels of c-Myc (mainly in the parotid gland). Interestingly, areas that expressed both transgenes appeared hyperplastic (salivary glands at 5 weeks) or tumorous (mammary glands at 3 weeks), indicating a requirement for both TGFα and c-Myc in tumorigenesis. In the salivary gland, this was quite clear since areas were found that expressed either TGFα, c-*myc*, both transgenes, or no transgenes. A progressive tumor onset was associated with a patchy expression of transgenes in the salivary glands. On the other hand, an extremely rapid

tumor onset was associated with a near uniform expression of transgenes in the mammary glands. This might suggest that additional events must occur in the course of the slower tumorigenesis in salivary glands. In the mammary glands, TGFα and c-Myc appear to be sufficient to mediate transformation, although additional events cannot be ruled out. In both glands, there appears to be a selective advantage to express increasing levels of the TGFα transgene in the course of malignant progression. The mechanism of this effect is not known, but an apparently similar phenomenon was observed previously in mouse skin carcinogenesis in transgenic TGFα mice (46).

In summary, TGFα and c-*myc* are extremely powerful, synergistic-acting genes in breast and salivary gland carcinogenesis in the mouse strains described here. Since TGFα and c-Myc cause uniform transformation of the mammary gland of transgenic mice, this model provides an ideal system to examine possible secondary events for malignant progression/metastasis and characterize the relevance of a deregulated TGFα/EGF receptor pathway in mammary tumorigenesis.

Materials and Methods

Transgenic Mice. The MMTV-c-*myc* mice used in this study were obtained from Charles River Laboratories (Wilmington, MA); experiments were carried out under a breeding license agreement with Du Pont Medical Products (Wilmington, DE). All mice were rederived and were free of adventious agents. Line MT100 has a mouse metallothionein I (MT) promoter driving expression of a human TGFα cDNA transgene in an FVB/N inbred genetic background (21). The MMTV-c-*myc* M line harbors a mouse c-*myc* gene driven by the mouse mammary tumor virus long terminal repeat promoter/enhancer (MMTV-LTR) in a CD-1 × C57BL/6J background (24). Both strains have been shown to form stochastic mammary gland tumors after a long latency period and multiple pregnancies. Double transgenic mice were generated by mating the MT-TGFα MT100 line to the MMTV-c-*myc* M line. Offspring were maintained on 50 mM $ZnCl_2$ drinking water from the time of weaning (3–4 weeks of age) to induce maximal expression of the TGFα transgene from the metallothionein promoter.

Genotyping of Offspring for Transgenes. DNA was isolated from 1-cm tail biopsies by an overnight proteinase K digestion at 55°C, followed by phenol/chloroform extractions and ethanol precipitation. For Southern analysis, 10 μg of tail DNA was digested overnight at 37°C with the following restriction enzymes: *Bgl*II for TGFα; and *Bam*HI and *Cla*I for c-*myc*. After electrophoresis through 0.8% agarose gels and transfer to nitrocellulose, blots were probed with random-primed ^{32}P-labeled cDNA probes for human TGFα and mouse c-*myc*. The TGFα probe was a 925-bp *Eco*RI fragment from the plasmid pTGFα, kindly provided by Dr. Francis Kern (Georgetown University, Washington, DC; Ref. 47). The c-*myc* probe was a 2400-bp *Eco*RI-*Xba*I fragment from the plasmid fpGV-1, generously provided by Dr. MaryLou Cutler (NIH, Bethesda, MD; Ref. 48).

For PCR, 3 μg DNA from tail biopsies was used as a template to amplify transgenes. 3′ primers were complementary to sequences in the TGFα and c-*myc* transgenes, and 5′ primers to sequences in the metallothionein (MT) and MMTV promoters, respectively: MT-TGFα 5′ primer, 5′-TCG TCC CCG AGC CAG TCG-3′; MT-TGFα 3′ primer, 5′-GTC CGT CTC TTT GCA GTT CTT-3′; MMTV-c-*myc* 5′ primer, 5′-CCC AAG GCT TAA GTA AGT TTT TGG-3′; and MMTV-c-*myc* 3′ primer, 5′-GGG CAT AAG CAC AGA TAA AAC ACT-3′. Primers were made by the Lombardi Cancer Center Macromolecular Synthesis and Sequencing Core facility (Georgetown University, Washington DC). PCR was performed using the Perkin Elmer Taq polymerase kit (Perkin Elmer, Norwalk, CT). The TGFα and c-*myc* transgenes were detected with Southern analysis and/or PCR.

Tumors and Histopathology. Mice were palpated biweekly for tumors and sacrificed before tumor sizes reached 10% of body weight. Location and size of each tumor were determined. Tumors were fixed in Bouin's solution for 5–12 h, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined to determine histopathological diagnoses. Transplantation of tumors into ovariectomized female NCR *nu/nu* mice was performed as follows. Tumor-bearing mice were anesthetized with methofane, and tumors were excised aseptically. Tumors were cut into about 1-mm^2 pieces and inserted s.c. (between nipples nos. 2 and 3) of nude mice under anesthesia.

Whole-Mount Staining. Animals were sacrificed, and the inguinal mammary glands were removed and fixed in 25% glacial acetic acid and 75% ethanol for 60 min at room temperature. After staining overnight in carmine alum solution [1 g carmine and 2.5 g aluminium potassium sulfate (both from Sigma Chemical Co.) in 500 ml water] glands were dehydrated in a series of ethanol washes and finally cleared in toluene. Glands were stored and photographed in methyl salicylate.

Estrogen Receptor Binding Assay. Frozen tumor samples (50–100 mg) were pulverized in liquid nitrogen and homogenized at 0°C in TEDG [10 mM Tris-OH (pH 7.4), 1 mM EDTA, 1 mM DTT, and 10% glycerol] plus 0.5 M NaCl and a cocktail of proteolysis inhibitors (leupeptin at 1 mg/ml, aprotinin at 77 μg/ml, and pepstatin A at 1 μg/ml). Homogenates were centrifuged at 105,000 × *g* at 4°C for 30 min to yield a whole-cell lysate, which was then adjusted to 2 mg/ml protein. Lysates were incubated with 10 nM [^{3}H]17β-estradiol with or without a 100-fold excess of unlabeled estradiol for 16 h at 4°C. Binding was assayed by adding dextran-coated charcoal to adsorb free hormone. After centrifugation, aliquots of supernatant were removed and counted in 10 ml of liquid scintillation fluid in a Beckman liquid scintillation counter. Estrogen receptor-positive control tumors were MCF-7 and MKL-4 breast cancer cell lines grown in nude mice (49). They were generously provided by Dr. Sandy McLeskey (Georgetown University, Washington DC).

RNA Isolation and Analysis. Total RNA was isolated by pulverizing frozen tumors in liquid nitrogen, followed by homogenization in guanidine thiocyanate, acid phenol extraction, and precipitation with isopropanol. TGFα and c-*myc* transgene expression was assessed by Northern blot hybridization; 15 μg total RNA were electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Amersham, Arlington Heights, IL), and probed with a ^{32}P-labeled random-primed probes. The c-*myc* probe was generated from the plasmid fpGV-1 (as described above) and the TGFα probe from the plasmid pTGFα-RP as described previously (50).

EGFR expression was determined using RNase protection assays in which ^{32}P-labeled antisense riboprobes (cRNA) were synthesized *in vitro* from the plasmid pME2.0 for the

EGFR, by linearizing with *Hin*dIII and transcribing with SP6 polymerase (51). It yields a 170-bp protected EGFR fragment. This plasmid was kindly provided by Dr. M. Rosner (University of Chicago, Chicago, IL). Total RNA (30 μg; EGFR) was hybridized for 12–16 h at 42°C to the ^{32}P-labeled cRNA probe and treated with RNase A for 30 min at 25°C. The radiolabeled riboprobes protected by total RNA were run on a 6% polyacrylamide/7M urea gel, which was subsequently dried and exposed to autoradiography.

***In Situ* Hybridization Analysis.** To detect localization of transgene expression, *in situ* hybridization analysis was performed on mammary and salivary glands from 3; 6; and 10-week old double transgenic TGFα/c-*myc* animals. Animals were sacrificed, and glands were fixed in 4% paraformaldehyde in PBS for 24 h. *In situ* hybridization analysis was performed by Molecular Histology, Inc. (Gaithersburg, MD Ref. 52). Probes were generated from plasmids; p.c.-*myc*20 was generously provided by Dr. S. Thorgeirsson (NIH, Bethesda, MD). For the antisense riboprobe, this plasmid was linearized with *Eco*RI and transcribed with T7 polymerase. For a sense control riboprobe, the same plasmid was linearized with *Hin*dIII and transcribed with SP6 polymerase. Plasmid pTGFα-pGem3Z was used to detect the TGFα transgene. For an antisense riboprobe, this plasmid was linearized with *Hin*dIII and transcribed with T7 polymerase. For a sense control riboprobe, the same plasmid was digested with *Eco*RI and transcribed with SP6 polymerase.

Immunostaining. Tissues were fixed in Bouin's solution for 5–12 h, embedded in paraffin, and sectioned. After treatment with 0.02% trypsin for 15 min, sections were incubated overnight at room temperature with a 1:20,000 dilution of a rabbit polyclonal antiserum generated against a rat pro-TGFα intracellular peptide (residues 137–159). The antibody was kindly provided by Dr. Larry Gentry (Medical College of Ohio, Toledo, OH). TGFα was localized using the Vectastain Rabbit Elite kit (Vector Laboratories, Burlingame, CA), as described previously (50). PCNA immunostaining was performed as described previously (53).

Acknowledgments

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