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13. ABSTRACT ***(Maximum 200 Words)***

This grant studied the nature of the synergistic interaction of the growth factor TGFα and the nuclear protoonco-protein product c-Myc for bitransgenic mouse mammary tumorigenesis. We found evidence of a multifactoral interaction of the two genes, involving cooperative stimulation of proliferation, anchorage independent colony formation, suppression of cell death (apoptosis), and chromosomal destabilization. In addition, we observed that the TGFα-related growth factors amphiregulin and cripto-1 were likely to have tumor promoting effects similar to TGFα in breast cancer. Further studies addressed the ability of c-Myc and TGFα to interact in a novel paracrine mammary system *in vivo*. In summary, this grant allowed the development of a new model system whereby the interactions of two important genes in breast cancer may be dissected *in vivo* at the molecular level.

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Robert B. D

PI - Signature Date

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Introduction

This project focussed on characterization of the potent tumorigenic interaction of c-Myc (a nuclear oncogene) and transforming growth factor α (TGFα, a growth factor) in the mammary gland. Both genes are commonly overexpressed in human breast cancer, and we established a novel bitransgenic mouse model for mechanistic studies of the results of their co-overexpression (1). Tumors were characterized for expression of the transgenes, state of differentiation, and expression of several other malignancy-associated genes. Detailed biochemical and molecular studies then assessed regulation of apoptosis (programmed cell death) and cell cycle aberrations as potential mechanisms of interaction of transgenes. Finally, xenograft transplantation of mammary cells expressing c-Myc were combined in variable proportions with cells expressing either no transgene or the TGFα transgene to assess the scope of paracrine interactions of TGFα and c-Myc *in vivo.*

Body

The revised and approved specific arms for this grant are included as an Appendix within the revised statement of work. Our overall hypothesis for the grant was that a growth factor commonly overexpressed in breast cancer (TGFα) and an oncoprotein commonly gene amplified and overexpressed in breast cancer (c-Myc) interact in a multifactoral fashon to facilitate initiation and/or progression of the disease.

Table I presents information concerning mammary tumor frequency and latency in our TGFα-c-Myc bitransgenic mouse model. Tumors arose in all males and virgin females containing the two transgenes; the latency was 66 days in each sex. In contrast, in virgin females, the mean tumor latency in single transgenic, Myc-bearing animals was nearly one year, and no tumors were ever seen in single transgenic TGFα-bearing animals. Table II presents the histopathologic characterization of all mammary lesions: bitransgenic tumors were all adenocarcinomas. We observed that although bitransgenic mammary (and salivary) tumors arose in male and female bitransgenic animals very rapidly and independent of pregnancy, the mammary tumors contained moderate levels of receptors for estrogen and progesterone. However, growth regulation of tumor cells *in vivo* and *in vitro* was independent of these steroids. In addition, the tumors were of epithelial morphology and expressed cytokeratins as detected with a pan-cytokeratin antibody. Tumors contained multiple copies of the expected transgenes, but were not amplified for genes encoding EGF receptor, the Mync partner Max (originally termed Myn in the mouse) or cyclins. These results were reported in the scientific literature (1, see Appendix).

We next set out to examine mRNA and protein expression in for the same genes bitransgenic tumors. Interestingly, we observed that co-overexpression of the two transgenes resulted in their expression at the mRNA level which was clearly in excess of expression of either transgene in single transgenic, long latency control tumors (Figure 1). This strongly suggested a selective advantage for co-overexpression of transgenes. Northern blot data were confirmed by *in situ* hybridization data in mammary and salivary gland tumors. When we examined Myn and Cyclin D1 expression; these two genes were also observed to be highly expressed at the mRNA level, consistent with a high degree of proliferation and malignant transformation of bitransgenic tumors. The EGF receptor (Figure 2) was expressed at moderate levels in bitransgenic and single transgenic tumors (1, see Appendix).

We were able to establish cell lines from the single and bitransgenic tumors in order to further address mechanisms of their interaction. We observed that, compared to single transgenic tumor cells, the bitransgenic tumor cells proliferated more rapidly and grew more readily in anchorage independent colonies. In addition, c-Myc single transgenic tumor cells exhibited a high degree of apoptosis (programmed cell death) compared to TGFα-expressing single and bitransgenic tumor lines (2, See Appendix).

Tables 1 and 2

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%

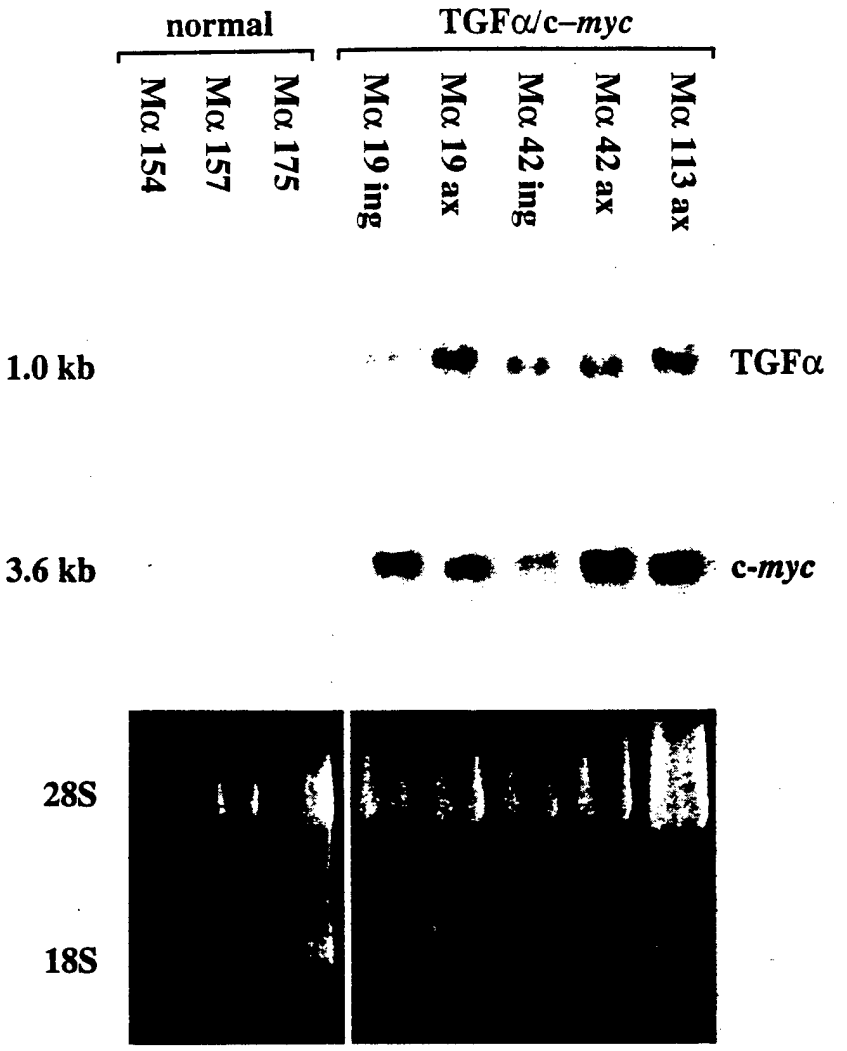
[a] Total offspring number is 157.
[b] One hundred % equals 20/20 animals; 3 animals died at very young ages.
NA, not available (tumors were not observed for more than 1 year).

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

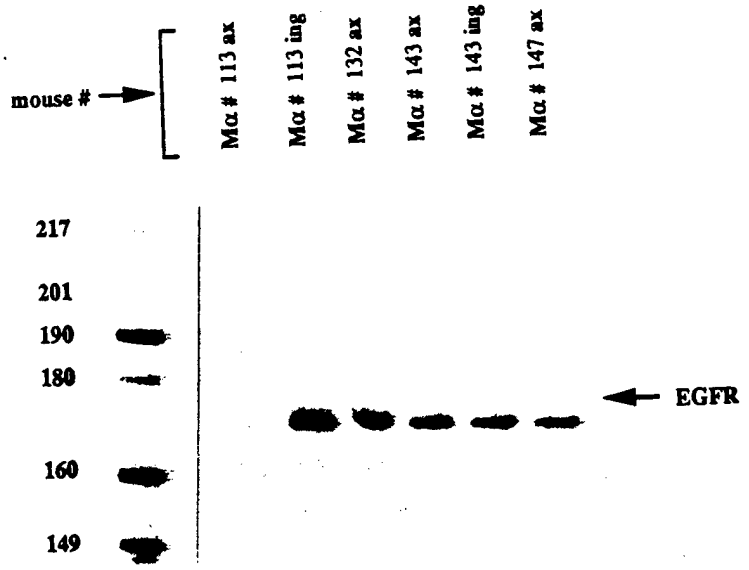
[a] Histopathology from both virgin females and males. All other diagnoses are from virgin females only. NA, not available (all animals of this genotype are deceased at this time point).

Figure 1

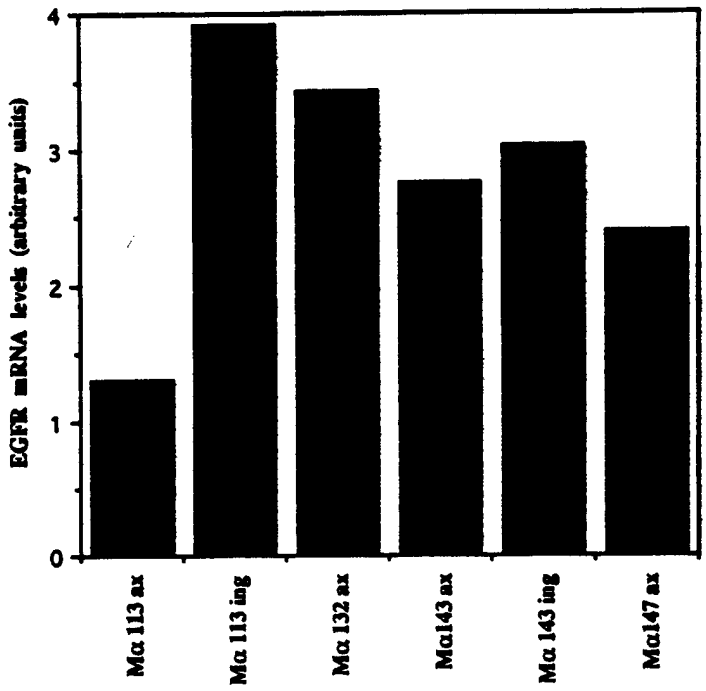


Northern blot analysis showing expression of the *TGFα* and *c-myc* transgenes in mammary gland tumors from double transgenic animals and in normal glands from single transgenic and wild-type animals. The endogenous, mouse TGFα transcript is not seen here. Loading controls are the 18S ribosomal RNA bands.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

Figure 2



b



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 Mα 147 denotes the number of each double transgenic mouse used in the assay. *Ax*, axillary gland tumor; *ing*, inguinal gland tumor.

The absence of the Myc transgene was associated with a lack of detectable apoptosis. Apoptosis was suppressed *in vitro* in c-Myc cells by addition of TGFα, EGF, FGF-2, and IGF-1; the EGF receptor-mediated effects were selectively blocked with an EGF receptor-selective tyrosine kinase inhibitor. Finally, using several assays, we confirmed the high level of c-Myc-associated apoptosis *in vitro*, selectively in single transgenic tumors. These results have been published (2, see Appendix). As a followup, we observed that TGFα induction of the survival factor, Bcl-X_L was closed associated with suppression of apoptosis in c-Myc-overexpressing mouse mammary tumor cells *in vitro* and *in vivo* (3, see Appendix). Next, we established that the action of c-Myc on the mammary epithelial cell cycle is to shorten the G1 phase and facilitate the G1/S transition. This appears to be due to diminished p27, activation of cdk2, and phosphorylation of Rb (4, see Appendix). These cell cycle defects are also associated with chromosomal instability (5, See Appendix).

The final goal of the study is to evaluate paracrine interactions of the two transgenes. In the first year of our work, we encountered a probable immunologic barrier to our cross-transplantation experiments due to different backgrounds of Myc and TGFα transgenic mice. However, Charles River breeders then backcrossed their Myc mice into the FVB strain (identical to the TGFα background). Using skin grafts, we then confirmed that c-Myc tissue is immunologically compatable when transplanted to the TGFα strain, and *vice versa*. These paracrine transplantation experiments have been all set up. Observations of the final two groups continue; tumors and glands will be analyzed in the coming months (See Chart 1, below, Reference 6, and Appendix).

Chart 1 - Ongoing Paracrine Interaction Studies

Experiment	Epithelial Source	Fatpad Source*	N	Status
1A	Myc (Tissue)	NT (cleared)	20	Completed, See Table III
1B	Myc (Tissue)	TGFα (cleared)	16	and Fig 3
2	Myc/TGFα (Tissue)	NT (cleared)	14	Completed, See Table III and Fig 3
3	Myc (Tissue)	NT (not cleared)	20	Completed, See Table III
	Myc (Tissue)	TGFα (not cleared)	20	Completed, See Table III
4A	Myc (cells) + TGFα (cells) (mixed 1:10, 1:1, 10:1)	NT (cleared)	9	2 months post-transplant, See Table III
4B	Myc (cells) + TGFα (cells) (mixed 1:10, 1:1, 10:1)	NT (cleared)	9	3 months post-transplant, See Table III

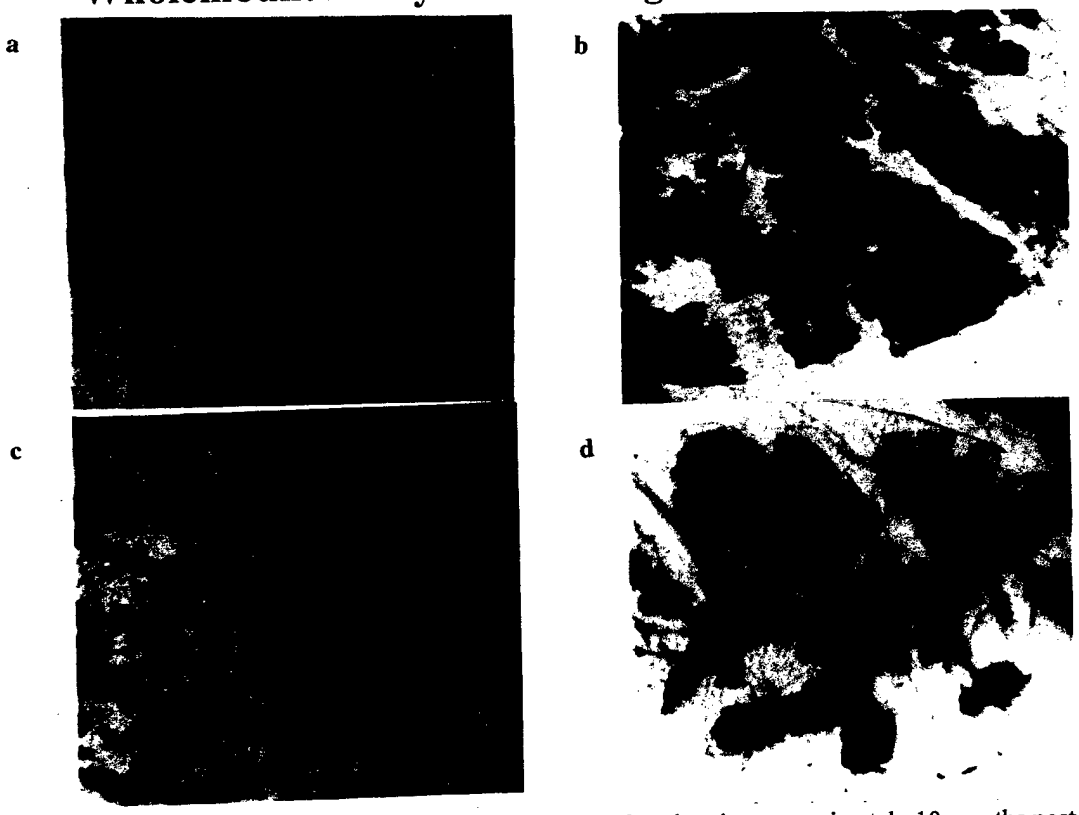
* NT = Non transgenic

Table 3
Latency and Frequency of Transgenic Gland Models

Epithelial Source	Fatpad Source *	Tumor Frequency**	Mean Tumor Latency (days)**
Myc tissue	NT (cleared)	5/28 (17.9%)	342 ± 67
Myc tissue	TGFα (cleared)	3/16 (18.8%)	382 ± 50
Myc/TGFα (tissue)	NT(cleared)	9/10 (90%)	50 ± 3
Myc tissue	NT (not cleared)	3/14 (21.2%)	316 ± 112
Myc tissue	TGFα (not cleared)	2/9 (22.0%)	318 ± 34
Myc/ TGFα (cells) [1:10, 1:1, 10:1]	NT (cleared)	ND	ND

*NT = non transgenic
**ND = data collection not yet completed

Figure 3
Wholemount Analysis of Transgenic Gland Models



Representative mammary gland whole mounts taken from adult virgin female mice approximately 10 months post transplant. **a)** #1 non-transplanted "host" gland from normal FVB. **b)** #4 gland from same animal (a) that was cleared of its epithelial rudiment, then transplanted with c-myc transgenic mammary tissue. **c)** #1 non-transplanted "host" gland from TGFα transgenic mouse. **d)** #4 gland from same animal (c) that was cleared of its epithelial rudiment, then transplanted with c-myc transgenic mammary tissue. No perceptable difference was observed in gross morphology between normal FVB and TGFα animals with c-myc tissue-repopulated glands.

The purpose of the experiments in Chart 1 is to test whether TGFα, released by stromal compared to epithelial cells in the mammary fatpad, is sufficient to drive tumorigenesis in Myc-overexpressing mammary epithelium. The first four of these experiments have now completed. No differences in Myc-induced tumor latency or frequency were observed relative to TGFα status of the cleared or uncleared host fatpads; the TGFα/myc positive control functioned as expected (Table 3). In addition, as seen in Fig 3, no TGFα-related differences were observed in the morphologies of the transgenic Myc glands which did not undergo tumorigenesis. The purpose of experiment 4 in chart 1 will be to determine if the transgenic, Myc-overexpressing epithelial cells can form tumors if they are exposed to transgenic TGFα-overexpressing cells in a contiguous developmental, paracrine context at a ratio of 1:1, 1:10, or 10:1. These experiments have all been set up; observations will continue and tumors will be processed according to protocols for one year post-implantation.

Additional progress has been made over the course of the grant on other studies closely related to our initial specific aims and now included in our approved revised aims or carried out in a collaborating laboratory (see Appendix). We observed that c-Myc and TGFα single transgenic tumors express aberrantly processed forms of the EGF family members amphiregulin, and cripto-1 (7, see Appendix). TGFα and its family members amphiregulin and cripto-1 were each capable of causing preneoplastic growth of the mouse mammary gland when applied as retroviral constructs to form a transgenic mammary gland (8,9, and unpublised data, see Appendix). In addition, we observed that in analogy to TGFα, amphiregulin (but not cripto-1) is inducable in human breast cancer cells, by both estrogen and by phorbol ester activators of protein kinase C (10, see Appendix). We also collaborated with another laboratory using our TGFα transgenic mice and observed that these mice exhibited preneoplastic-appearing female reproductive tract lesions. However, these lesions were non-progressing; DES induced fully malignant lesions and positively interacted in this respect in the reproductive tract (but not mammary glands) with TGFα (11,12, see Appendix). Finally, we published four review articles on various aspects of the subject of this grant (13-16, see Appendix).

Key Research Accomplishments

- Characterization of the first *in vivo* transgenic model of c-Myc-growth factor interaction in cancer (TGFα-c-Myc)
- First demonstration that the EGF receptor can deliver a survival signal to cells (through Bcl-X_L).
- First characterization of the effects of c-Myc overexpression on the cell cycle and cell death of epithelial cells (human and mouse mammary epithelial cells)
- First application of multicolor spectral karyotype analysis (SKY) to mouse chromosomes (for study of c-Myc-induced chromosomal instability)
- First application of retroviral technology to study growth factor - mammary gland interactions *in vivo* (amphiregulin, cripto-1, and TGFα)
- First demonstration that TGFα interacts with diethylstilbestrol (DES) to promote tumorigenesis (female reproductive tract, but not mammary gland)

Reportable Outcomes

- **Manuscripts**

1. 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 salivary gland tumorigenesis. Cell Growth and Different, 6:737-748, 1995.

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- **Presentations at Meetings**

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14. Nass SJ, Amundadottir LT, Johnson MD, and Dickson RB: Interaction of c-myc and transforming growth factor α/epidermal growth factor in breast tumor progression, US Army Breast Cancer Meeting, Washington DC, 1997.

- **Degrees obtained that were supported by this award:**

  PhD in Cell Biology awarded to Laufey T. Amundadottir
  PhD in Cell Biology awarded to Isabel Martinez-Lacaci
  PhD in Cell Biology awarded to Sharyl Nass

- **Development of Cell Lines**

  Myc 83, Myc 5, Myc 7, Myc 9, Myc α75, MT100

- **Animal Models**

  MMTV-c-myc X MT-TGFα cross

- **Funding applied for (and obtained) based on work supported by this award**

  NIH R01 AG 1496, "Apoptosis and malignant progression in mammary tumor cells"
  NIH R01 CA 72460, "EGF family induction of mammary hyperplasias and tumors"

- **Employment/research opportunities applied for (and obtained) based on experiences/training supported by this award:**

  Postdoctoral position for Laufey T. Amundadottir, PhD with Dr. Philip Leder at Harvard Univesity
  Postdoctoral position for Isabel Martinez-Lacaci, PhD with Dr. David Salomon at NCI, NIH
  Postdoctoral position for Sharyl Nass, PhD with Dr. Nancy Davidson at Johns Hopkins University
  Senior Staff Fellow position for Nicholas Kenny, PhD with Dr. Carl Barrett at NIEHS, NIH.

Conclusions

1. Bitransgenic TGFα/Myc tumors are derived from mammary epithelial cells and contain estrogen, progesterone, and EGF receptors.

2. While multiple copies of the two transgenes were detected, other related growth control genes were not amplified in the bitransgenic tumors.

3. Bitransgenic tumors provide a strong selection for further overexpression at the mRNA level of transgenes and expression of high levels of Max (Myn) and Cyclin D1.

4. The mechanisms of the tumorigenic interaction of TGFα and Myc included cooperative stimulation of proliferation, anchorage independent colony formation, and suppression of apoptosis.

5. One aspect of TGFα-suppression of apoptosis involved induction of the survival-promoting Bcl-X_L gene.

6. Another important aspect of Myc-initiated tumors involved shortening of the G1 phase of the cell cycle; this appeared due to p27 down-regulation, cdk-2 activation, and Rb phosphorylation. Both Myc and Myc/TGFα tumors exhibited chromosomal instability.

7. Transgenic tumors also expressed the TGFα-family growth factors amphiregulin and cripto-1, but in a variety of unusual isoforms.

8. Amphiregulin and cripto-1, like TGFα, were capable of induction of preneoplastic outgrowths of the mouse mammary gland.

9. Amphiregulin mRNA and protein synthesis are upregulated by estrogen and by protein kinase C in human breast cancer cells, in analogy to the previously-described regulation of TGFα.

10. Female TGFα transgenic mice are susceptable to reproductive tract abnormalities and to DES-induced neoplasias, in addition to Myc-induced mammary tumors. However, TGFα and DES did not interact to promote tumors in the mammary glands.

11. In summary, these results strongly support the initial premises of the grant; that the c-Myc transcription factor and the TGFα family of growth factors are each potent modulators of mammary tumor onset and progression. Binary interaction of Myc and TGFα in the mouse mammary gland allows rapid tumor development due to a synergistic, multifactorial interaction, that encompasses disregulation of the all cycle, apoptosis, and chromosomal stability.These studies should lead to better understanding of the pathophysiology of breast cancer and could highlight new opportunities for prevention and therapy of the disease.

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Appendix

Revised Statement of work (approved by USARMC 1/5/98)

Final Bibliography

Published Articles.

Personnel Receiving Pay from this Research Effort

DAMD-17-J-4257
Statement of Work
(Revised)

Year 1:

a. Establish the time course of bitransgenic c-myc-and TGFα -induced hyperplasias and tumor development (status: completed)

b. Evaluate salivary tumorigenesis in bitransgenic myc- TGFα tumors (status: completed)

c. Examine expression of amphiregulin, and other TGFα family members in transgenic mammary tumorigenesis (status completed)

Year 2:

a. Characterize tumors for steroid receptors and differentiation markers (status: completed)

b. Characterize tumors for amplification in genes encoding TGFα, Myc, EGF receptor, myn and cyclins (status: completed)

c. Evaluate mRNA and protein expression for TGFα , myc, EGF receptor, myn, and cyclins (Status: completed)

d. Complete skin graft tissue compatability experiment (status: completed)

Year 3:

a. Establish the molecular basis for TGFα -myc interaction at the levels of cell cycle, apoptosis, and genetic instability (status: completed)

b. Set up long term Year 4a experiments (status: completed)

Year 4:

a. Establish the range of paracrine models whereby TGFα stroma or epithelial cells interact with myc-expressing epithelial cells to modulate tumorigenesis (status: experimental setup completed)

Year 5:

a. Carry out detailed comparison of genetic changes and gene expression in male versus female and autocrine versus paracrine TGFα -myc models (status: experimental setup completed and observations ongoing).

Final Bibligraphy

Publications

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cancer, Breast J., 3:15-25, 1997.

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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

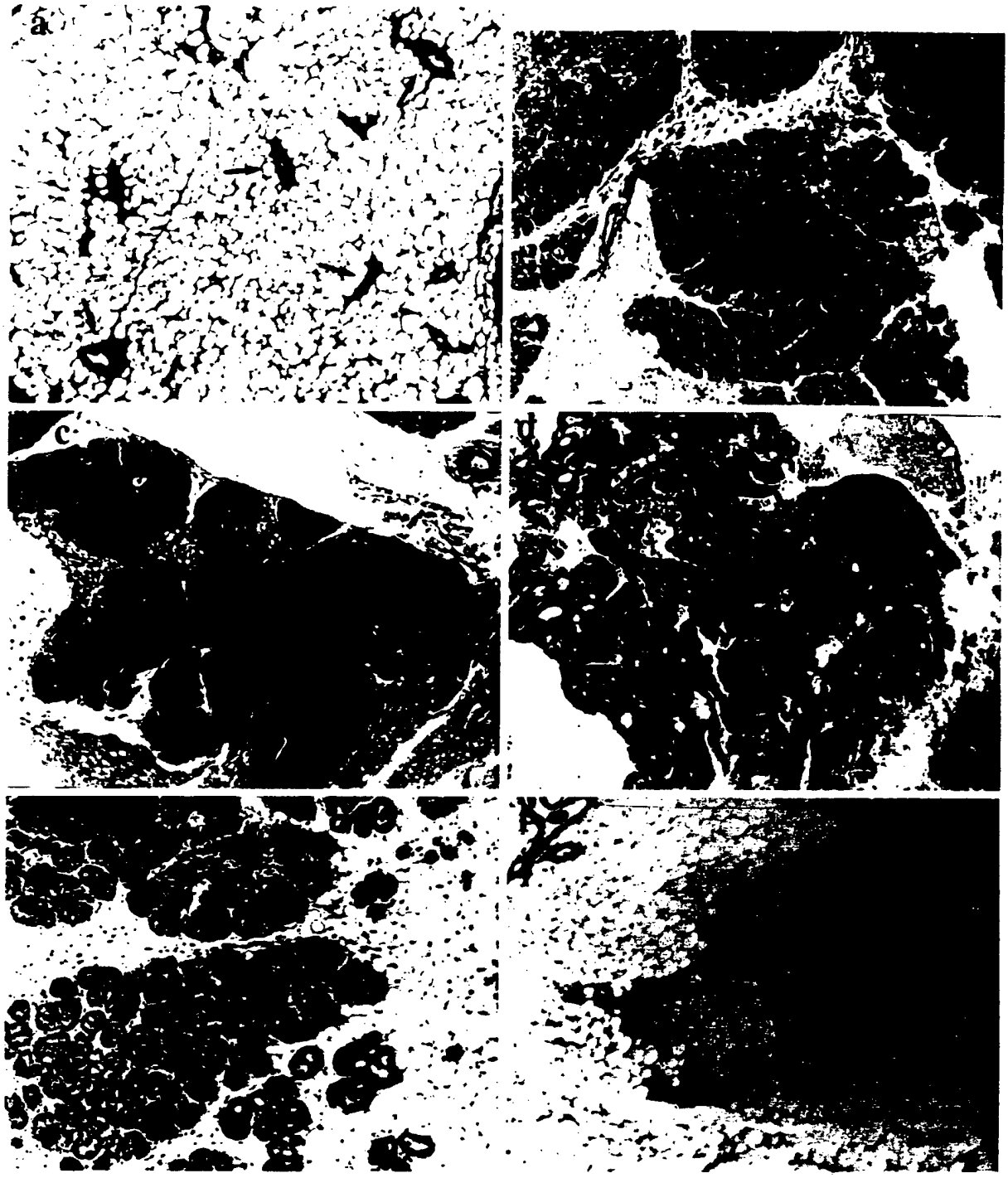


Fig. 1. Hematoxylin & eosin-stained sections of mammary glands. *a*, a normal virgin gland at 3 months of age. It is representative of TGFα and c-*myc* single transgenic animals and of nontransgenic littermates at this age. *Arrows*, epithelial ducts. Panels *b–d*, mammary gland tumors from double transgenic 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

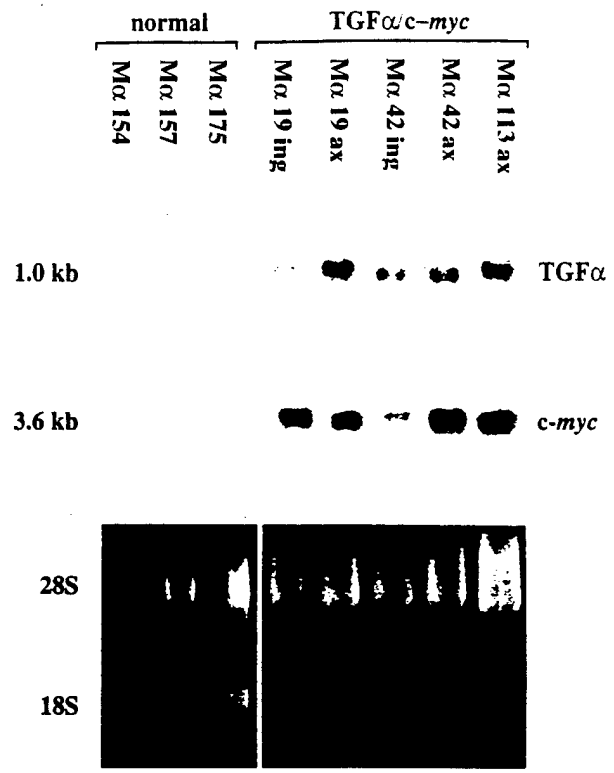


Fig. 3. Northern analysis showing expression of the TGFα and c-*myc* transgenes in mammary gland tumors from double transgenic animals and in normal glands from single transgenic and wild-type animals. The TGFα transcript is 1.0 kb, and the c-*myc* transcript is 3.6 kb. Note that the endogenous 2.3-kb 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,

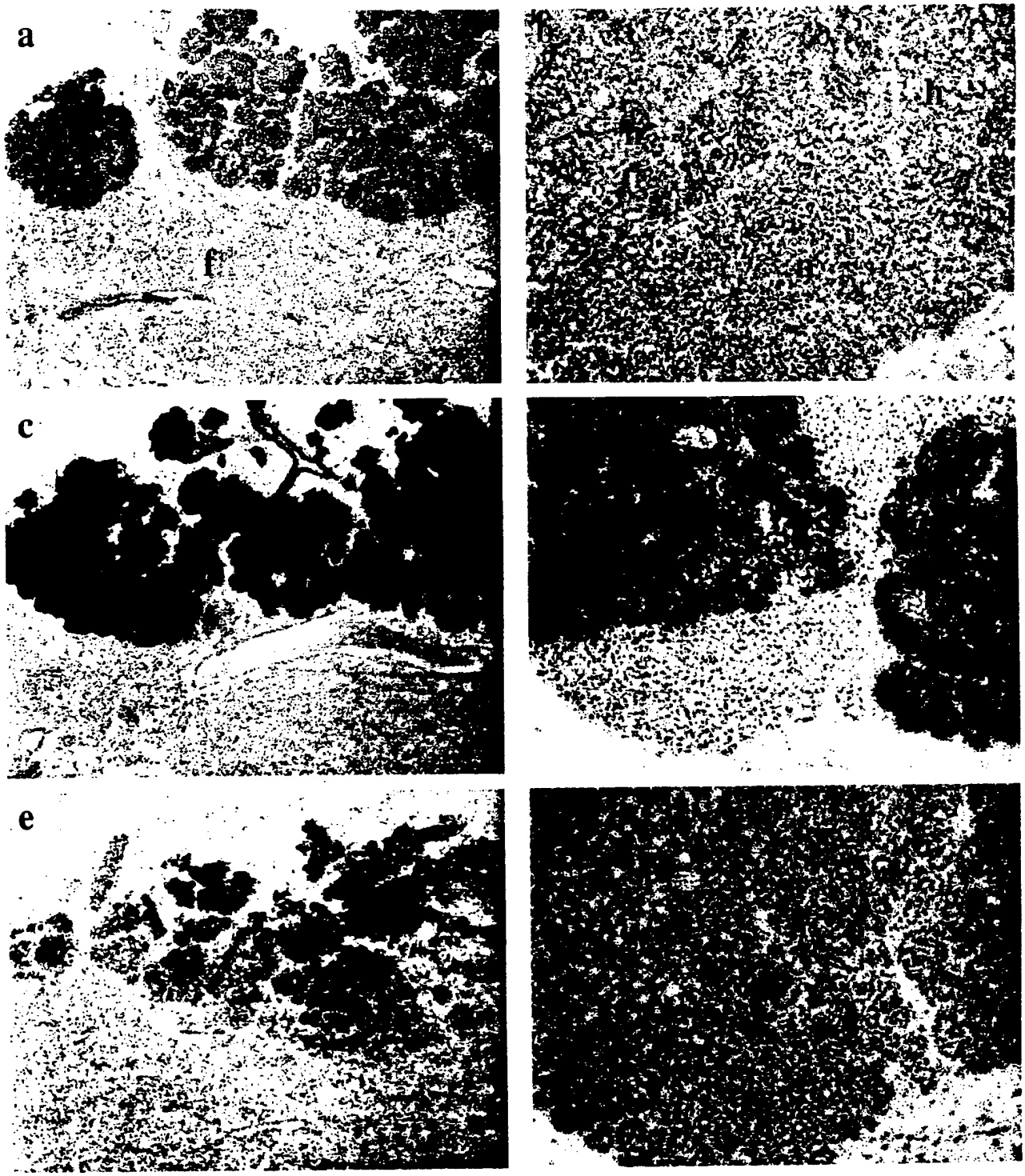


Fig. 4. In situ hybridization analysis of mammary and salivary glands from TGFα/c-*myc* animals. Sequential paraffin-embedded tissue sections were hybridized to ^{35}S-labeled riboprobes generated against the TGFα and c-*myc* transgenes. *a* and *b*, control sections hybridized to sense riboprobes. *c* 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.

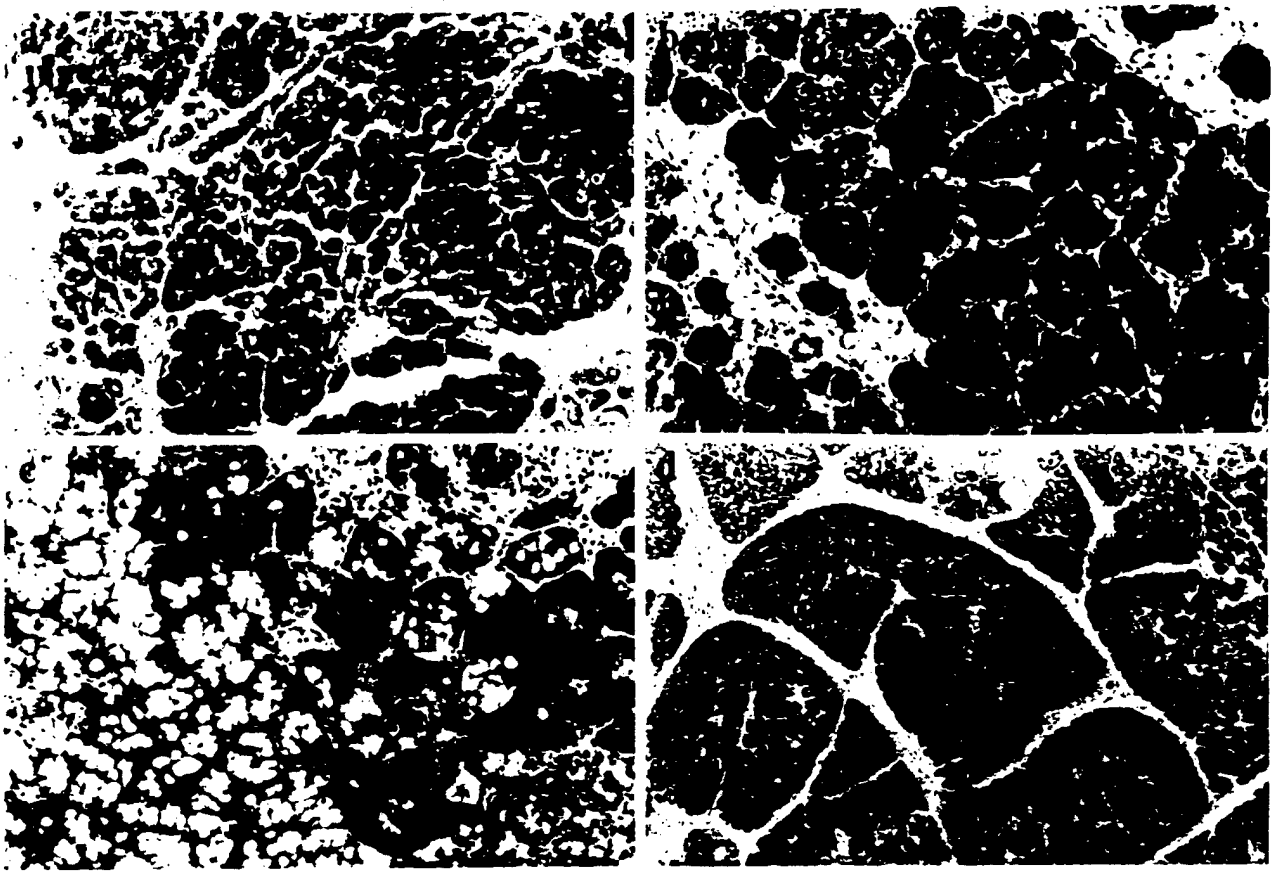


Fig. 5. Immunohistochemical staining of mammary and salivary glands from TGFα/c-*myc* double transgenic animals with an anti-TGFα polyclonal antiserum. *a*, a mammary gland tumor from a 3-month-old animal and *b*, from a 5-week-old animal. Both have a scattered pattern of strong TGFα staining. *c*, an 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.

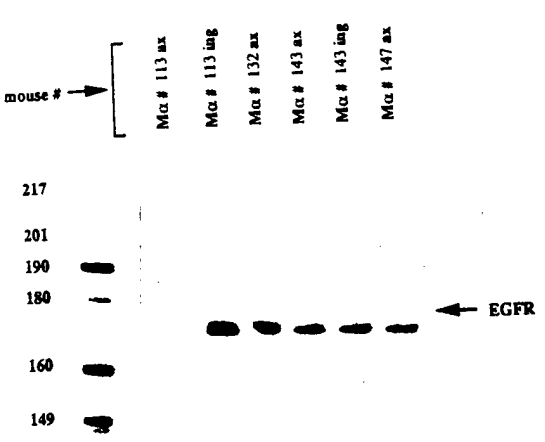
and adenoma and adenocarcinoma in the parotid gland at the same age. Salivary glands of single transgenic TGFα animals showed minimal ductule hyperplasia, but single transgenic c-*myc* mice and transgene-negative littermates were free of pathological abnormalities at 3 months of age (Table 3; Fig 7).

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*



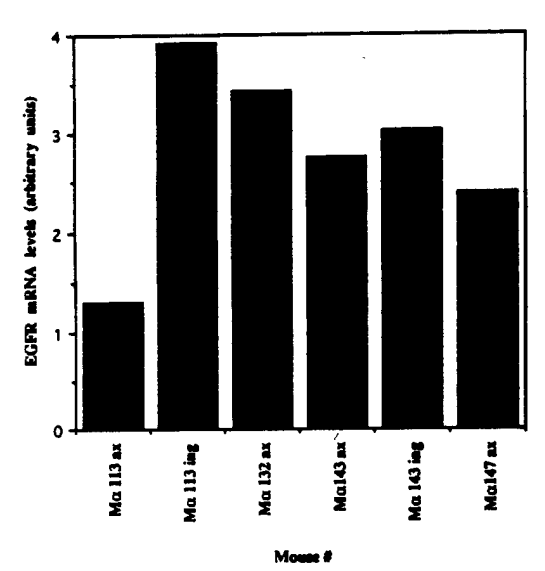


Fig. 6. Expression of the endogenous EGFR mRNA measured by RNase protection assay. RNA levels were compared between mammary gland tumors from double transgenic animals. *b*, a scanned version of the data in *a*. Mα113 through Mα147 denotes the number of each double transgenic mouse used in the assay.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

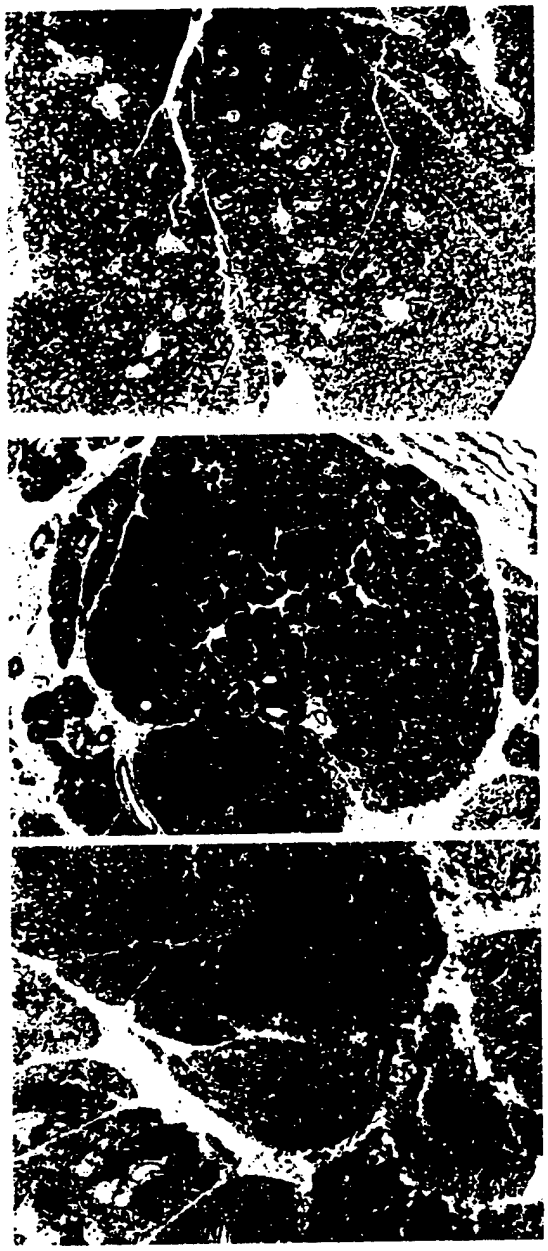


Fig. 7. Hematoxylin & eosin-stained sections of salivary glands. *a*, a normal parotid gland from a wild-type mouse. *b*, a parotid adenoma and *c*, an adenocarcinoma from TGFα/c-*myc* double transgenic animals. All animals are about 3 months old. ×100.

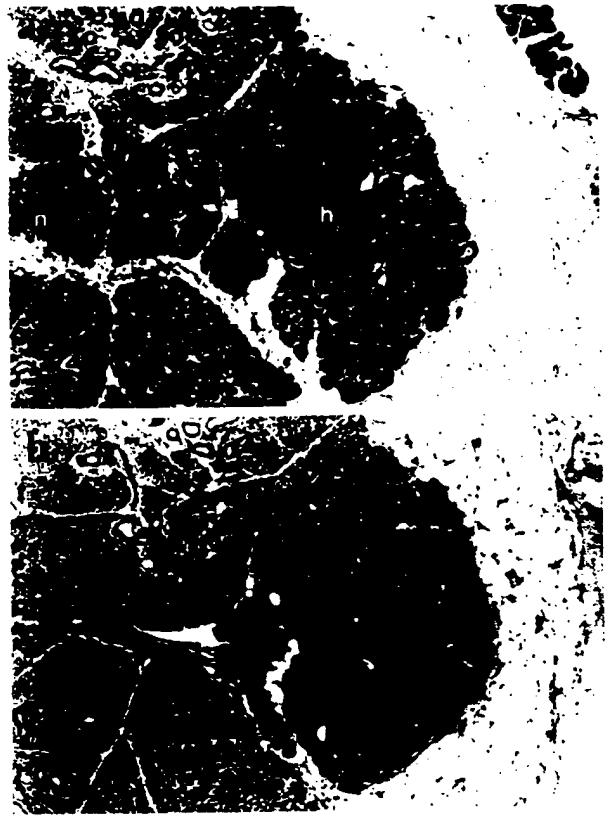


Fig. 8. Immunohistochemical staining of proliferating cell nuclear antigen in salivary glands from a 6-week-old double transgenic TGFα/c-*myc* animal. *a*, a hematoxylin & eosin-stained section of the salivary gland and *b*, a sequential section stained for proliferating cell nuclear antigen. Note intense 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.

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.

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).

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Cooperation of TGFα and c-Myc in mouse mammary tumorigenesis: coordinated stimulation of growth and suppression of apoptosis

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We have previously shown that TGFα and c-Myc interact in a strong, synergistic fashion to induce mammary gland tumors in double transgenic mice. Here we show this interaction can be explained, at least in part, by a cooperative growth stimulus by the two proteins, and by TGFα-mediated inhibition of c-Myc-induced apoptosis. We initially compared rapidly progressing mammary tumors from double transgenic mice to long latency tumors from single transgenic mice and observed a striking difference in the occurrence of apoptosis among the three groups. Tumors exhibiting apoptosis were derived exclusively from mice that expressed the c-*myc* transgene in the absence of the TGFα transgene, indicating that TGFα might protect c-Myc-overexpressing cells from programmed cell death. Cell lines were derived from single and double transgenic mammary tumors to examine further the mechanism underlying the cooperative interaction between the two gene products. In accordance with our *in vivo* data, apoptosis was only detected when the c-*myc* transgene was expressed without the TGFα transgene. Furthermore, exogenous addition of TGFα inhibited apoptosis in cells overexpressing c-Myc alone. In addition, tumor-derived cells that overexpressed both TGFα and c-Myc exhibited faster growth rates *in vitro* and *in vivo* and were less sensitive to the inhibitory effects of TGFβ *in vitro* compared to cell lines expressing only one of the transgenes. Based on our findings we propose that TGFα acts both as a proliferative and a survival factor for c-Myc-expressing tumor cells. Our results indicate that TGFα and c-Myc cooperate in tumorigenesis *via* a dual mechanism: TGFα can inhibit c-Myc-induced apoptosis and both proteins provide a growth stimulus.

Keywords: c-Myc; TGFα; apoptosis; mammary tumorigenesis

Introduction

It is well documented that overexpression of the proto-oncogene c-*myc* can induce proliferation, transformation, and apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992; Kato and Dang, 1992; Khazaie *et al.*, 1991; Marcu *et al.*, 1992; Meichle *et al.*, 1992; Telang *et al.*, 1990; Valverius *et al.*, 1990). It has also been reported by a number of investigators that c-Myc can cooperate with growth factors such as transforming growth factor alpha (TGFα) or epidermal growth factor (EGF) to promote a transformed phenotype *in vitro* (Khazaie *et al.*, 1991; Stern *et al.*, 1986; Telang *et al.*, 1990; Valverius *et al.*, 1990). We and others have recently shown that c-Myc and TGFα synergize in an extremely strong way to induce mouse mammary gland tumors in transgenic mice *in vivo* as well (Amundadottir *et al.*, 1995; Sandgren *et al.*, 1995). In order to understand the mechanisms responsible for this interaction, we were interested in examining proliferation, anchorage independent growth, and apoptosis as possible points of interaction between TGFα and c-Myc that may enhance tumorigenesis in the mammary gland.

Both gene products have been implicated in the genesis of many human cancers, including breast tumors. The c-*myc* gene is frequently found amplified and/or overexpressed in human breast cancer (Bonilla *et al.*, 1988; Escot *et al.*, 1986; Garcia *et al.*, 1989; Mariani-Costantini *et al.*, 1988). Although TGFα is not amplified at the gene level in human breast cancer, its expression (and that of other EGF family members) is frequently increased compared to the normal gland (Artega *et al.*, 1988; Bates *et al.*, 1988; Derynck *et al.*, 1987; Perroteau *et al.*, 1986; Travers, 1988). In addition, various groups have reported a tumorigenic action of these genes when overexpressed in the mammary gland of transgenic mice (Jhappan *et al.*, 1990; Leder *et al.*, 1986; Matsui *et al.*, 1990; Sandgren *et al.*, 1990; Schoenenberger *et al.*, 1988; Steward *et al.*, 1984).

Apoptosis is an active process whereby the cell is programmed to carry out a series of events that eventually lead to its auto-destruction. When apoptosis is initiated, cells undergo various biochemical and morphological changes which result in the degradation of genomic DNA and fragmentation of the cell into apoptotic bodies (Bellamy *et al.*, 1995). Apoptosis occurs during development as well as in adult organisms and can be activated or inhibited by specific agents, such as hormones or growth factors (Schwartzman and Cidlowski, 1993). Inhibition of apoptosis can also contribute to tumorigenesis.

The c-Myc protein has been implicated in the regulation of apoptosis. When c-Myc expression is deregulated, cells are prone to enter an apoptotic pathway, depending on the cell environment (Askew *et al.*, 1991; Evan *et al.*, 1992). A number of survival factors which protect cells from c-Myc-mediated

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apoptosis have been identified (Askew *et al*., 1991; Harrington *et al*., 1994). In mouse fibroblasts for example, apoptosis induced by c-Myc was inhibited by various growth factors, such as insulin-like growth factors and PDGF, whereas EGF and bFGF were ineffective. In the current study, we show that TGFα can function as a survival factor for mammary epithelial cells which overexpress c-Myc. We observed that apoptosis occurred in mouse mammary gland tumors and in their derived epithelial cell lines only in cases where c-Myc was overexpressed in the absence of TGFα overexpression. Furthermore, when c-Myc overexpressing cells were treated with TGFα *in vitro*, apoptosis was greatly decreased. Our results suggest an explanation for the cooperative interaction between TGFα and c-Myc in tumorigenesis: both factors stimulate anchorage dependent proliferation and anchorage independent growth, and in addition, TGFα suppresses c-Myc-induced apoptosis.

Results

*Programmed cell death occurs only in mammary gland tumors from c-*myc *single transgenic mice*

To examine whether apoptosis was a factor in the cooperation between TGFα and c-Myc in tumorigenesis, we measured apoptosis in five mammary gland tumors from each of the three transgenic mouse strains (double transgenic TGFα/c-*myc* mice, and single transgenic TGFα and c-*myc* mice). *In situ* nick end-labeling of nucleosomal fragments by Klenow DNA polymerase I revealed that apoptosis was occurring in mammary gland tumors from c-*myc* transgenic animals and not in mammary tumors from single transgenic TGFα animals or double transgenic TGFα/c-*myc* animals. As shown in Figure 1c, only tumors from c-*myc* mice exhibited scattered cells with positive staining. When apoptosis was quantitated by counting apoptotic cells in 20 random fields of each tumor type (400 × magnification) we observed that tumors from c-*myc* mice had 23.0 ± 2.8 apoptotic cells per field whereas tumors from TGFα and TGFα/c-myc animals had 0.3 ± 0.2 and 1.2 ± 0.5 labeled cells per field respectively.

Figure 1 Detection of apoptosis in mammary gland tumors from transgenic mice. Tumor sections were analysed by *in situ* nick end-labeling of DNA fragments. (**a**) shows a tumor from a double transgenic virgin TGFα/c-*myc* mouse, (**b**) a tumor from a single transgenic multiparous TGFα mouse and (**c**) a tumor from a single transgenic c-*myc* mouse. Note cytoplasmic staining in scattered cells of the tumor from a c-*myc* single transgenic animal (arrows, **c**) indicating DNA fragmentation

Generation of cell lines from mammary gland tumors

We have generated three cell lines from mammary gland tumors arising in double and single transgenic mice. Our intent was to use them to verify our findings in mammary gland tumors *in vivo* and examine further the molecular mechanisms underlying the cooperation between TGFα and c-Myc. The following nonclonal cell lines were generated: TGFα/Myc#75 was derived from a tumor arising in a double transgenic virgin female (TGFα/c-*myc* animal number 75), Myc#83 was derived from a c-*myc* virgin single transgenic female (c-*myc* animal number 83) and TGFα#13 from a multiparous TGFα single transgenic female (TGFα animal number 13). Additional cell lines from tumors arising in c-*myc* and TGFα/c-*myc* animals have recently been isolated and were used where noted to confirm our findings with the first three cell lines.

Expression of cytokeratins and morphology of cell lines grown as subcutaneous tumors in nude mice was used to verify epithelial origins of the cell lines. The single transgenic cell lines TGFα#13 and Myc#83 were positive for keratin 14 at the mRNA level (not shown). TGFα/Myc#75 cells apparently did not express keratin 14, but positive immunofluroescent signal was observed in these cells with a pan-keratin antibody (not shown). All three lines also gave rise to tumors in nude mice that had a very distinct epithelial morphology. We therefore conclude that all three lines are epithelial, but that line TGFα/Myc#75 has probably lost expression of some of its keratins. This is not without precedent, since human breast

carcinoma cell lines, especially hormone independent lines, have been described to do the same (Sommers *et al*., 1989, 1992).

Expression of the TGFα and c-*myc* transgenes was also assessed by Northern analysis (not shown). The single transgenic lines, TGFα#13 and Myc#83, expressed only the TGFα or c-*myc* transgenes, respectively. In contrast, the double transgenic cell line, TGFα/Myc#75, expressed both transgenes. Therefore, all three cell lines expressed the expected transgenes at the mRNA level. In addition, expression of the TGFα transgene was upregulated by ZnC12 and CdC12 as expected, since the TGFα transgene is expressed from the heavy metal-inducible metallothionein promoter (Jhappan *et al*., 1990). The endogenous c-*myc* gene was downregulated in the two cell lines expressing the c-*myc* transgene (TGFα/Myc#75 and Myc#83), consistent with a negative autoregulation of the c-Myc protein on its own promoter, as has been described previously (Penn *et al*., 1990).

In order to assess ploidy of the cell lines, cells were stained with propidium iodide and analysed by FACS. Each cell line was tested at two or three different timepoints (between passages 6 and 31). Only the double transgenic line TGFα/Myc#75 was found to be aneuploid. It was tetraploid at all three timepoints tested. In contrast, the two single transgenic lines were diploid at the time points tested (not shown).

Apoptosis occurs in c-Myc overexpressing mammary tumor cells in vitro

Tumor derived cell lines were tested for their ability to undergo apoptosis by two independent methods. For the first method, cytoplasmic DNA fragments were isolated and run on agarose gels. In this assay, the Myc#83 line was positive, with a characteristic nucleosomal ladder whereas both TGFα#13 and TGFα/Myc#75 were negative (Figure 2a). Those results were confirmed by an ELISA apoptosis assay that is based on detecting histone-associated DNA in cytoplasmic cell lysates via a peroxidase catalyzed color change (A_{405}). CEM cells (a T cell leukemia cell line) treated with 10^{-7} M dexamethasone served as a positive control (Catchpoole and Stewart, 1993; not shown). The Myc#83 line showed a high degree of apoptosis, whereas the other two had levels close to background (Figure 2b). Five additional cell lines derived from Myc-single transgenic tumors also showed a propensity to undergo apoptosis as determined by the apoptosis ELISA (not shown). The appearance of apoptotic Myc#83 cells under conditions of EGF deprivation or TGFβ1 treatment was further confirmed by observing morphological changes which are characteristic of apoptosis (Figure 2c). The cells displayed prominent apoptotic bodies with concomitant reduction of cytoplasm and altered nuclear morphology.

When the Myc#83 line was treated with the growth factors TGFα, EGF, IGF-I or bFGF, apoptosis was inhibited up to 75% as measured by the ELISA assay (Figure 3a). In contrast, treatment with TGFβ1 resulted in elevated levels of apoptotic DNA, even in the presence of EGF. The effects of both TGFα and TGFβ1 on apoptosis were concentration dependent (Figure 3b and c), with maximal responses at 10 ng/ml and 100 pM, respectively.

Additional evidence for the importance of the TGFα/EGF receptor system was provided by using a synthetic inhibitor of EGF receptor tyrosine kinase activity (PD153035). PD153035 has been shown to

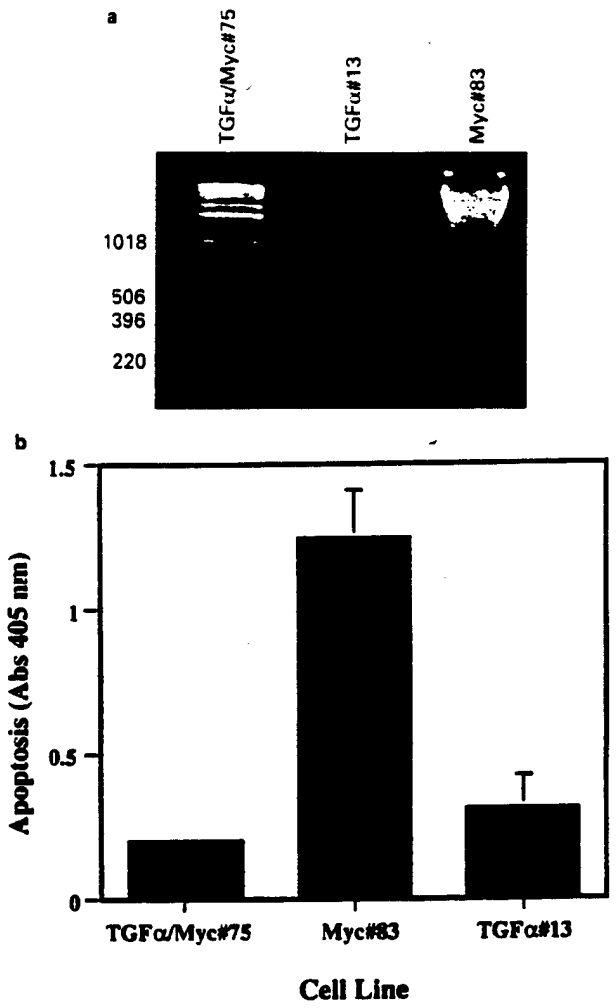
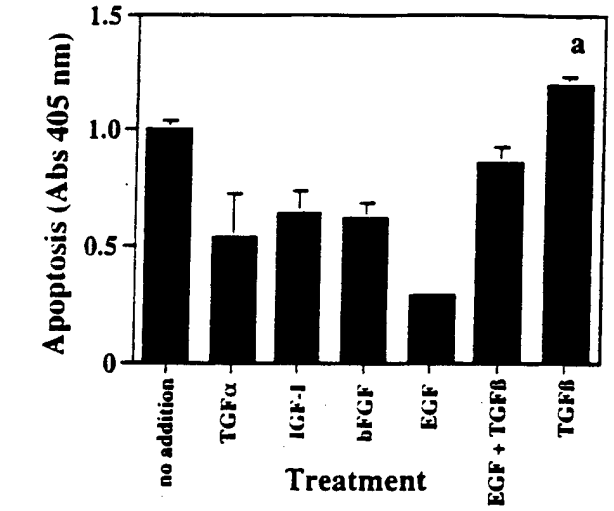
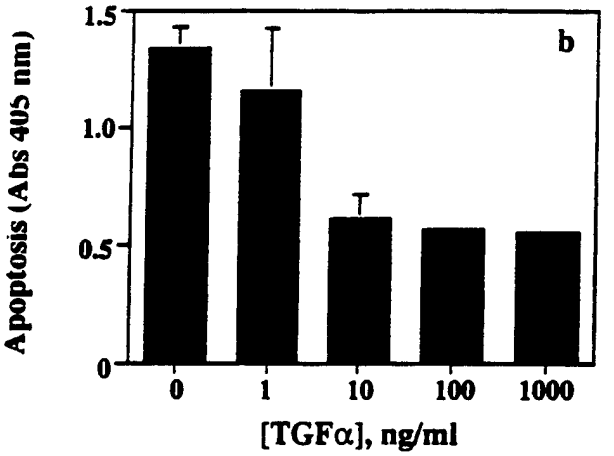


Figure 2 Detection of apoptosis in tumor-derived cell lines. (**a**): Nucleosomal ladder. DNA was isolated from cytoplasmic fractions of transgenic tumor cell lines and run on a 1.8% agarose gel which was stained with ethidium bromide. Note characteristic nucleosomal DNA with a size interval of approximately 180 bp. (**b**): ELISA-apoptosis assay. Apoptotic DNA fragments were detected in cytoplasmic lysates *via* a histone-DNA ELISA. Peroxidase substrate conversion was quantitated by measuring absorbance at 405 nm. (**c**): Morphological changes in apoptotic Myc#83 cells. Cells on the left side were grown in media containing EGF. The right side is representative of cells which have become apoptotic via EGF deprivation or TGFβ1 treatment, for 24 h. Note the characteristic reduction in cell size due to cytoplasmic blebbing (apoptotic bodies)





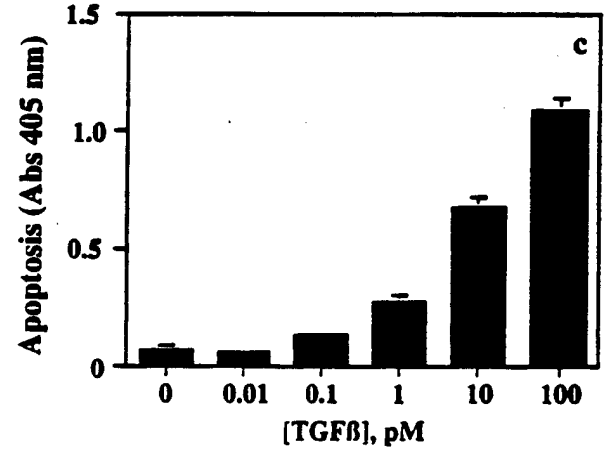


Figure 3 Effects of growth factors on apoptosis in the Myc#83 line. (a) shows the effects of TGFα (5 ng/ml), IGF-I (50 mM), bFGF (8 ng/ml), EGF (10 ng/ml), TGFβ1 (100 pM) or EGF + TGFβ on apoptosis. (b) shows that the inhibitory effect of TGFα on apoptosis in the Myc#83 cell line is concentration dependent. (c) demonstrates that the stimulatory effect of TGFβ1 on apoptosis is also concentration dependent. In all three panels, cells were treated for 24 h prior to harvest for apoptosis ELISA. Each point represents the mean (±SE) of two determinations

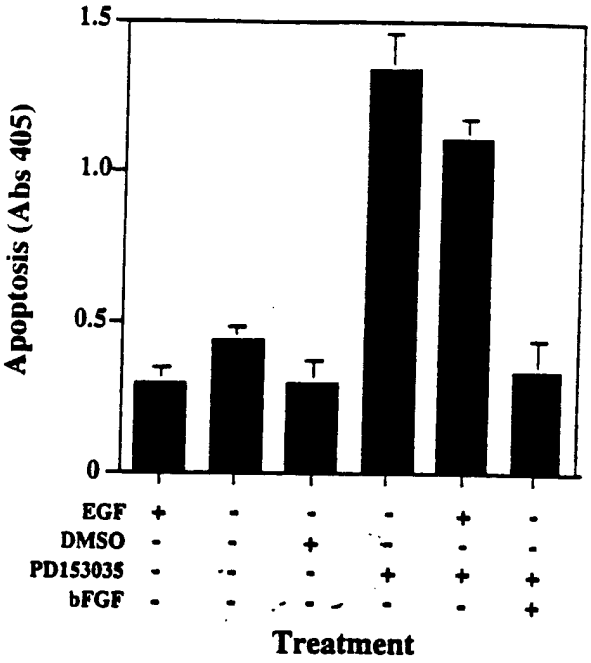


Figure 4 Induction of apoptosis in the TGFα/Myc#75 line by PD153035, a specific inhibitor of EGF receptor tyrosine kinase activity. Cells were incubated for 24 h with the indicated additions (10 ng/ml EGF or bFGF, 10 μM PD153035, 1 μl/ml DMSO as a control) and apoptosis was measured via ELISA assay. $n=4$ (±SE)

specifically downregulate the tyrosine phosphorylation status of the EGFR (Fry *et al.*, 1994). We verified this in our cell system by an anti-phosphotyrosine Western blot which showed that a 170 kb species was reduced greater than 90% in cells treated with the compound, while other phosphotyrosine bands remained constant (not shown). The TGFα/Myc#75 cells became apoptotic when exposed to PD153035 for 24 h (Figure 4). Removing EGF from the growth media of these cells did not affect viability, but exposure to PD153035 in either the presence or absence of EGF induced apoptosis. In contrast, bFGF, which acts through a different receptor tyrosine kinase, could rescue the cells from the effects of the drug.

Anchorage dependent (ADG) and anchorage independent (AIG) growth analysis of tumor derived cell lines

The TGFα/Myc#75 double transgenic cell line had the fastest ADG growth rate *in vitro* under normal growth conditions (doubling time of 16.7 h±0.4 h). Myc#83 and TGFα#13 had much longer doubling times of 33.4 h (±1.7) and 35.0 h (±0.82) respectively when grown in normal media containing EGF (Figure 6a). Growth rates of all three cell lines were similar when EGF was replaced with TGFα (not shown). The two cell lines that overexpress TGFα (TGFα/Myc#75 and TGFα#13) were able to grow in the absence of exogenous EGF, but with a significantly reduced growth rate (Figure 5a). Two additional TGFα/Myc cell lines also exhibited relatively fast growth rates and were not dependent on exogenous EGF for growth or survival, similar to TGFα/Myc#75 (not shown). In

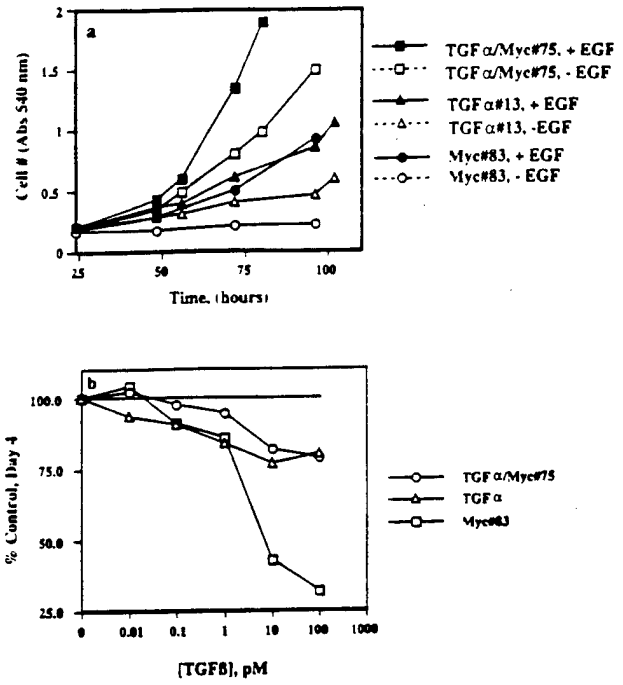


Figure 5 Co-expression of TGFα and Myc results in cooperative growth stimulus under anchorage dependent conditions. (**a**): Cells were grown in the presence or absence of EGF in 96 well plates for the indicated times and stained with crystal violet. (**b**): Cells were grown in the presence of EGF and increasing concentrations of TGFβ1 for 3 (TGFα/Myc#75) or 4 (Myc#83, TGFα#13) days and then stained with crystal violet. For (**a**) and (**b**), $n=8$ ($\pm$SE)

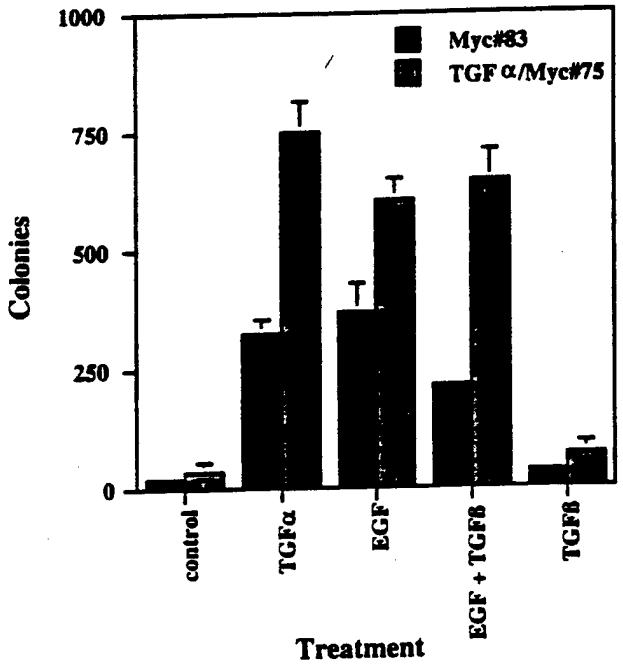


Figure 6 Co-expression of TGFα and Myc results in cooperative growth stimulus under anchorage independent conditions. Cells were suspended in 0.3% agar with 10% FCS and the following additions: TGFα (5 ng/ml), EGF (10 ng/ml), TGFβ1 (100 pM) or EGF + TGFβ1. Colonies were counted on day 7 (TGFα/Myc#75) or day 10 (Myc#83). $n=3$ ($\pm$SD). Results for TGFα#13 are not shown since that cell line did not grow well in soft agar under any of the above conditions

contrast, Myc#83 cells were completely dependent on exogenous EGF and showed no significant growth in its absence. However, FACS analysis demonstrated that Myc#83 cells were not arrested in G0/G1 when deprived of EGF (not shown). Those results are consistent with the hypothesis that Myc overexpressing cells are unable to withdraw from the cell cycle and undergo apoptosis in the absence of EGF.

All three cell lines exhibited concentration dependent inhibition by TGFβ1 under ADG conditions, but sensitivity to the growth factor varied (Figure 5b). At the highest concentrations (10–100 pM), TGFα/Myc#75 and TGFα#13 were marginally responsive to TGFβ, with about 20% fewer cells in treated wells than in untreated controls after 3–4 days in culture. In contrast, Myc#83 cells were quite sensitive to high concentrations of TGFβ1, reflecting the observation that TGFβ induces apoptosis in these cells (Figure 3a,c).

TGFα/Myc#75 and Myc#83 cells both grew well under AIG conditions in the presence of exogenous EGF or TGFα (Figure 6), whereas TGFα#13 cells grew poorly in soft agar. The effects of those growth factors on Myc#83 cells are similar to published results for other Myc-overexpressing breast cells (Telang *et al.*, 1990; Valverius *et al.*, 1990). The rate of colony formation and growth was much higher for the TGFαMyc#75 cells, and a dose response curve showed that those cells were extremely sensitive to TGFα, with optimal induction by only 0.1 ng/ml of the growth factor. Maximal colony formation by the Myc#83 line occurred with 10–30 ng/ml TGFα (not shown). Addition of TGFβ1 significantly reduced the number of Myc#83 colonies stimulated by addition of EGF, but had no effect on TGFα/Myc#75 colony formation (Figure 6).

Tumorigenicity of tumor derived cell lines

Cells were injected into female *nu/nu* mice to establish their *in vivo* tumorigenicity and growth rate. All three cell lines grew readily in nude mice, but with different latency times. The double transgenic line TGFα/Myc#75 formed tumors with a latency period of only 4 weeks, while the single transgenic TGFα#13 and Myc#83 lines formed tumors with a latency period of about 9 weeks. None of the cell lines appeared to have metastatic capabilities over the period of time the tumors were allowed to grow (About 2 months for TGFα/Myc#75 and 3 months for TGFα#13 and Myc#83).

Discussion

Myc overexpression (achieved by gene amplification, translocations and other means) has been strongly implicated in the genesis of many types of human tumors including breast cancer (Bonilla *et al.*, 1988; Cole, 1986; Escot *et al.*, 1986; Garcia *et al.*, 1989; Mariani-Constantini *et al.*, 1988). However, since deregulated c-Myc expression can promote cell death *via* apoptosis, it is likely that the apoptotic pathway(s) induced by c-Myc must be inhibited or inactivated to achieve aggressive tumor formation. That may be accomplished, directly or indirectly, by secondary

events which alter the cell environment (such as growth factor secretion) or gene expression (such as mutations in downstream effectors). In accordance with that hypothesis, we detected apoptosis only in mammary gland tumors that expressed the c-*myc* transgene alone without the TGFα transgene. Tumors from TGFα single transgenic or TGFα/c-*myc* double transgenic mice did not contain apoptotic cells. Van Dyke and co-workers have proposed a similar 'multi-hit' hypothesis of tumor formation based on studies of SV40 T antigen-induced brain tumors (Symonds *et al.*, 1994). In that system they found that wild type T antigen induced rapidly growing, aggressive tumors, whereas a mutated form of the protein which only interfered with pRb function produced very slow growing tumors which displayed a high percentage of apoptotic cells. In contrast, expression of the mutant T antigen in a p53-null background resulted in tumors which were indistinguishable from those induced by the wild type protein. Taken together, the results suggest that the first event in cancer initiation stimulates both proliferation and apoptosis and that a secondary event which blocks apoptosis is necessary for aggressive tumor formation.

Cell lines derived from the tumors provided an *in vitro* confirmation of our *in vivo* observations. Apoptosis was observed only in the cell line derived from a Myc single transgenic animal (Myc#83), while the two cell lines overexpressing TGFα (TGFα/Myc#75 and TGFα#13) did not undergo apoptosis under normal culture conditions. TGFα/Myc#75 cells only became apoptotic when exposed to a specific inhibitor of EGF receptor tyrosine kinase activity (PD153035), suggesting that these cells were dependent on autocrine stimulation by TGFα for survival. Analogously, exogenous TGFα inhibited apoptosis in the Myc#83 cells. Results from both mammary gland tumors and their derived cell lines are therefore in good agreement and mirror previous studies which have shown that apoptosis was induced when c-Myc was overexpressed (Askew *et al.*, 1991; Evan *et al.*, 1992).

Our findings could provide at least a partial explanation for why TGFα and c-Myc cooperate in mammary gland tumorigenesis in the powerful way we described previously (Amundadottir *et al.*, 1995). In that study, single transgenic virgin c-Myc mice developed mammary gland tumors at around 10 months of age and virgin single transgenic TGFα animals never developed mammary gland tumors. In contrast, double transgenic TGFα/c-*myc* mice exhibited a tumor latency that was shortened to only 66 days, and mammary gland tissue from mice as young as 3 weeks grew readily as a tumor in nude mice. A similar synergism was observed in a WAP-TGFα × WAP-Myc double transgenic model (Sandgren *et al.*, 1995). In that report, as well as a study involving the MT100TGFα strain used in our model (Smith *et al.*, 1995), it was also observed that TGFα overexpression inhibited post-lactational involution, a process dependent on apoptosis. Those oberservations lend further credence to the hypothesis that TGFα can act as a survival factor in the mammary gland.

TGFα has not been shown previously to inhibit c-Myc-mediated apoptosis but insulin like growth factors (IGF-I and IGF-II) and platelet derived growth factor (PDGF) acted as survival factors for Rat-1 fibroblasts which overexpressed c-Myc (Harrington *et al.*, 1994). Interestingly, EGF could not inhibit c-Myc-induced apoptosis in those cells, even though they expressed functional EGF receptor. Although the reasons for this discrepancy are not known, it is most likely the result of cell type specificity. That assumption is supported by the observation that EGF could act as a survival factor for nontransformed mammary epithelial cells which were serum starved or grown to confluency (Merlo *et al.*, 1995). Our data from the Myc#83 tumor cell line indicate that EGF, bFGF and IGF-I can also inhibit c-Myc-mediated apoptosis, suggesting that they could potentially cooperate with c-Myc in mammary tumorigenesis as well.

Mutations in the p53 gene may also cooperate with c-Myc in tumorigenesis, since p53 has been shown to be required for Myc-mediated apoptosis in some, but not all cases (Sakamuro *et al.*, 1995; Hsu *et al.*, 1995; Hermeking and Eick, 1994). In addition, upregulation of *bcl*-2 gene expression (a death suppressor), downregulation of *bax* gene expression (a death promotor) or abrogated expression of other proteins involved in Myc-induced apoptosis might be involved. Bcl-2 has been shown to cooperate with c-Myc in transformation *in vitro* and *in vivo* (Bissonette *et al.*, 1992; Fanidi *et al.*, 1992; Strasser *et al.*, 1990; Wagner *et al.*, 1993), and Bax gene expression may be regulated by c-Myc, since its promotor contains several putative Myc binding sites (Miyashita and Reed, 1995). However it is not known whether TGFα can directly influence the apoptotic machinery of the cells. The effects of TGFα and TGFβ1 on expression of p53, Bcl-2, Bax and other proteins involved in apoptosis are currently being investigated.

A cooperative growth stimulus also appears to contribute to the synergism between TGFα and c-Myc in mammary tumorigenesis. The doubling time of TGFα/Myc#75 cells was approximately half that of cells expressing only one of the transgenes, and their growth in soft agar and nude mice was much more aggressive than the other two cell lines. Taken together, the data suggest that one aspect of the positive interaction between TGFα and c-Myc in tumorigenesis might be via upregulation of genes that control progression through the cell cycle. Collectively, these gene products might account for high growth rates and malignant progression. Potentially, coexpression of TGFα and c-Myc could also alleviate negative control on growth and transformation.

In vitro studies with the tumor-derived cell lines suggest that may be the case. TGFβ1 inhibits the growth of most epithelial cells, including mammary epithelial cells (Daniel *et al.*, 1989; Jhappan *et al.*, 1993; Pierce *et al.*, 1993; Silberstein and Daniel, 1987; Valverius *et al.*, 1989; Zugmaier *et al.*, 1989). However, the TGFα/Myc#75 line was only marginally responsive to TGFβ1 in ADG assays and was insensitive to the growth factor under anchorage independent conditions. In contrast, Myc#83 cells grown on plastic were quite sensitive to TGFβ, and their rate of colony formation in soft agar was significantly reduced in the presence of TGFβ. Apoptosis assays revealed that Myc#83 cells were not merely growth-inhibited by TGFβ, but rather they were stimulated to undergo apoptosis, even in the presence of a survival factor (EGF). Induction of apoptosis by TGFβ has previously been reported for

some other cell types, including normal and malignant ovarian epithelial cells (Havrilesky *et al.*, 1995), endometrial cells (Rotello *et al.*, 1991), rat prostate cells (Martikianen *et al.*, 1990), normal and transformed hepatocytes (Oberhammer *et al.*, 1992), and leukemia cells (Selvakumaran *et al.*, 1994a,b; Taetle *et al.*, 1993). Furthermore, mammary glands from pregnant WAP-TGFβ transgenic mice showed high levels of apoptosis with a subsequent lack of secretory lobule development (Korden *et al.*, 1995). Since TGFβ expression is elevated in human tumor cells compared to normal mammary tissue and protein levels are positively correlated with disease progression (Gorsch *et al.*, 1992), breast tumor cells must develop the ability to grow in the presence of relatively high concentrations of TGFβ. The results from our *in vitro* studies indicate that cells which overexpress only c-Myc would not have that ability.

Our results suggest a new role for TGFα as a survival factor in breast cancer. We therefore conclude that the strong synergism of TGFα and Myc in mammary gland tumorigenesis is in fact due not only to a dual growth stimulus, but to the ability of TGFα to suppress a negative aspect of Myc overexpression.

Materials and methods

Transgenic animals

Transgenic mice were generated as described previously by mating the MT100 TGFα strain to the MMTV-c-*myc* M strain (Amundadottir *et al.*, 1995). The four resulting genotypes were: TGFα/c-*myc* double transgenic mice, TGFα single transgenic mice, c-*myc* single transgenic mice, and wild type mice. Tumors were observed to form in each strain as follows: in TGFα/c-*myc* virgin females and males with a latency of about 66 days; in multiparous single transgenic TGFα females with a latency of about 10 months; and in virgin or multiparous single transgenic c-*myc* females with a latency of about 10 months.

Detection of apoptosis in tumors

The occurrence of apoptosis in mammary gland tumors was detected by *in situ* nick end-labeling of nucleosomal DNA fragments (Ansari *et al.*, 1993). Paraffin embedded tumor sections were deparafinized in a series of xylene and ethanol washes. This was followed by a 0.3% H_2O_2 treatment for 30 min to inactivate endogenous peroxidases, after which slides were immersed in buffer A for 5 min (50 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.76 mM 2-mercaptoethanol and 0.005% BSA). Subsequently, slides were incubated with Klenow enzyme (50 U/ml, Boehringer Mannheim, Indianapolis, IN), 5 μM biotinylated dUTP (Boehringer Mannheim) and 2 μM dATP, dGTP and dCTP (Promega, Madison, WI) in buffer A for 60 min at 37°C. After washing slides in PBS, they were incubated with solution AB (ABC kit, Biomeda, Foster City, CA), rewashed in PBS and stained with diamino benzidin (DAB, Sigma, St. Louis, MO). Finally, the slides were counterstained with aqueous methyl green (Sigma), dehydrated and mounted.

Primary cultures from tumors

Tumor bearing transgenic animals were sacrificed and tumors were excised aseptically. Tumors were then cut into about 1 mm^3 pieces and digested overnight at 37°C in DMEM-F12 (Biofluids, Rockville, MD) with 10% fetal calf serum (FCS, Biofluids), 5 ng/ml EGF (Upstate Biotechnology Incorporated[UBI], Lake Placid NY), 10 μg/ml insulin (Biofluids) supplemented with 1 mg/ml collagenase type 1A (Sigma), antibiotics and fungizone (Biofluids). The following day cells were pelleted by centrifugation and washed three times in growth media (DMEM-F12 with 2.5% FCS, 5 ng/ml EGF, 10 μg/ml insulin and antibiotics). Cells were plated at $1-2\times10^6$ cells per T75 flask in growth medium. Fungizone was used in the cell medium for the first 2–3 weeks to prevent fungal contamination. Media were changed every 2–3 days and fibroblast overgrowth was prevented by differential trypsination of cultures until fibroblasts were no longer observed (based on morphology). When epithelial cells were about 60–70% confluent (after 2–3 months of growth), the cultures were passed at 1:2 dilutions with dispase (Boehringer Mannheim). At later passages cells were split with trypsin (Gibco BRL, Gaithersburg, MD) twice a week at 1:5 to 1:50, depending on the line.

RNA isolation and analysis

Cultured cells were harvested by rocking plates with guanidine thiocyanate for 5–10 min. RNA was extracted with acid phenol and precipitated with isopropanol. Ten 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 ^{32}P-labeled riboprobes generated with the Riboprobe II Core System (Promega) from the following plasmids: pTGFα-pGEM3Z linearized with HindIII and transcribed with T7; c-*myc*-pGEM4Z linearized with EcoRI and transcribed with T7; and pmK14-pGEM3 (mouse keratin#14) linearized with HindIII and transcribed with SP6 polymerase. Labeled pBluescript polylinker was hybridized with the 28S RNA as an internal loading control for Northern analysis (Witkiewicz *et al.*, 1993).

Growth assays

Anchorage dependent growth assays were performed in 96-well plates (Costar, Cambridge, MA). Cells were plated at a density of 1500 cells per well and were cultured in normal growth media, with or without EGF (10 ng/ml). At various time points (two per day for 4 days), the plates were stained with crystal violet (Sigma, 0.5% in 30% MeOH), rinsed with water and dried. At the end of the experiment, the dye was redissolved in 0.1 M sodium citrate in 50% ETOH and the absorbance at 540 nm was measured with an MR700 plate reader (Dynatech Laboratories Inc). Doubling times were calculated from the slope of the line generated by plotting log (absorbance) vs time. In order to test the sensitivity of the cells to TGFβ1, cells were also plated in normal growth media with EGF plus TGFβ1 (0.01–100 pM [R&D Systems, Minneapolis, MN]). When TGFβ was used as a treatment, the media were changed every other day and cell number was measured on day 3 (TGFα/Myc#75) or 4 (TGFα#13 and Myc#83).

The cell lines were also tested for their ability to grow under anchorage independent conditions. Cells (10^4) were suspended in 0.3% Bactoagar (Difco, Detroit MI) and seeded into 35 mm dishes over a 0.8% agar base layer in IMEM plus 10% FCS with the following additions: TGFα (10 ng/ml, UBI), EGF (10 ng/ml), TGFβ1 (100 pM), or TGFβ1 and EGF together. Every other day, 300 μl of media with growth factors was added to each plate. After 7–10 days, colonies larger than 40 μm in diameter were counted with an Omnicron 3600 image analysis system (Artek Systems Corp., Farmingdale NY).

Tumorigenicity of cell lines

Cell lines were injected into female NCR *nu/nu* mice in order to determine whether they retained tumorigenic potential. About 10^6 cells were injected subcutaneously (between nipples number 2 and 3, and 4 and 5) into nude mice under anesthesia. Two to four sites were injected per animal.

Detection of apoptosis in cell lines

Apoptosis in the cell lines was detected by an apoptotic cell death ELISA assay (Boehringer Mannheim) and by visualization of nucleosomal laddering in cytoplasmic fractions of cells (Kamesaki *et al.*, 1993). The ELISA detects cytoplasmic nucleosomal DNA fragments with antibodies directed against histones and DNA. Cells were plated in 6-well plates (1.7×10^5 cells/well) and treated 24 h later. Treatments consisted of normal growth media without EGF plus the following additions: TGFα (1–1000 ng/ml), bFGF (10 ng ml, UBI), IGF-I (50 mM, UBI), EGF (10 ng/ml), or EGF plus TGFβ (10 ng/ml and 0.01–100 pM, respectively). The TGFα/Myc#75 cells were also treated with PD 153035 (10 μM, Park Davis), a specific inhibitor of EGF receptor tyrosine kinase activity (Fry *et al.*, 1994). Treatment with DMSO (μl/ml) served as a negative control since the stock drug was suspended in DMSO. Twenty-four hours later, cytoplasmic lysates were prepared from the cells. The ELISA plate was coated overnight (4°C) with the first antibody (anti-histone) and then incubated with lysis buffer for 30 min at room temperature (RT). The wells were washed three times and then incubated with 100 μl cytoplasmic lysate for 90 min (RT). Wells were washed again and incubated with the peroxidase-linked second antibody (anti-DNA). Following the final wash, ABTS peroxidase substrate was added and color development was detected by measuring absorbance at 410 nm. CEM cells (T cell leukemia cell line) treated with dexamethasone (Catchpoole and Steward, 1993) served as a positive control.

Internucleosomal cleavage of the DNA is a hallmark of apoptosis and can be observed as a 'ladder' in agarose gels. DNA was isolated from cytoplasmic fractions of the cells and was run out on 1.8% agarose gel which was stained with ethidium bromide to visualize the DNA ladder (Kamesaki *et al.*, 1993). Cells that are undergoing apoptosis show a characteristic DNA 'ladder' in this assay whereas other cells do not contain DNA in their cytoplasm and are therefore negative.

FACS analysis

Cell nuclei were analysed by the detergent-trypsin method (Vindelov *et al.*, 1983) with a Fluorescent Activated Cell Sorter (FACS) to obtain cell cycle histograms and to determine ploidy. Approximately 10^6 cells were pelleted and resuspended in 100 μl of citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 0.05% v/v DMSO, pH 7.6) and stored at −70°C before analysis. For cell cycle analysis, cells were plated in normal growth media with EGF. After 24 h, the cells were switched to media without EGF and then harvested at various time points to determine whether the cells were growth arrested in G_0/G_1. For ploidy analysis, tumor cells (passages 6–31) were analysed alone and also mixed with normal primary mouse fibroblast cultures (passage 2). The normal fibroblasts served as a control to establish a diploid mouse histogram.

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Role for Bcl-x_L in the Regulation of Apoptosis by EGF and TGFβ1 in c-myc Overexpressing Mammary Epithelial Cells

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We previously showed that TGFα synergizes with c-myc in mammary tumorigenesis through inhibition of Myc-induced apoptosis. We therefore examined the effects of growth factors on apoptosis induction in several cell lines from MMTV-*myc* mammary tumors. When EGF was withdrawn or TGFβ1 was added, cells became apoptotic after 15 h (by ELISA and morphology). Northern and Western analysis revealed high levels of Bax and p53, and low or undetectable levels of Bcl-2 and Bcl-x_S under all treatment conditions. In contrast, Bcl-x_L expression was highest in the presence of EGF or TGFα, with a significant reduction upon removal of EGF or exposure to TGFβ. In mouse mammary tumors, the relative Bcl-x_L/Bax ratio was higher in TGFα/Myc double transgenics than in Myc single transgenics, in agreement with the *in vitro* data. Our results suggest a role for Bcl-x_L in the regulation of apoptosis by EGF and TGFβ in mammary epithelial cells.

The proto-oncogene c-myc encodes a transcription factor which forms a heterodimer with Max (1). Although the Myc/Max targets are not well defined, Myc is believed to have an important regulatory function in cell proliferation. Myc expression is tightly regulated and correlated with the proliferative state of the cell. Reduced c-myc levels due to disruption of one allele results in a lengthened G1 phase (2), while inhibition of c-myc expression blocks cell cycle progression and leads to G1 arrest (3). Conversely, cells which constitutively express c-*myc* cannot arrest in G1 and thus continue to proliferate or undergo apoptosis (4).

Activation of c-myc is thought to play a role in the development of breast cancer since it is commonly amplified and/or overexpressed in human breast tumors (5, 6). c-myc amplification is associated with a high proliferation index in mammary tumors and is correlated with poor prognosis. In addition, Myc confers tumorigenicity when overexpressed in the mammary gland of transgenic mice (7). Recent results indicate that overexpression of both c-*myc* and TGFα cooperate strongly in mammary tumorigenesis (8, 9). The contribution of TGFα may be due, at least in part, to the suppression of myc-induced apoptosis (10).

Apoptosis is regulated by the bcl family of proteins which either promote or inhibit cell death (11). The death suppressor Bcl-2, initially recognized for its role in lymphoma, has since been found to be highly expressed in a variety of tumors (12). When overexpressed, Bcl-2 can protect cells from many apoptotic signals, including unregulated myc expression (4, 11, 12). Bcl-x is a unique family member in that the mRNA can be alternately spliced to produce 2 different proteins: a death suppressor (Bcl-x_L) and a death inducer (Bcl-x_S). The suppressive activity of Bcl-2 and Bcl-x_L can be modulated by death inducers such as Bax, a family member which forms heterodimers with the two former proteins. The ratio of Bcl inducers to suppressors

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determines the fate of the cell (11). Bcl-x and Bax are both expressed in breast tissue and play an important role in the normal apoptotic process of mammary gland involution (13, 14).

The tumor suppressor p53 has also been implicated in the regulation of apoptosis and, like Myc, can regulate growth as well as death. Expression of p53 leads to cell cycle arrest or apoptosis, depending on the cell type and environment. In many, but not all systems, p53 activity is required for apoptosis. In the mammary gland, apoptosis can occur in the absence of p53 (14, 15), but it is not known whether myc-induced apoptosis requires wild type p53 in that cell type.

For this study, we wished to investigate the regulation of apoptosis in mammary epithelial cells which overexpress c-myc. Mammary tumors which arise in MMTV-myc transgenic mice, as well as epithelial cell lines derived from such tumors, both show a high propensity to undergo apoptosis (10). However, mammary tumors and cell lines which express both Myc and TGFα exhibit very low levels of apoptosis and therefore grow much faster *in vitro* and *in vivo*. Furthermore, apoptosis in the Myc single transgenic cell lines can be inhibited by exogenous TGFα or EGF and accelerated by the growth inhibitor TGFβ. We therefore examined expression of apoptotic pathway genes in the presence or absence of those growth factors.

MATERIALS AND METHODS

Transgenic mice. The TGFα transgenic mice (MT100) were provided by Dr. Glenn Merlino (NIH, Bethesda, MD). The c-Myc mice (MMTV-c-*myc* M) were developed by Dr. Philip Leder (Harvard Medical School, Boston, MA) and obtained from Charles River Breeding through a breeding license with DuPont. Double transgenic mice were generated as described previously by mating the TGFα strain to the c-Myc strain (8). Mammary tumors were excised and frozen at −70°C as they spontaneously arose in each strain.

Cell lines. The cell lines Myc#83, Myc#7, and Myc#9 were established from mammary tumors of single transgenic mice as previously described (10). The cells were routinely grown in IMEM (Gibco-BRL) containing 2.5% FCS, 10 ng/ml /EGF (Upstate Biotechnology Incorporated) and 5 μg/ml insulin (Biofluids).

Cell death ELISA. Cytoplasmic nucleosomal DNA fragments were detected using an apoptotic cell death ELISA (Boehringer Mannheim) with antibodies directed against histones and DNA, as previously described (66). Cells were plated in 12-well plates (6.7×10^4 cells/well) and were treated for 3-48 hours prior to lysis with the following growth factors: EGF (10 ng/ml), TGFβ1 (100 pM, R & D Systems), or EGF plus TGFβ.

Northern analysis. Cells were plated (5×10^5 cells/ 60 mm plate) and treated as above for 24 h. Total RNA was harvested by the guanidine thiocyanate-acid phenol method. Total RNA (15 μg) was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (Amersham). Blots were sequentially hybridized overnight with the following ^{32}P-labeled, random-primed murine probes: bcl-2 (nt 1635-1945), bcl-x_{L+S} (mRNA nt 110-394), bax (mRNA nt 138-389), and p53 (nt 97-1407).

RT-PCR. The relative amounts of bcl-x_L and bcl-x_S mRNA were measured by an RT-PCR assay. One μg RNA from each sample was reverse transcribed with random primers and the cDNA for bcl-x_{L+S} was amplified for 31 cycles using a pair of primers that amplify the nucleotide sequence containing the region differentially spliced in the bcl-x_L and bcl-x_S mRNAs. 5′ primer (mRNA nt 466-488): 5′-GCG CGG GAG GTG ATT CCC ATG GC-3′; 3′ primer (nt 891-870): 5′-CAT GCC CGT CAG GAA CCA GCG G-3′. PCR products were fractionated on a 2% agarose gel and transferred to a nylon membrane which was sequentially hybridized with a ^{32}P-labeled random-primed probe for bcl-x_L (Bcl-x mRNA nt 466-891) and an oligonucleotide specific for the splice site within the 237-bp bcl-x_S product (5′-CAG AGC TTT GAG CAG GAC ACT TTT GTG G-3′).

Western analysis. Cells were plated (1.3×10^6 cells/100 mm plate) and treated as above for 24 h before lysis in RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). Frozen mouse mammary tumors were pulverized in liquid nitrogen and homogenized in Tris-SDS (10 mM, pH 7.4, 1%). Twenty μg of protein were separated on 14% SDS-PAGE gels (or 10% for p53) and transferred to nitrocellulose. Blots were blocked with 5% milk in Tris-buffered saline with Tween-20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween-20) for 1 h and then incubated in TBST with 1% BSA and the following antibodies (diluted 1/400): Bcl-x_{L+S} (S-18), Bax (N-20), Bcl-2 (N-19) (Santa Cruz Biotechnology); or p53 (Ab-1, Oncogene Sciences). Proteins were visualized with an HRP-linked second antibody (1/500 in TBST with 1% BSA) and a chemiluminescent detection system (Pierce).

RESULTS

Three mammary epithelial cell (MEC) lines derived from tumors of MMTV-myc mice were tested for their apoptotic response to EGF and TGFβ. Southern analysis demonstrated

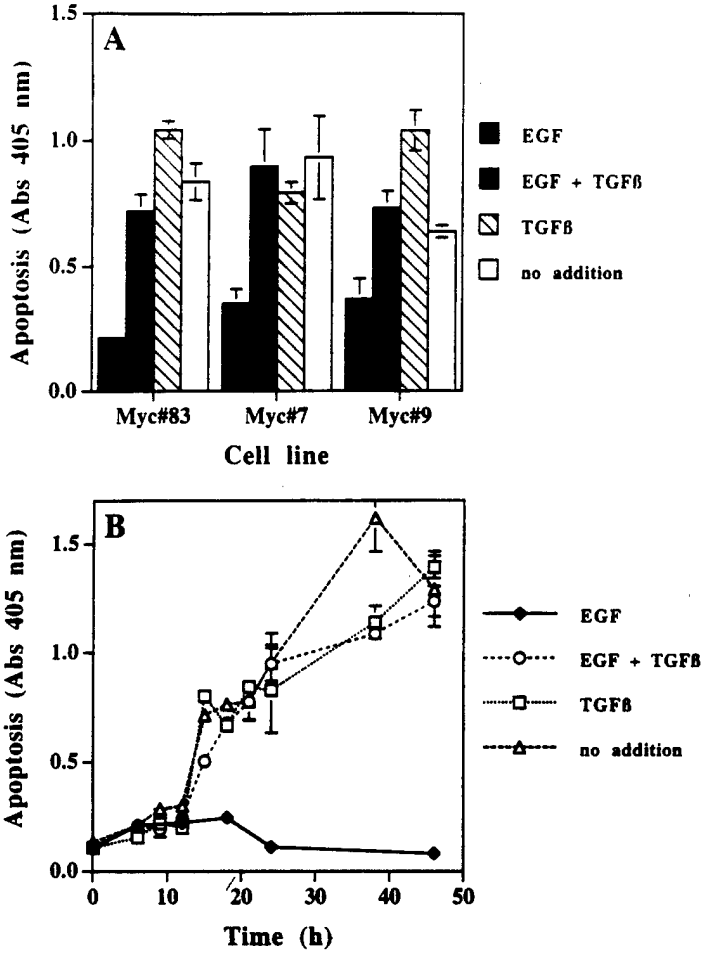

FIG. 1. Apoptosis in MECs which overexpress c-myc. (A) Three cell lines derived from mammary tumors of MMTV-myc transgenic mice were treated for 24 h with: EGF (10 ng/ml), TGFβ1 (100 pM), EGF + TGFβ, or no addition. Apoptotic DNA fragments were detected in cytoplasmic lysates via histone-DNA ELISA. n=2 (+/−SE). B) Time course of apoptosis induction in Myc#83 cells. Cells were treated as in A and lysates were harvested at three hour intervals for ELISA. n=4 (+/−SE).

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that the cell lines retained the myc transgene, and expression *in vitro* was confirmed by Northern and Western blots (not shown). Cells were treated for 24 h before preparing cytoplasmic lysates for assessing apoptosis via histone-DNA ELISA. For each cell line, the occurrence of apoptosis was lowest in the presence of EGF (Figure 1A). The level of apoptosis increased dramatically following EGF withdrawal or exposure to TGFβ. However, there was no apparent synergism for apoptosis induction between addition of TGFβ and removal of EGF.

The time course of apoptosis induction was assessed in the Myc#83 cells. At all time points, cells treated with EGF showed very low, basal levels of apoptosis (Figure 1B). In contrast, cells deprived of EGF or treated with TGFβ showed a very similar temporal pattern of cell death. They began to exhibit cytoplasmic histone-DNA complexes at about 15 hours after treatment and achieved maximal levels between 24 and 48 hours.

The ELISA results were confirmed by observing morphological changes in Myc#83 cells undergoing apoptosis (not shown). Cells grown with EGF appeared healthy and displayed

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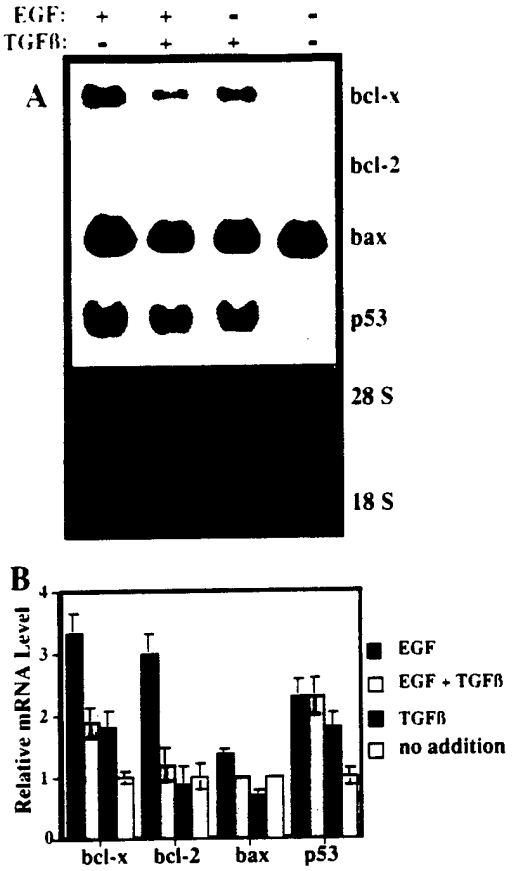

FIG. 2. Northern Analysis. Myc#83 cells were treated for 24 h as in Fig. 1A before harvesting total RNA. (A) Representative results. Northern blots were sequentially hybridized with random-primed probes for mouse bcl-2, bcl-x, bax, and p53. 28S and 18S ribosomal RNA bands are shown as a loading standard. Autoradiography exposure times were as follows: bcl-2, 7 d; bcl-x_{L+S}, 5 d; bax, 3 d; p53, 2 d. (B) Cumulative data. The mean band intensity for untreated cells was assigned a value of 1 and all other values were calculated as a relative increase or decrease. n=8 (+/−SE).

many mitotic figures. In contrast, cells exposed to TGFβ or deprived of EGF exhibited a very typical apoptotic morphology, with DNA condensation at the nuclear membrane and prominent apoptotic bodies. Furthermore, mitotic structures were rare under conditions which favored apoptosis.

Based on the results in Fig. 1B, a 24 h treatment period was chosen to assess changes in gene expression. Northern blots were sequentially hybridized with murine probes for bcl-2, bcl-x, bax, and p53 (Figure 2). Bcl-x showed the greatest variation in expression. Levels were highest in EGF treated cells and were significantly decreased following TGFβ treatment or EGF withdrawal. Expression of bcl-2 was clearly much lower than bcl-x, but the pattern of expression was similar, with the strongest signal for cells grown in the presence of EGF. In contrast, bax RNA was easily detectable and was relatively uniform across treatments. Expression of p53 RNA was also consistent across treatments, with the notable exception of EGF deprivation (50% reduction).

RT-PCR was used to distinguish the long and short forms of bcl-x RNA (Figure 3). A single band corresponding to bcl-x_L was visible on ethidium bromide stained gels of the PCR products. When the DNA was transferred to nylon and hybridized with a random-primed

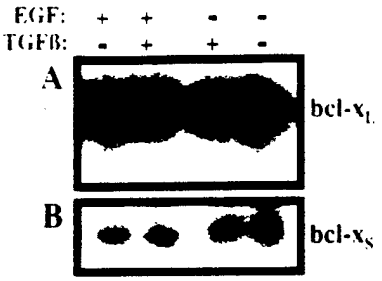

FIG. 3. Bcl-x_L & x_S RT-PCR. RNA samples were reverse-transcribed and then amplified by PCR. The PCR products were separated on an agarose gel and transferred to nylon for Southern analysis. (A) The blot was hybridized with a random-primed probe (Bcl-x mRNA nt 466-891) (3 h exposure). (B) The blot was hybridized with an oligonucleotide probe specific for the short form of *bcl-x* (20 h exposure).

probe, a strong signal for bcl-x_L was observed. Hybridization with a bcl-x_S specific oligonucleotide revealed a much weaker band for the short form, with little fluctuation among treatments. The results suggest that the bcl-x signal observed by Northern analysis was largely due to expression of bcl-x_L rather than bcl-x_S.

Western blots were used to examine expression at the protein level (Figure 4). Bcl-x_L protein levels varied dramatically, with highest expression in EGF-treated cells. Bcl-x_L expression was similar in EGF- and TGFα-treated cells (not shown). Following TGFβ treatment or EGF withdrawal, there was an 80% reduction in Bcl-x_L protein. Analysis of lysates prepared at various time points indicated that the decrease in Bcl-x_L protein preceded the onset of DNA degradation (not shown). Neither Bcl-x_S nor Bcl-2 could be detected by

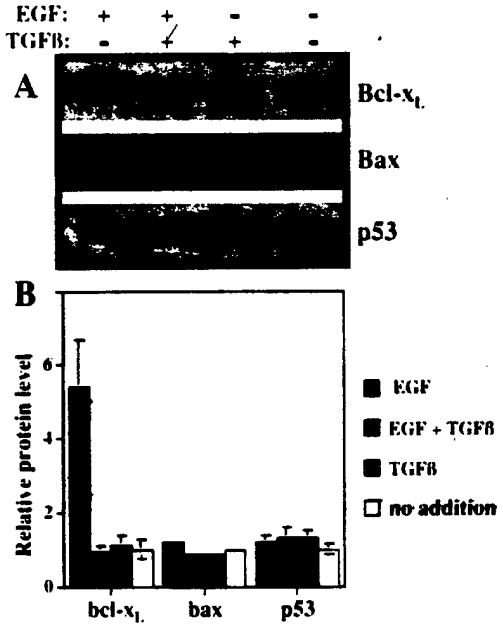

FIG. 4. Western analysis of Myc#83 cells. Cells were treated for 24 h as in Fig 1A before preparing total cell lysates. 20 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose. (A) Representative results. Individual blots were incubated with antibodies against Bcl-x, Bax, and p53. (B) Cumulative data. The mean band intensity for untreated cells was assigned a value of 1 and all other values were calculated as a relative increase or decrease. n=6 (+/−SE).

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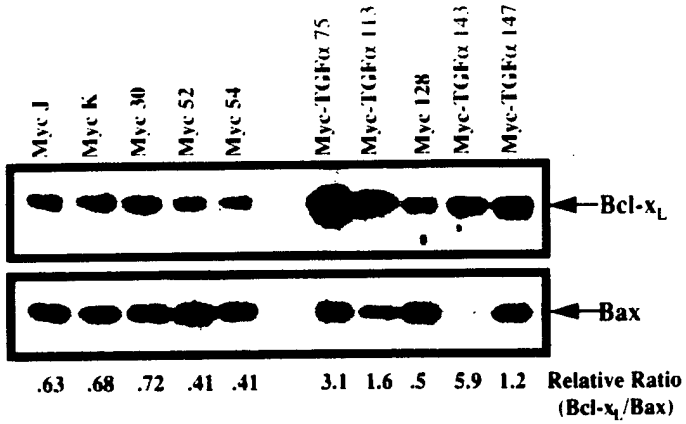

FIG. 5. Western analysis of mammary tumors from single Myc and double Myc/TGFα transgenic mice. Lysates were prepared from frozen tumors and 20 μg of protein were separated on 14% polyacrylamide gels. The blot was probed first with an antibody against Bcl-x and then stripped and re-probed with Bax antibody. Relative ratios were calculated from the band intensities as measured by densitometry.

Western analysis. Bax and p53 protein levels were relatively high and showed little variation across treatments.

The relative ratios of Bcl-x_L to Bax were compared in mammary tumors from single (MMTV-myc) or double (MMTV-myc X MT-TGFα) transgenic mice. Bcl-x_L and Bax were detected by Western analysis and relative ratios of the signal intensity for the two proteins were calculated from the values obtained by densitometry (Figure 5). Lysates from Myc single transgenic tumors consistently showed a relative ratio of Bcl-x_L to Bax of approximately 0.5 (+/− 0.06). Ratios for the double transgenic tumors were more variable, but were consistently higher than the single transgenics, with a mean value of 2.9 (+/− 1.0). Bcl-2 was not detected in the tumor lysates.

DISCUSSION

Our results suggest a role for Bcl-x_L in growth factor-mediated regulation of apoptosis in MECs which overexpress c-Myc. Bcl-x_L expression showed the greatest variation in response to EGF and TGFβ. Bcl-x_L mRNA and protein levels were dramatically elevated in Myc#83 cells grown in the presence of EGF or TGFα compared to cells which had been deprived of EGF or treated with TGFβ. Consistent with the *in vitro* results, the relative ratio of Bcl-x_L to Bax was higher *in vivo* in mouse mammary tumors which expressed TGFα and Myc together compared to those which overexpressed Myc alone.

The data indicate that MECs which overexpress c-Myc are dependent on EGF receptor signaling for survival and growth. We and others have recently demonstrated that EGF and TGFα can act as survival factors for mammary epithelial cells *in vitro* (16) and *in vivo* (10, 17). However, in the aforementioned *in vitro* study (16), much harsher conditions were used to induce apoptosis in MECs than in our study (high cell density combined with serum deprivation rather than simple withdrawal of EGF from subconfluent cells in the presence of 2.5% FCS and 10 μg/ml insulin). Many MEC lines, both human and mouse, are dependent on EGF for growth, but deprivation of EGF under normal culture conditions results in reversible growth arrest rather than apoptosis (18; personal communication, Dr. Daniel Medina, Baylor College of Medicine, Houston, TX). Those results imply that Myc-overexpressing MECs are very susceptible to the induction of apoptosis and are especially dependent on EGF receptor pathway(s) for survival. Our cell lines therefore provide an excellent model for studying

growth/survival factor regulation of Myc-induced apoptosis in MECs. The results presented here indicate that increased expression of Bcl-x_L may, at least in part, explain the mechanism by which EGF and TGFα function as survival factors.

The manner in which EGF regulates apoptosis in MECs may therefore be analogous to that observed in hematopoietic cells following cytokine withdrawal. Two recent reports implicate Bcl-x_L in the control of cell survival in normal activated T cells and in myeloid leukemia cells (19, 20). In the former study, expression of Bcl-x_L in activated T cells was significantly reduced following interleukin-2 withdrawal, while bax and bcl-2 levels did not change. The latter study showed a reduction of Bcl-x_L expression in leukemia cells following survival factor withdrawal.

Some hematopoietic cancer cells also undergo apoptosis in response to TGFβ, as in our MEC system. TGFβ induced apoptosis in leukemia cells with a concomitant decrease in Bcl-2 expression, but no change in Bax expression (21), while in lymphoma B cells, apoptosis induction by TGFβ was not accompanied by changes in Bcl-2 expression (22), suggesting that regulation of cell death by TGFβ may be cell type specific. Bcl-x_L expression was not examined in those studies.

Increased TGFβ expression has been observed in MECs which have been stimulated to undergo apoptosis (23, 24). However, it was not determined whether TGFβ secretion was required for apoptosis induction. *In vitro*, TGFβ has previously been shown to inhibit growth of MECs (25, 26), but it has not been reported to induce apoptosis. In contrast, overexpression of TGFβ1 *in vivo* has been associated with increased occurrence of apoptosis in normal MECs (27). Since the levels of growth/survival factors such as EGF and estrogen are relatively high in the mammary gland during pregnancy, the effects of TGFβ are presumed to be dominant over those of the positive survival signals, analogous to our *in vitro* observations. Our results suggest that TGFβ may block the induction of Bcl-x_L expression by survival factors like EGF.

In contrast to Bcl-x_L, expression of Bax and p53 were relatively constant in the MECs across the various treatments examined. Since c-Myc can transactivate the p53 promoter (28), and the *bax* gene promoter also contains a putative c-Myc response element (29), those two genes may be continuously activated when Myc is constitutively expressed. That could explain the propensity of Myc-overexpressing cells to undergo apoptosis when challenged with a negative growth signal. High levels of Bax may determine the "set point" of the cells so that any decrease in the protective Bcl family members due to removal of growth/survival signals will push cells toward apoptosis. Myc may activate the p53 and Bax pathways as a safeguard to prevent the survival of cells with oncogenic activation. The mechanism by which Myc triggers cell death is not universal however, since the induction of apoptosis by myc overexpression is dependent on wild type p53 in some, but not all systems (4, 30). The role of p53 in our MEC system is currently being examined.

Bcl-2 and Bcl-x_S apparently do not play a significant role in the MEC system examined here. The RNA levels for both were quite low and the proteins were undetectable on Western blots. Bcl-x_L and Bcl-2 regulate a common pathway (31), but the tissue specific expression patterns of the two genes do not always overlap (32), perhaps allowing for cell specific responses to different stimuli. For example, in the human breast, Bcl-2 is not expressed in functionally differentiated secretory cells, although it can be detected in non-secretory epithelial cells (33). Alternatively, the low Bcl-2 levels may be secondary to the expression of p53 in Myc#83 cells. The 5′ untranslated region of the bcl-2 gene contains a p53-dependent negative response element (34) and p53 can down-regulate bcl-2 expression in human breast cancer cells (35).

In summary, we have demonstrated a potential role for the cell survival-promoting protein Bcl-x_L in MEC apoptosis driven by c-Myc overexpression. Myc activation is common in

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breast cancer, but it is well documented that Myc overexpression alone is insufficient for transformation. Since Myc can induce apoptosis as well as growth, it is likely that secondary events will block cell death, thereby allowing the stimulatory effects of Myc to predominate. We suggest that changes in Bcl-x_L expression, either directly *via* genetic alteration, or indirectly *via* increased production of survival factors, may promote tumorigenesis of cells which overexpress Myc.

ACKNOWLEDGMENTS

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Epidermal Growth Factor-dependent Cell Cycle Progression Is Altered in Mammary Epithelial Cells That Overexpress c-*myc*[1]

Sharyl J. Nass[2] and Robert B. Dickson[3]

Department of Cell Biology and Vincent T. Lombardi Cancer Center, Georgetown University, Washington, DC 20007

ABSTRACT

Amplification and overexpression of the c-*myc* gene are common in primary human breast cancers and have been correlated with highly proliferative tumors. Components of the epidermal growth factor (EGF) receptor signaling pathway are also often overexpressed and/or activated in human breast tumors, and transgenic mouse models have demonstrated that c-*myc* and transforming growth factor α (a member of the EGF family) strongly synergize to induce mammary tumors. These bitransgenic mammary tumors exhibit a higher proliferation rate than do tumors arising in single transgenics. We, therefore, chose to investigate EGF-dependent cell cycle progression in mouse and human mammary epithelial cells with constitutive c-*myc* expression. In both species, c-*myc* overexpression decreased the doubling time of mammary epithelial cells by ~6 h, compared to parental lines. The faster growth rate was not due to increased sensitivity to EGF but rather to a shortening of the G_1 phase of the cell cycle following EGF-induced proliferation. In cells with exogenous c-*myc* expression, retinoblastoma (Rb) was constitutively hyperphosphorylated, regardless of whether the cells were growth-arrested by EGF withdrawal or were traversing the cell cycle following EGF stimulation. In contrast, the parental cells exhibited a typical Rb phosphorylation shift during G_1 progression in response to EGF. The abnormal phosphorylation status of Rb in c-*myc*-overexpressing cells was associated with premature activation of cdk2 kinase activity, reduced p27 expression, and early onset of cyclin E expression. These results provide one explanation for the strong tumorigenic synergism between deregulated c-*myc* expression and EGF receptor signal transduction in the mammary tissue of transgenic mice. In addition, they suggest a possible tumorigenic mechanism for c-myc deregulation in human breast cancer.

INTRODUCTION

The proto-oncogene c-*myc* encodes a highly conserved nuclear phosphoprotein with domains that are common to many transcription factors (1–6). When bound to its heterodimeric partner Max, Myc protein binds specifically to DNA and can activate transcription. However, the physiologically relevant targets of *myc* regulation are not well defined, and thus, its mode of action is not fully understood, despite intense investigation.

Myc has been implicated in the regulation of cell proliferation, differentiation, and death by apoptosis (reviewed in Refs. 1–6). Because aberration of any of those normal processes can contribute to tumorigenesis, it is not surprising that deregulated expression of the c-*myc* gene is often associated with neoplasia. *In vitro*, c-*myc* overexpression can cooperate with other oncogenes such as Ras to transform cells, and *in vivo*, the ability of inappropriately expressed Myc to promote tumorigenesis has been clearly demonstrated by transgenic mouse models (7).

Classified as an immediate early gene, c-*myc* expression is tightly regulated and correlated with the proliferative state of the cell (8). In normal quiescent cells, Myc protein levels are very low, and its expression is strongly induced following mitogen stimulation. Similarly, its expression decreases as cells become growth-arrested or undergo differentiation. A reduction in c-*myc* levels due to disruption of one copy of the gene results in a lengthened G_1 cell cycle phase (9), whereas inhibition of c-*myc* expression blocks cell cycle progression and leads to G_1 arrest (10, 11). Conversely, when c-*myc* expression is deregulated, cells may grow at a faster rate and are often unable to withdraw from the cell cycle when signaled to do so (12–14).

On the basis of the above observations, c-*myc* has long been thought to control key aspects of the proliferative response. Because passage through the cell cycle is orchestrated by the cyclins and their associated cdks[4] (reviewed in Ref. 15), these regulatory proteins would be logical targets for such a proposed action of Myc. Normally, expression of the various cyclins is tightly regulated and is characteristic of specific stages of the cell cycle. Several studies in fibroblasts and hematopoietic cells, in fact, suggest that expression or activity of some cyclins and cdks may be altered when c-*myc* expression is deregulated (9, 16–22).

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[4] The abbreviations used are: cdk, cyclin-dependent kinase; TGF, transforming growth factor; EGF, epidermal growth factor; EGFR, EGF receptor; MEC, mammary epithelial cell; FACS, fluorescence-activated cell sorting; Rb, retinoblastoma; CAK, cdk-activating kinase.

Although some mechanistic details of the action of Myc have been studied in rodent fibroblasts, there is considerable interest in further elucidating the mechanism(s) of malignant transformation by Myc in human epithelial malignancies, in which the oncoprotein has a clear pathophysiological function. Overexpression of c-*myc* is thought to play a role in the development of breast cancer because it is commonly amplified and/or overexpressed in human breast tumors (reviewed in Ref. 23). Amplification of the c-*myc* gene is often associated with highly proliferative tumors and poor prognosis. In addition, Myc confers tumorigenicity when it is overexpressed in the mammary gland of transgenic mice. Recent results from our laboratory (24) and others (25) showed that overexpression of TGF-α (which is also common in primary human breast tumors; Refs. 26 and 27) can strongly synergize with c-*myc* in transgenic mice to promote mammary tumor development *in vivo*, confirming previous *in vitro* observations that Myc can cooperate with growth factors such as TGF-α or EGF to transform MECs (28, 29). The contribution of TGF-α may be partly due to the suppression of Myc-induced apoptosis via increased expression of Bcl-x_L (30, 31). However, tumors and cell lines derived from the double transgenic mice also showed an accelerated growth rate compared to those from single transgenic mice (24, 30). Those results suggest that c-myc may also cooperate with the EGFR signaling pathway to promote aberrant cell cycle progression in MECs. Although a variety of changes in the expression of cell cycle regulators have been identified in human breast cancer cell lines and primary tumors (reviewed in Ref. 32), little is known about the causes or consequences of cell cycle deregulation in breast cancer. Thus, the purpose of this study was to identify changes in cell cycle regulation during EGF-dependent growth of MECs that overexpress c-*myc*.

MATERIALS AND METHODS

Cell Lines. A pair of human MEC lines (184A1N4 and 184A1N4-myc) were used to study the effects of c-*myc* overexpression on cell cycle regulation. The parental cell line, A1N4, was derived from normal mammary tissue obtained by reduction mammoplasty and was immortalized with benzo(*a*)pyrene (33). The A1N4-myc line (29) was established via retroviral infection of A1N4 cells with a construct containing mouse c-*myc* under the control of the Moloney mouse leukemia virus long terminal repeat. Retention and expression of the c-*myc* transgene were confirmed by Southern and Western analysis, respectively (data not shown). Both cells lines were maintained in Iscove's MEM (Life Technologies, Inc., Gaithersburg, MD) containing 0.5% FCS, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin (Biofluids, Rockville, MD), and 10 ng/ml EGF (Upstate Biotechnology Inc., Lake Placid, NY). The cells arrest in G_1 in the absence of EGF (34).

Two pairs of mouse mammary cell lines (HC14 and HC14-myc and MMEC and MMEC-myc) were also used in preliminary experiments. The HC14 line was established from a mid-pregnant mammary gland, whereas the MMEC line was derived from an 8-week-old virgin mammary gland. Both cell lines were transfected with a c-*myc* expression construct driven by the Moloney mouse leukemia virus long terminal repeat (28, 35).

Growth Assays. Cells were plated in 96-well plates (Costar, Cambridge, MA) at a density of 1000–2000 cells/well. At various time points, plates were stained as described previously (36) with crystal violet (0.5% in 30% methanol; Sigma Chemical Co., St. Louis, MO), rinsed with water, and dried. At the end of the experiment, the dye was redissolved in 0.1 M sodium citrate in 50% EtOH, and $A_{540\ nm}$ was measured with an MR700 plate reader (Dynatech Laboratories Inc.). Doubling times were calculated from the slope of the line generated by plotting log(*A*) *versus* time.

FACS Analysis. Cells were plated (5×10^5 cells/plate) in 10-cm dishes (Falcon 3003; VWR Scientific, Philadelphia, PA) in normal growth medium containing EGF. The next day, the cells were changed to EGF-free medium to arrest them in G_1. After 48 h, the cells were restimulated with EGF (10 ng/ml), and cells were harvested at 3-h intervals. Nuclei were isolated and stained with propidium iodide for cell cycle analysis according to the method of Vindelov *et al.* (37).

Western Analysis. Cells were plated, arrested, and restimulated with EGF as described for FACS analysis. At 1.5- or 3-h intervals following EGF stimulation, total cell lysates were prepared. Cells were washed with cold PBS and then scraped into cold lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3O_4V, 100 mM NaF, 10 mM inorganic phosphate, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. After a 10-min incubation on ice, lysates were spun for 10 min in a cold microcentrifuge to remove cellular debris and were frozen at −70°C. Twenty μg of protein from each sample were separated by SDS-PAGE and transferred to either nitrocellulose or polyvinylidene fluoride membranes. Acrylamide concentrations varied depending on the target protein as follows; Rb, 6%; cyclin D1, cyclin E, cdk2, and cdk4, 10%; and p27, 14%. Blots were blocked in 4% milk-1% BSA in Tris-buffered saline with Tween 20 [TBST; 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.5% Tween 20] for 1 h at room temperature and then incubated in TBST with 1% BSA and the following antibodies (1 μg/ml): Rb (PharMingen, San Diego, CA), cyclins D1 and E (Upstate Biotechnology Inc.), cdk2 and cdk4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and p27 (Santa Cruz). Proteins were visualized with a horseradish peroxidase-linked second antibody (1:2000 in TBST, with 1% BSA) and a chemiluminescent detection system (Pierce, Rockford, IL). In all cases, proteins from the A1N4-myc cells, and the parental A1N4 cells were analyzed in parallel on the same film to allow direct comparison of specific protein levels between the two cell lines. Amido black or India ink staining of the membranes demonstrated equal loading and transfer of the samples. Because an appropriate antibody for Cdc25A was not commercially available, we chose to examine its expression at the RNA level only (see below).

Kinase Assays. Cell lysates (100 μg) were incubated with 1 μg of anti-cdk2 antibody for 2 h (4°C) prior to precipitation with Agarose A beads (Santa Cruz). Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer [50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 1 mM DTT, 0.3 mM β-glycerophosphate, 1 mM Na_3O_4V, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml

Table 1 Doubling times for one human pair and two mouse pairs of cell lines

Pair = c-*myc*-overexpressing line and its parental line. The last column indicates the decrease in doubling time of the Myc line, compared to its parental line.

Cell line	Doubling time (h)	Difference (h)
A1N4	27.4	5.9
A1N4-myc	21.5	
HC14	25.1	6.3
HC14-myc	18.8	
MMEC	24.9	5.8
MMEC-myc	19.1	

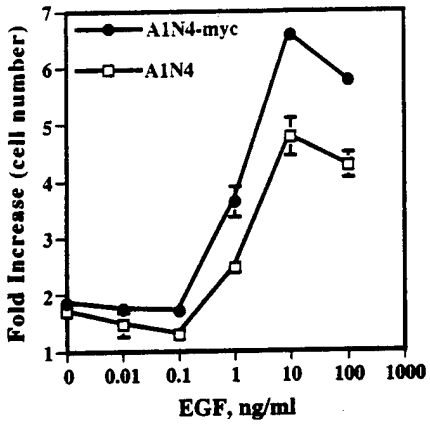

Fig. 1 Growth of A1N4 and A1N4-myc cells in response to EGF is concentration dependent. Cells were plated in 96-well plates with increasing concentrations of EGF and incubated for 3 days before being stained with crystal violet. Note that the two curves are parallel. $n = 8$. *Bars*, SE.

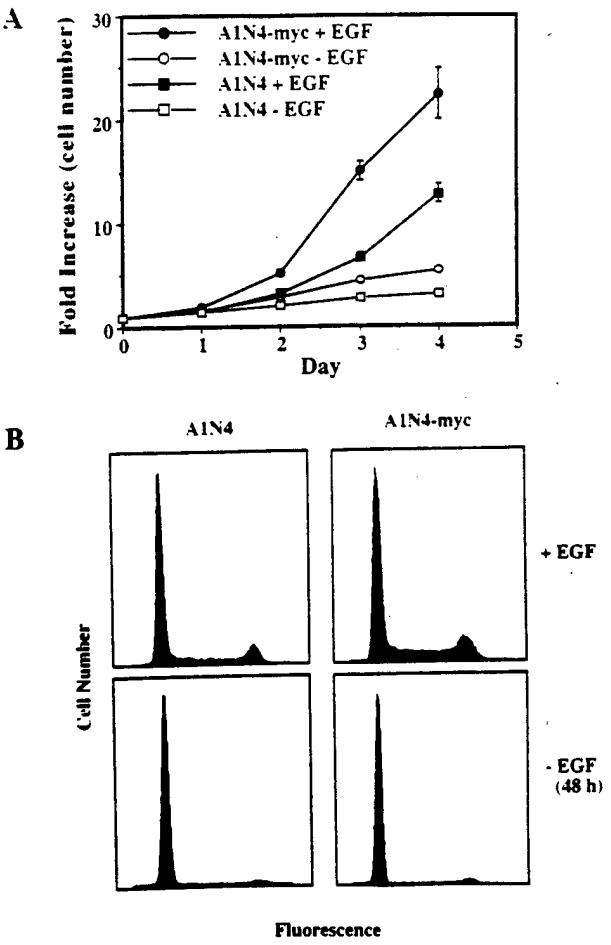

Fig. 2 A1N4 and A1N4-myc cells arrest in G_1 in the absence of EGF. *A*, growth of both A1N4 and A1N4-myc cells is dependent on EGF. Cells were plated in 96-well plates (10^3 cells/well) in the presence or absence of EGF and were stained with crystal violet at the indicated times. Relative cell number was then measured as $A_{540\ nm}$. $n = 8$. *B*, cell cycle histograms for unsynchronized cells grown in normal medium with EGF, and arrested A1N4 and A1N4-myc cells that had been deprived of EGF for 48 h.

aprotinin, and 10 μg/ml leupeptin]. The beads were then resuspended in 30 μl of kinase buffer, and the reaction was started by adding ATP (200 μM), [γ-^{32}P]ATP (5 μCi) and histone H1 (1 μg). Samples were incubated at 30°C for 15 min before the reaction was stopped with 2× loading buffer [62.5 mM Tris (pH 6.8), 10% sucrose, 2% SDS, 5% β-mercaptoethanol, and 1% bromphenol blue]. Labeled proteins were run on a 10% polyacrylamide gel that was dried prior to visualization with a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA).

RNA Analysis. A1N4 and A1N4-myc cells were plated sparsely (1.5×10^6 cells) in culture flasks (225 cm^2; Costar) and growth-arrested as described above. Following restimulation with EGF (10 ng/ml), total RNA was harvested at 3-h intervals by the guanidine thiocyanate-acid phenol method (38). Expression of Cdc25A RNA was measured by Northern analysis. Total RNA (12 μg) was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (Amersham). Blots were hybridized overnight with a 700 bp of ^{32}P-labeled, random-primed human probe for Cdc25A (nucleotides 936–1637). Bands were detected with a PhosphorImager 445 SI. Changes in cyclin RNA expression were examined using a nonradioactive RNase protection assay, as described previously (39).

RESULTS

We began our studies by comparing the effect of constitutive Myc expression on the growth rate of human and mouse MEC lines. In both mouse [HC14-myc and MMEC-myc (28)] and human (A1N4-myc) MECs, c-*myc* overexpression decreased the doubling time by ~6 h compared to parental lines (Table 1). To determine whether the faster growth rate was simply due to increased sensitivity to growth factors, the two human cell lines were grown in the presence of various concentrations of EGF for 3 days. The two resulting concentration-response curves were parallel, with the A1N4-myc cells growing faster than the parental cells at all concentrations tested (Fig. 1).

The A1N4 and A1N4-myc cells were used to further investigate the observed change in growth rate. In the absence of EGF, neither the parental nor the c-*myc*-infected cell line showed significant growth (Fig. 2*A*). That observation was due

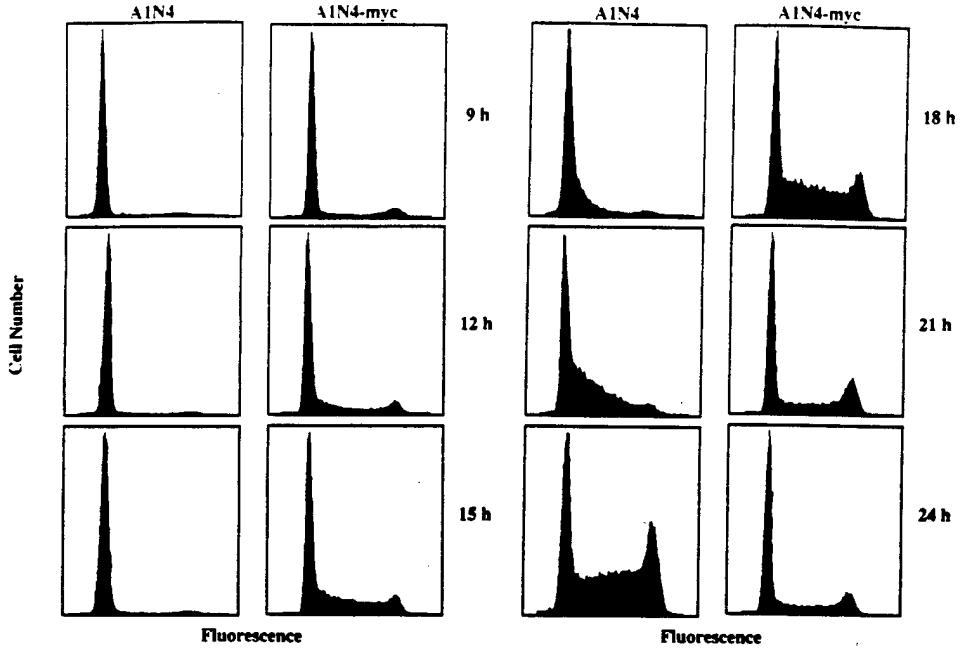

Fig. 3 Cell cycle analysis of A1N4 and A1N4-myc cells re-stimulated with EGF following growth arrest for 48 h. Arrested cells were treated with 10 ng/ml EGF and harvested at 3-h intervals. Propidium iodide staining and FACS analysis were performed with isolated nuclei.

to the fact that both cell lines arrested in G_1 upon EGF deprivation (Fig. 2*B*). To determine the kinetics of cell cycle progression in the two lines, cells were arrested in G_1 in the absence of EGF and were allowed to reenter the cell cycle by replacing EGF. FACS analysis demonstrated that the A1N4-myc cells began to enter S phase 12 h after EGF addition and that the percentage of cells in S phase peaked at 18 h (Fig. 3). In contrast, parental cells did not enter S phase until 18 h, and they peaked at 24 h. The results suggested that the difference in doubling time was due to a shortened G_1 phase of the cell cycle.

Because Rb is believed to play an important role in the G_1 phase of the cell cycle, we next examined Rb expression and phosphorylation and observed a significant difference between the two cell lines. In arrested A1N4 cells, Rb expression was relatively low, and the protein was present only in the hypophosphorylated state (Fig. 4). About 6 h after EGF stimulation, ~50% of the protein was found in the hyperphosphorylated state. At all time points beyond 6 h, Rb protein levels were greatly increased, and most of the protein was hyperphosphorylated. In contrast, Rb was highly expressed and phosphorylated at all time points tested in A1N4-myc cells. However, because Rb contains many phosphorylation sites that regulate its activity, it is quite possible that restimulation with EGF does actually increase the level of Rb phosphorylation, although we were unable to detect it. Using Western analysis to measure a change in migration due to phosphorylation is not sensitive enough to distinguish between partial and full phosphorylation. Nonetheless, it is interesting to note that, in both cell lines, entry into S-phase following EGF addition began ~12 h after hyperphosphorylated Rb could be detected.

We then examined the levels of several proteins that are known to be involved in the regulation of G_1 progression and have been implicated in the phosphorylation of Rb (Fig. 5). Cyclin D1 protein expression was absent in arrested cells, was rapidly induced following EGF stimulation, and remained elevated throughout the remainder of the cell cycle. Cyclin D1 levels were maximal at 6 h after stimulation in A1N4 cells and at 3 h in A1N4-myc cells. Cyclin E protein was detectable in arrested cells of both lines, with moderately higher levels in A1N4-myc cells. Following EGF addition, cyclin E was further stimulated and then down-regulated later in the cell cycle. Peak levels of this cyclin were observed between 9 and 15 h in parental cells and from 3 to 6 h in c-*myc*-expressing cells. Expression of two cdks that interact with cyclins D1 and E were also examined. cdk4 expression was constant throughout the cell cycle in both cell lines, but protein levels were higher in A1N4-myc cells. Western analysis of cdk2 demonstrated a dramatic mobility shift in the protein as cells progressed through the cell cycle. This type of bandshift has previously been shown to be the result of phosphorylation by the enzyme termed CAK (40). In A1N4 cells, the shift was observed about 12 h after EGF addition, whereas A1N4-myc cells already contained some phosphorylated cdk2 protein, even when arrested, with a maximal shift at ~6–9 h poststimulation. As expected, those mobility shifts corresponded to the time of maximal cyclin E induction. CAK is believed to be constitutively active, but it can only phosphorylate cdks that are complexed with a cyclin (40). Finally, expression of a cdk inhibitor, p27, was analyzed. Protein levels were quite high in arrested A1N4 cells and decreased as the cells progressed through the cell cycle. In contrast, p27 was barely detectable in arrested A1N4-myc cells and was rapidly eliminated following EGF addition.

Taken together, the results presented in Fig. 5 suggested that differences in cdk2 activity might play a role in the shortened G_1 phase in c-*myc*-overexpressing cells because p27 and cyclin E are also known to regulate cdk2 activity, in addition to the activation of cdk2 by CAK phosphorylation. We, therefore, directly examined activation of cdk2 in the cells with an *in vitro*

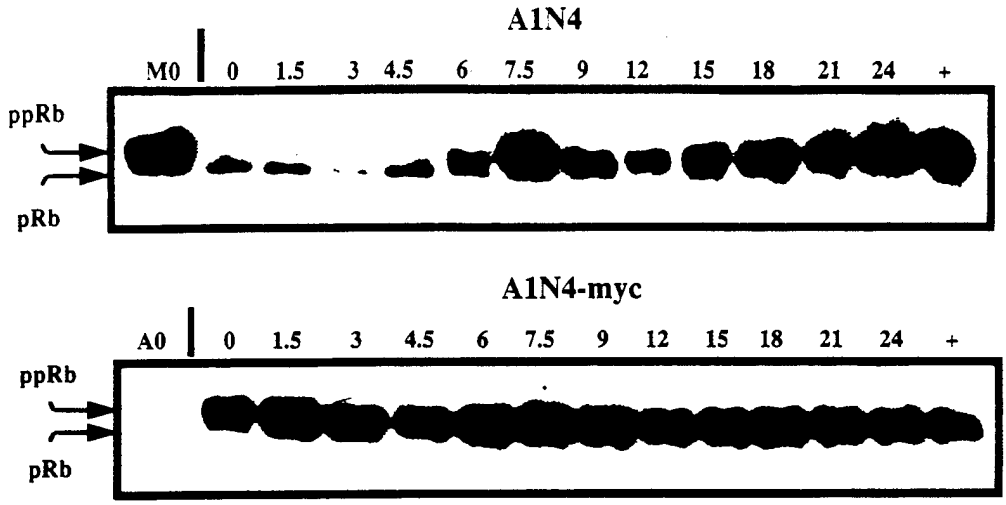

Fig. 4 Expression and phosphorylation of Rb in synchronized cells (A1N4 and A1N4-myc). Arrested cells were restimulated with EGF, and whole-cell lysates were prepared at the times indicated. Twenty μg of protein were separated on a 6% gel before transfer to nitrocellulose for Western analysis. The faster moving band is due to hypophosphorylated (inhibitory) Rb, and the upper band contains hyperphosphorylated Rb. *Numbers* above each lane indicate time (in h) after EGF addition; *Lane* +, unsynchronized cells; *Lane M0*, A1N4-myc at time 0; *Lane A0*, A1N4 at time 0. *Lane A0* was included (*bottom*) to indicate the migration position of the hypophosphorylated Rb.

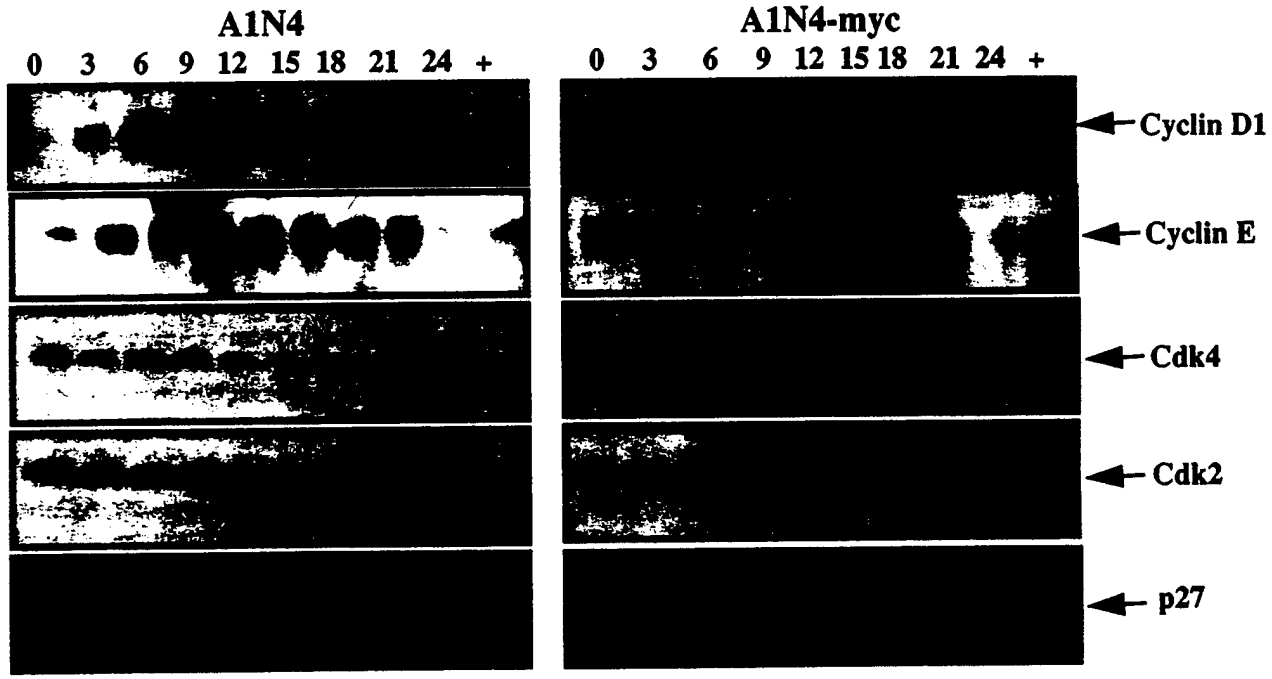

Fig. 5 Expression of the G_1 cyclins D1 and E, their associated kinases cdk4 and cdk2, and the cdk inhibitor p27 in synchronized A1N4 and A1N4-myc cells. Lysates were harvested as in Fig. 4 and were separated on 10% acrylamide gels prior to transfer to nitrocellulose for Western analysis. In all cases, proteins from the A1N4-myc cells and the parental A1N4 cells were analyzed in parallel on the same film to allow direct comparison of specific protein levels between the two cell lines. In the case of cdk2, phosphorylation by CAK leads to a downward shift in mobility, producing the observed doublet (22). *Numbers* above each lane indicate time (in h) after EGF addition; *Lane* +, unsynchronized cells.

kinase assay (Fig. 6). As predicted, arrested parental A1N4 cells contained very little active cdk2, and a significant increase in activity was observed 12 h after EGF stimulation, the time at which cyclin E was maximally expressed, p27 levels were reduced, and cdk2 was phosphorylated by CAK. In contrast, cdk2 activity was relatively high even in EGF-deprived A1N4-myc cells, with maximal activity observed at 6 h after EGF stimulation. In fact, the nearly constitutive nature of cdk2 ac-

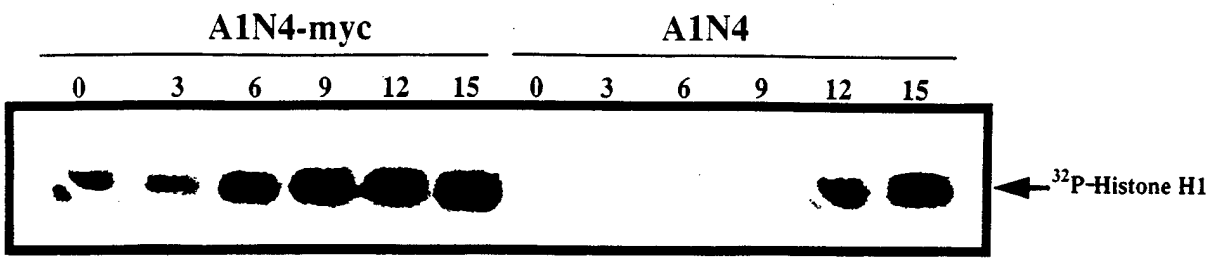

Fig. 6 Kinase activity of cdk2 in synchronized A1N4 and A1N4-myc cells. cdk2 was immunoprecipitated from whole cell lysates at the indicated times following EGF restimulation. The precipitates were then incubated for 15 min at 30°C in the presence of histone H1 and [γ-^{32}P]ATP. Labeled substrate was detected by PhosphorImager analysis following fractionation on a 10% polyacrylamide gel. *Numbers* above each lane indicate time (in h) after EGF addition.

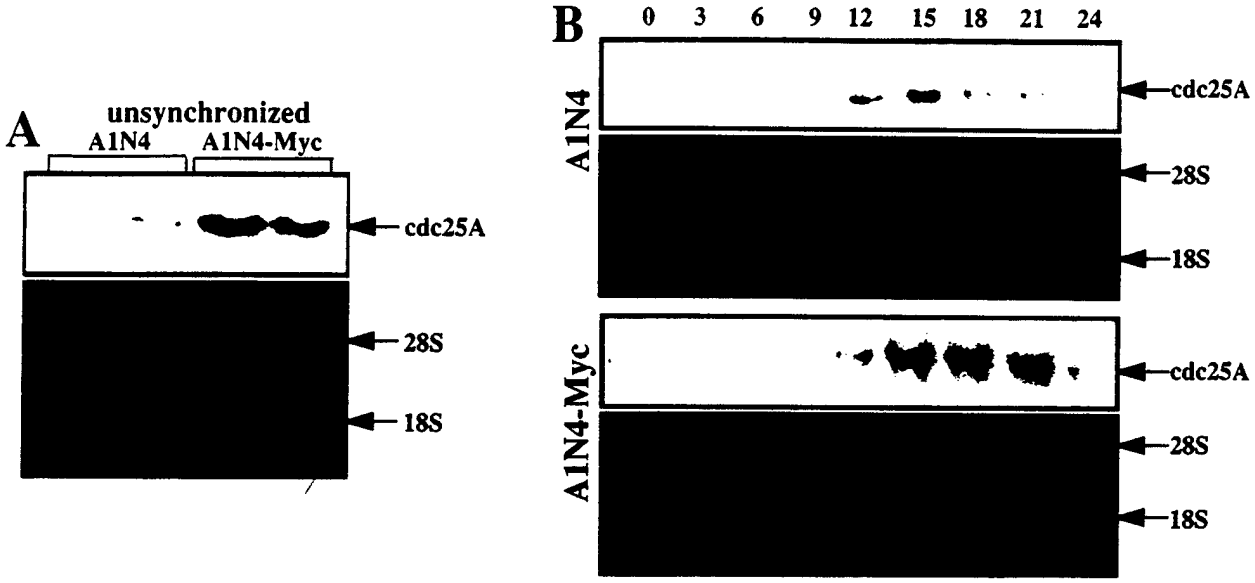

Fig. 7 Northern analysis of Cdc25A RNA in A1N4 and A1N4-myc cells. *A*, expression in unsynchronized cells. Cells were grown in the presence of EGF and were harvested at ~75% confluence. Results are shown in duplicate for each cell line. *B*, cell cycle-dependent expression. Cells were arrested and restimulated by addition of EGF, as in Fig. 3. *Numbers* above each lane indicate time (in h) after EGF addition.

tivity in the A1N4-myc cells correlated well with the constitutive hyperphosphorylation of Rb and was even more striking than could have been predicted from the results in Fig. 5.

Because a recent study identified the cdk2 phosphatase Cdc25A as a potential direct transcriptional target of myc, we also wished to examine its expression in our MEC system. In unsynchronized cells, Cdc25A RNA was elevated compared to parental cells (Fig. 7*A*). However, despite the elevated RNA levels, the time of Cdc25A induction in synchronized cells was similar in both cell lines, beginning at 12 h and reaching maximal levels between 15 and 21 h after EGF stimulation (Fig. 7*B*).

The shortened G_1 phase did not appear to be a consequence of any gross changes in cyclin A or D1 RNA expression because no significant differences in the RNA levels of these two cyclins were detected between the two cells lines during asynchronous growth (data not shown). Cyclin A and D1 RNA was undetectable in arrested cells, and induction was closely correlated with changes in cell cycle phase. In both cell lines, cyclin D1 expression was detectable by 3 h after EGF treatment and levels remained relatively constant throughout the cell cycle, in agreement with the results by Western blot. In the A1N4-myc cells, cyclin A RNA expression began ~9 h after EGF stimulation, with a peak at 18 h (39).

DISCUSSION

Human breast tumors often overexpress the proto-oncogene c-*myc*, as well as components of the EGFR signaling pathway (23, 26, 27). Transgenic mouse models have demonstrated that these two characteristics can dramatically synergize to induce early-onset, multifocal mammary tumors with a rapid growth rate (24). To begin to define a mechanism for the high proliferation rates observed in those double transgenic tumors, we chose to examine the ability of c-*myc* overexpression, in cooperation with EGF, to abrogate cell cycle regulation in an *in*

vitro MEC model system. The results presented here show that constitutive, elevated expression of c-*myc* in MECs is not sufficient to force the cells through the cell cycle but rather leads to altered cell cycle progression in response to EGF, with accelerated passage through G_1. The faster growth rate of c-*myc*-expressing MECs (A1N4-myc cells) compared to parental cells (A1N4) was correlated with constitutive phosphorylation of Rb and increased cdk2 activity. Furthermore, the elevated cdk2 activity in arrested and synchronized A1N4-myc cells compared to parental cells was associated with diminished expression of the cdk inhibitor p27 and with early onset of cyclin E expression.

Rb and the G_1 cdks. In its hypophosphorylated state, the Rb protein prevents cells from exiting the G_1 phase of the cell cycle (reviewed in Ref. 41). Normally, as cells progress through G_1, Rb becomes increasingly phosphorylated, allowing the cells to proceed into S phase to complete the rest of the cycle. We have shown that growth-arrested A1N4 cells exhibit a normal shift in Rb phosphorylation during passage through G_1 in response to EGF. In contrast, hypophosphorylated Rb was not observed in A1N4-myc cells during any phase of the cell cycle, regardless of whether the cells were proliferating in response to EGF or were arrested by EGF deprivation. Thus, the Myc-expressing cells appear to have lost an important negative regulatory mechanism for G_1 progression. However, this loss was not sufficient to allow the cells to progress into S phase in the absence of EGF.

The high levels of phosphorylated Rb in A1N4-myc cells may be due to premature cdk2 activity, which was significant even in arrested cells. *In vitro*, several cyclin/cdk complexes can phosphorylate Rb, but *in vivo*, the mechanism of Rb phosphorylation is not fully understood. Both cyclin D- and cyclin E-associated kinases have been implicated in Rb phosphorylation (41–46), but the timing of the major shift in Rb hyperphosphorylation in normal cells most closely corresponds with the activation of cyclin E/cdk2 (41, 47–50). Indeed, it has been proposed that cyclin D1-associated kinase activity may promote a low, basal level of Rb phosphorylation during the early portion of G_1 in preparation for the sudden change in Rb hyperphosphorylation via cdk2 activation late in G_1 (51).

Because constitutively hyperphosphorylated Rb in A1N4-myc cells should be inactive with respect to growth inhibition, it is interesting to note that cells prepared from Rb knockout mouse embryos also exhibit a shortened G_1 phase compared to wild-type cells, and like the A1N4-myc cells, the Rb-deficient cells are still dependent on an external growth signal and can be arrested in G_1 by serum withdrawal (52). Furthermore, the Rb-negative cells display premature and elevated expression of cyclin E but comparatively insignificant changes (either quantitative or temporal) in the expression of several other cell cycle regulated genes, including cyclin D1. Those results reiterate the likely connection between Rb function and cyclin E expression and, thus, cdk2 activity.

Cyclin D1 expression was absent in arrested cells of both cell lines and was rapidly induced by EGF stimulation, in agreement with the hypothesis that Myc and cyclin D1 function in complementary rather than linear pathways (53). No changes in cdk4 protein expression or phosphorylation were observed in either cell line under our experimental conditions, but cdk4 levels appeared to be elevated in A1N4-myc cells compared to parental cells. Thus, cyclin D1/cdk4 complexes may contribute to the accelerated growth rate of Myc-expressing cells, but clearly the high level of hyperphosphorylated Rb in arrested A1N4-myc cells cannot be attributed to cyclin D1-associated kinase activity. The results suggest that cyclin D1-associated activity may be necessary for some other aspect of G_1 progression besides Rb phosphorylation. Recently, a novel target of cdk4 and cdk6 was identified in a human breast cancer cell line (54), and certainly, there could be other, as yet undefined, targets of cyclin D1-associated kinases.

Control of Cyclin E/cdk2 Activity by p27. In normal cells, p27 protein levels undergo cell cycle-dependent oscillations, with highest levels in G_1 (55). The protein is also induced by several conditions that facilitate G_1 arrest (55–57). In the parental A1N4 line, p27 was expressed in arrested cells and was down-regulated following EGF addition. The A1N4-myc cells, in contrast, had markedly reduced p27 protein levels, even in the absence of EGF stimulation. It is thought that p27 associates with cyclin E/cdk2 until cyclin D levels are high enough to sequester the inhibitor in cyclin D/cdk complexes. p27 may, thereby, determine the order of cdk activation by inhibiting cdk2 activity until the cyclin D level (and, therefore, cdk4 activity) is maximal (32, 57). Our observations, therefore, indicate that this level of regulation is reduced or eliminated in MECs that overexpress c-*myc*, perhaps explaining the premature activity of cdk2 observed in A1N4-myc cells.

Because p27 levels are known to be controlled primarily through translational and posttranslational mechanisms, our results suggest that Myc may target an ubiquitin-dependent protein degradation pathway. However, these results appear to contrast with the findings of a recent study in fibroblasts (58). It was reported that Rat1 cells infected with a p27 retrovirus had inactive cyclin E/cdk2 complexes and arrested in G_1. Coexpression of Myc with p27 promoted cdk2 activation and released the cells from the G_1 arrest without altering the p27 protein levels. The authors proposed that Myc indirectly promoted the sequestration and inactivation of p27 rather than its degradation.

Role of Cdc25A. The Cdc25 family of phosphatases has also been implicated in the regulation of cdk activity because these enzymes remove inhibitory phosphate groups on cdks (40). The A and B forms of Cdc25 clearly play an important role in growth regulation because they can function as transforming oncogenes in cooperation with activated Ha-*ras* or loss of Rb (59). Although it has been reported that Cdc25A expression may be directly induced by Myc in fibroblasts (22), our results indicate that forced expression of c-*myc* in MECs is not sufficient to induce Cdc25A RNA expression. In our MEC system, Cdc25A steady-state RNA levels were elevated by Myc overexpression, but the timing of Cdc25A expression induction following exposure to EGF was quite similar in the two MEC lines. Cdc25A RNA was first detected 12 h after EGF addition, suggesting that other factors in addition to Myc are required for Cdc25A expression. However, even if Myc could directly induce Cdc25A expression, EGF or a related signal might still be required for full enzymatic activity because a recent study found that the phosphatase can be activated by Raf1 kinase, a target of the EGFR pathway (60).

Distinguishing the Roles of Myc in Cell Cycle, Apoptosis, and Malignant Progression. In contrast to fibroblast models in which c-*myc* expression was sufficient to force quiescent cells to reenter the cell cycle (13, 14), c-*myc* overexpression was not sufficient to drive the MECs through the cell cycle in the absence of a growth stimulus (EGF). That difference may be due to cell type specificity, but it should also be pointed out that although the fibroblasts in these previous studies reentered the cell cycle, they were executing an apoptotic pathway rather than a proliferative response. The A1N4-myc cells, like the parental A1N4 cell line, reversibly arrest in G_1 in the absence of EGF rather than undergoing apoptosis. In primary mouse tumor MECs, deregulated c-*myc* expression can induce apoptosis in the absence of growth/survival factors (30, 31). That observation suggests that A1N4 cells, perhaps during the process of immortalization, have undergone some change that makes them incapable of executing the apoptotic pathway in response to Myc. Thus, these cell lines provide an excellent model for studying alterations in cell cycle control due to c-*myc* overexpression in the absence of the confounding effects of apoptosis induction. This is an important distinction to make because a recent study indicated that the effects of Myc on cell cycle progression and apoptosis are, indeed, distinct (61).

Although our studies have focused on an MEC model that is particularly dependent on EGF for growth, a variety of growth factors have been implicated in the regulation of breast cancer cell growth (reviewed in Ref. 62), and several are known to cooperate with c-myc to promote a transformed phenotype in MECs (29). Thus, our results may be representative of a more general phenomenon that could occur when myc-overexpressing MECs are exposed to growth factors.

In summary, our results provide one explanation as to why Myc and EGF can cooperate to transform MECs and, similarly, why there is such a strong synergism between Myc and TGF-α in mammary tumorigenesis, as demonstrated by transgenic mouse models. We have previously shown that EGF can act as a survival factor for mammary tumor cells that overexpress Myc (30, 31). The current results reported here indicate that Myc overexpression, in conjunction with EGFR stimulation, can also force MECs through G_1 at a faster rate, resulting in accelerated growth. Taken together, these two characteristics may allow epithelial cells within the mammary gland to survive and proliferate under some conditions that would normally prevent DNA replication through the induction of apoptosis or a G_1 arrest. Thus, increased genetic instability may also be a consequence of such a phenotype, analogous to the phenomenon that was demonstrated for p53 mutations (63, 64). Indeed, it has been demonstrated that Rat1a cells with prolonged Myc overexpression exhibit a variety of genetic aberrations, including numerical changes, chromosome breakage and fusions, and extrachromosomal elements (65). In further support of that hypothesis, a recent study demonstrated that Myc overexpression blocks G_1 cell cycle arrest in response to PALA and permits *CAD* gene amplification (66).

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Multicolour spectral karyotyping of mouse chromosomes

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Murine models of human carcinogenesis are exceedingly valuable tools to understand genetic mechanisms of neoplastic growth. The identification of recurrent chromosomal rearrangements by cytogenetic techniques serves as an initial screening test for tumour specific aberrations. In murine models of human carcinogenesis, however, karyotype analysis is technically demanding because mouse chromosomes are acrocentric and of similar size. Fluorescence *in situ* hybridization (FISH) with mouse chromosome specific painting probes[1] can complement conventional banding analysis. Although sensitive and specific, FISH analyses are restricted to the visualization of only a few mouse chromosomes at a time. Here we apply a novel imaging technique[2] that we developed recently for the visualization of human chromosomes[3] to the simultaneous discernment of all mouse chromosomes. The approach is based on spectral imaging to measure chromosome-specific spectra after FISH with differentially labelled mouse chromosome painting probes. Utilizing a combination of Fourier spectroscopy, CCD-imaging and conventional optical microscopy, spectral imaging allows simultaneous measurement of the fluorescence emission spectrum at all sample points. A spectrum-based classification algorithm has been adapted to karyotype mouse chromosomes. We have applied spectral karyotyping (SKY) to chemically induced plasmocytomas, mammary gland tumours from transgenic mice overexpressing the c-*myc* oncogene and thymomas from mice deficient for the ataxia telangiectasia (Atm) gene. Results from these analyses demonstrate the potential of SKY to identify complex chromosomal aberrations in mouse models of human carcinogenesis.

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We performed spectral karyotyping (SKY) on a normal mouse metaphase spread prepared from splenocytes of the FVB mouse (Fig. 1). The chromosome specific painting probes were generated by high resolution flow sorting[1] and were labelled either singly with Spectrum Green, Cy3, Texas Red, Cy5, or Cy5.5 or in combinations (Table 1). The differentially labelled probe pools were combined and hybridized together with an excess of unlabelled Cot-1 fraction of mouse genomic DNA to mouse metaphase chromosome preparations. Chromosome-specific spectra were measured using spectral imaging. Spectral imaging combines Fourier spectroscopy, CCD-imaging and optical microscopy to measure the fluorescence emission spectrum simultaneously at all image points (pixels) after a single exposure[2,3]. Therefore, spectral imaging is significantly different from conventional fluorescence imaging techniques for FISH experiments based on fluorochrome-specific optical filters and multiple subsequent exposures[4]. The spectral measurement of the hybridization was visualized by assigning a red, green and blue (RGB) look-up table to specific sections of the emission spectrum (Fig. 1*b*, see Methods). For instance, the X-chromosome, labelled only with Spectrum Green appears blue, chromosome 16 (Cy3) appears green, and chromosome 2 (Cy5 and Cy5.5) appears red (Fig. 1*b*). The RGB display allows the assessment of important parameters of the hybridization, e.g., intensity and homogeneity (Fig. 1*b*). Based on the measurement of discrete emission spectra at all pixels of the image, the hybridization colours are then converted by applying a spectral classification algorithm that results in the assignment of a discrete colour to all pixels with identical spectra (Fig. 1*c*). The spectral classification is the basis for chromosome identification (Fig. 1*c*) and spectral karyotyping (Fig. 1*d*).

Table 1 Fluorescence labelling scheme of mouse chromosomes

Chromosome	Spec. green	Cy5	Cy3	Texas red	Cy5.5
1	+	+	+		
2		+			+
3			+		+
4	+		+	+	
5		+	+		
6	+		+		+
7	+			+	
8			+	+	
9					+
10		+			
11		+		+	
12	+	+			+
13		+	+	+	
14		+	+		+
15	+		+		
16			+		
17				+	+
18	+	+		+	
19	+				+
X	+				
Y				+	

Murine models of human carcinogenesis are widely used to delineate genetic mechanisms that determine tumour initiation and progression[5]. The study of chromosomal aberrations in mouse models, however, is extremely demanding because mouse chromosomes are difficult to discern and subtle aberrations often remain unrecognized. The potential of spectral karyotyping as a genome scanning method for detection of chromosomal aberrations in mouse metaphase spreads was explored by analysing metaphase chromosomes from different mouse models of human carcinogenesis: (i) in chemically induced plasmocytomas in BALB/c mice, (ii) in mammary gland tumours that developed in transgenic animals that overexpress the c-*myc* gene under the control of the MMTV-promotor and (iii) in thymomas that occured in mice deficient for the ataxia telangiectasia (*Atm*) gene.

Mice from susceptible strains (BALB/c) treated with pristane oil develop, in a multistep process, tumours that are histomorphologically and immunologically similar to human plasmocytomas[6]. The cytogenetic hallmark of

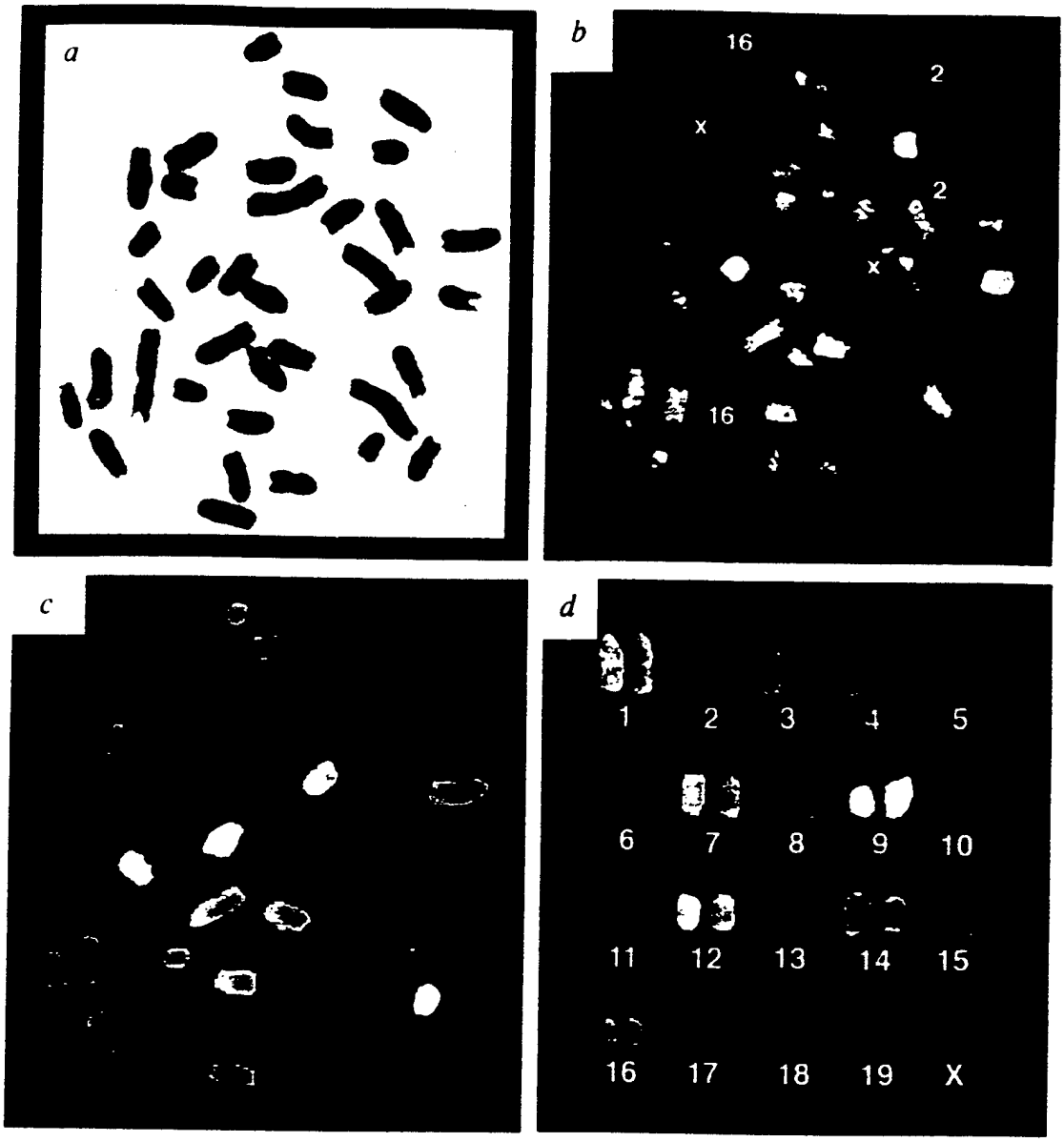


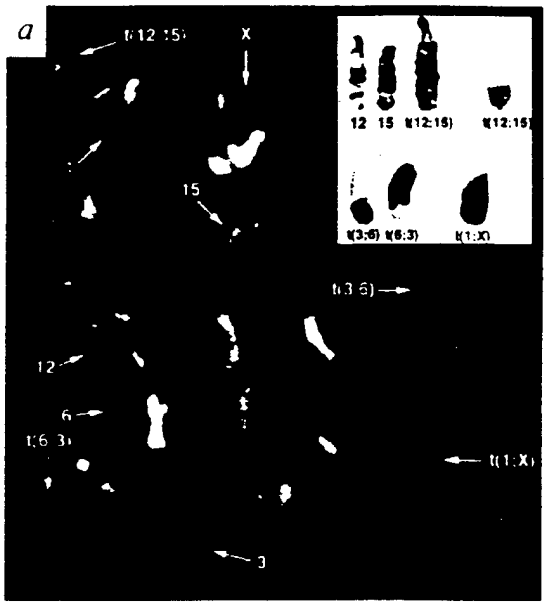
Fig. 1 Spectral karyotyping (SKY) of mouse chromosomes. *a*, Metaphase spread from normal mouse splenocytes (Strain: FVB). The inverted image of the DAPI-stained chromosomes results in a G-like banding pattern. *b*, Same metaphase as in (*a*) after simultaneous hybridization of 21 differentially labelled mouse chromosome painting probes. The display colours allow discernment of most chromosomes in different colours. However, chromosome painting probes that were labelled with similar fluorochromes appear in similar colours. The numbers denote chromosomes that were labelled with green (X-chromosome), red (chromosome 16) or near infrared (chromosome 2) fluorochromes only. *c*, Spectral classification of the same metaphase spread shown in (*a*, *b*). Based on pixel by pixel measurement of the spectra of the entire image, the display colours in (*b*) were converted to classification colours. Here, all pixels that have identical spectra were assigned the same classification colour. *d*, The spectral classification forms the basis for colour karyotyping of mouse chromosomes. All mouse chromosomes can be discerned according to a specific colour.

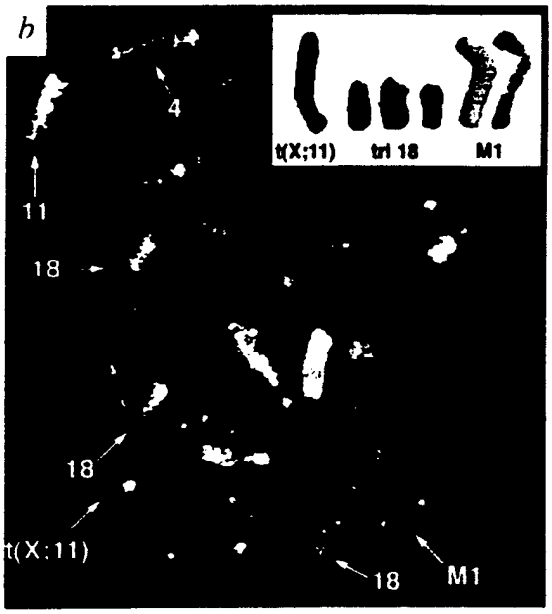
pristane-induced plasmocytoma in mice is the chromosomal translocation t(12;15) that juxtaposes the c-*myc* oncogene in the vicinity of promoters for the immunoglobulin heavy chain genes on mouse chromosome 12 (ref. 6). The genetic consequence of the mouse translocation t(12;15) is therefore similar to the translocation t(8;14) observed in human Burkitt's lymphoma. However, it is not clear whether tumour progression depends on additional chromosomal aberrations and conventional cytogenetic analysis has not detected further recurrent chromosomal aberrations. We examined the entire chromosome complement from a mouse plasmocytoma (X24) by SKY on metaphase chromosomes. In this hypotetraploid tumour, the translocation t(12;15), identified by G-banding, was readily confirmed. In addition, unrecognized rearrangements were detected. In all cells analysed ($n = 9$) a reciprocal translocation t(3;6) and a translocation t(1;X) were identified (Fig. 2*a*). The analysis of a larger number of tumours using SKY will reveal if similar aberrations occur consistently in mouse plasmocytomas, and the fine mapping of the translocation breakpoints will guide the search for candidate genes.

The analysis of a sequence of chromosomal aberrations that occur during the development of human breast carcinomas is arduous. This is in strong contrast to, for example, colon carcinogenesis[7] and is mainly attributable to the difficulty of retrieving tumour mate-

rial at defined stages of human breast carcinogenesis. It is clear, however, that a recurrent pattern of chromosomal aberrations occurs in breast carcinomas and that the gain of function of certain oncogenes, such as the c-*myc* oncogene, is a crucial genetic aberration[8]. Transgenic mice that overexpress oncogenes hold great promise in unravelling genomic effects of oncogene gain-of-function mutations because they will allow the study of chromosomal aberrations that occur during tumour initiation and progression[9]. Transgenic mice that express the c-*myc* oncogene under MMTV-promotor control develop mammary gland tumours at the age of 9 to 12 months[10]. We analysed metaphase spreads from one of these tumours[11] by SKY in order to identify and map chromosomal aberrations (Fig. 2*b*). Numerous rearrangements were identified that occured in the majority (90%) of the cells ($n = 11$): a loss of the X chromosome, trisomy for chromosome 18, a translocation t(X;11) and a dicentric marker chromosome that is derived from chromosome 4. The identification of this dicentric marker chromosome is particularly interesting because it indicates that the formation of a dicentric marker does not preclude proper chromosome segregation during mitotic cell division. Compared to G-banding alone, SKY will allow large numbers of tumours to be analysed reliably and rapidly. This may provide insight into c-*myc* induced chromosomal changes in murine mammary gland tumorigenesis.

The biological and genetic significance of tumour suppressor gene function can be elegantly studied in mice[12]. For example, we have studied tumours that developed in mice that have homozygous disruption of the *Atm* gene[13]. Humans afflicted with ataxia telangiectasia (AT) have, among a plethora of other symptoms, an increased risk for the development of malignant tumours, most commonly lymphomas. *Atm*-deficient mice have virtually all of the findings of AT patients. Of note, thymic lymphomas were observed invariably between 2 and 4 months of age[13]. We analysed the chromosomal consequences of *Atm* inactivation in one of the thymomas using SKY (Fig. 2*c*). We examined 12 metaphases; increased genomic instability was reflected by a marked heterogeneity of chromosomal aberrations in metaphase spreads of this tumour. We detected an involvement of chromosomes 14 in all metaphase spreads, suggesting an important role of genes on this chromosome in





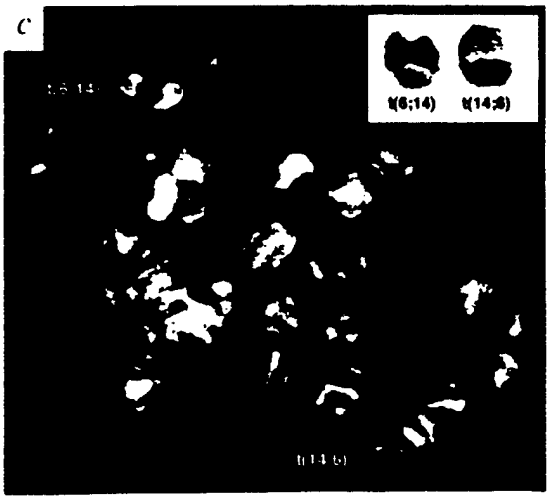


Fig. 2 Applications of SKY to identify chromosomal aberrations in murine models of carcinogenesis. *a*, Metaphase spread from a mouse plasmocytoma (XRPC24). Several chromosomal translocations were detected. SKY confirmed the presence of a translocation t(12;15) which had already been identified by G-banding analysis (inset). Previously unrecognized chromosomal aberrations not detected by chromosome banding alone include a reciprocal translocation involving chromosomes 3 and 6 as well as a translocation t(1;X). Arrows denote the aberrant chromosomes in metaphase and their normal homologues. The spectra based classification of all aberrant chromosomes is presented (inset). *b*, Metaphase spread from a cell line established from a mammary gland carcinoma that developed in c-*myc* transgenic mice. Several previously unknown aberrations were identified: translocation t(X;11), trisomy 18, and a marker chromosome (M1) derived from chromosome 4. Arrows denote the aberrant chromosomes in metaphase and their normal homologues. The spectrum-based classification and the DAPI-banding of the marker M1 is shown (inset). *c*, Identification of chromosomal aberrations in metaphase cells of primary cultures from a thymoma that developed in mice with homozygous inactivation of the ataxia telangiectasia (*Atm*) gene. The reciprocal translocation t(6;14) was readily identified (arrows). The aberration was confirmed by spectra-based classification (inset).

tumorigenesis (Fig. 2*c*). Chromosome 14 carries the genes for T-cell receptor chains α and δ. Similar involvement of T-cell receptor genes was observed in lymphomas diagnosed in AT-patients[14]. This suggests common genetic mechanisms in human AT and murine *Atm*-deficiency. Based on the SKY-results, a targeted analysis of candidate genes will allow the further elucidation of genetic pathways involved in the genesis of malignant thymomas.

We have shown that spectral karyotyping is a reliable and robust screening test that is broadly applicable to the detection of chromosomal aberrations in murine models of tumorigenesis. SKY will assist in the identification of regions that are recurrently altered in mouse model systems by delineating consistent chromosomal breakpoints. Other applications of SKY will extend to the interspecies analysis of chromosomal aberrations in related animal models, such as hamster or rat, and will become an important tool to automate the analysis of a large number of metaphase spreads for mutagenicity testing in experimental toxicology.

Methods

Preparation of mouse metaphase chromosomes. Mouse metaphase chromosomes were prepared from LPS-stimulated spleen cultures, from cell lines established from transgenic mice, or from short term cultures according to standard procedures, and fixed in methanol acetic acid. The suspensions were dropped onto precleaned slides and rinsed with acetic acid to remove excess cytoplasm. The preparations were dehydrated through an ethanol series and stored at room temperature.

Amplification and labelling of flow sorted mouse chromosomes. Mouse chromosomes were isolated by high resolution flow sorting as described[1]. Chromosomes were amplified using degenerate oligonucleotide primed PCR (DOP-PCR)[15]. The individual probe pools were labelled using DOP-PCR by direct incorporation of haptenized or fluorochrome conjugated nucleotides in the combination described in Table 1.

***In situ* hybridization and detection.** Approximately 100 ng of the labelled probe pools for each mouse chromosome were precipitated in the presence of 30 μg of the Cot-1 fraction of mouse genomic DNA (Bethesda Research Laboratories). The probe cocktail was resuspended in 50% formamide, 10% dextran sulfate, 2× SSC and denatured (5 min, 80 °C) followed by a pre-annealing step for 1 h at 37 °C. After hybridization at 37 °C for 2 days, the slides were washed, and the haptenized probe sequences detected: biotin labelled sequences were detected using avidin Cy5, and the digoxigenin labelled sequences were detected using a mouse anti-digoxigenin antibody followed by a goat anti-mouse antibody conjugated to Cy5.5 (Amersham Life Sciences). Samples were counterstained with DAPI and embedded in an antifade reagent (para-Phenylenediamine).

Spectral imaging. The spectral analysis is based on the spectral cube system (SD200, Applied Spectral Imaging). Imaging and analysis were performed as described[2,3]. The SD200 imaging system was attached to an inverted microscope (Leica DMIRBE) via a C-mount. It consists of an optical head with a special Fourier transform spectrometer (Sagnac common path interferometer) to measure the spectrum and a cooled CCD camera (Princeton Instruments) for imaging. The samples were illuminated with a Xenon lamp (OptiQuip 770/1600) and imaged with a 63x oil immersion objective through a custom designed filter set (Chroma Technology) with broad emission bands (excitation filter: 486/28, 565/16, 642/22; emission filter: 524/44, 600/38, 720/113; beamsplitter: reflection 421–480, 561–572, 631–651, transmission 495–564, 580–620, 660–740). The use of this filter allows the simultaneous excitement of all dyes and the measurement of their emission spectra. Therefore, a single exposure (approximately 1 min depending on the brightness of the preparation) is sufficient to acquire the entire image. The conversion of emission spectra to visualize the spectral image in display colours is achieved by assigning a different colour (blue, green, red) to specific spectral ranges. The intensity for each colour is proportional to the integrated intensity in the corresponding spectral range (Fig. 1*b*). The spectral classification that is independent of signal intensities enables multiple different spectra in the image to be identified and highlighted in classification-colours (Fig. 1*c*). This allows assignment of a specific classification-colour to all mouse chromosomes based on their spectra alone (Fig. 1*d*). The algorithm is described in detail[3].

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ETIOLOGY OF BREAST AND GYNECOLOGICAL CANCERS

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MECHANISMS OF DES CARCINOGENICITY: EFFECTS OF THE TGFα TRANSGENE*

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ABSTRACT

Inappropriate estrogen exposure during critical periods of development will cause numerous abnormalities in the female reproductive tract. Epigenetic effects on the expression of estrogen-regulated genes is proposed to be one of the mechanisms by which neonatal estrogen elicits teratogenic and carcinogenic effects. Of note is the existence of an integral relationship between the regulation of members of the epidermal growth factor (EGF) gene family and estrogen effects on the growth and differentiation of the reproductive tract. To determine whether the EGF gene family plays a critical role in mediating the pathogenic effects of estrogen, we have used transforming growth factor-alpha (TGFα) transgenic mice to investigate the effects of constitutive TGFα expression in the reproductive tract and whether TGFα would potentiate carcinogenesis induced by the potent estrogen, diethylstilbestrol (DES), and by the carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA). The animals were homozygous TGFα transgenic female mice from the MT42 line and the parental CD-1 outbred mice. Constitutive TGFα expression was found to augment the effects of both DES and DMBA in eliciting hyperplastic and differentiation changes in the reproductive tract. The presence of the TGFα transgene significantly increased the incidence of DES-induced vaginal adenosis, uterine endometrial hyperplasia, hypospadia, and benign ovarian cysts. In addtion,

TGFα potentiated the effects of DMBA in eliciting uterine polyps and benign ovarian cysts, and in the retention of Wolffian Duct remnants. However, the incidence of reproductive tract neoplasia was not promoted by the presence of the TGFα transgene. This study indicates that TGFα plays a role in the developmental and morphogenic events of both the Müllerian duct and urogenital sinus, and that deregulation is associated with pathogenesis of these tissues. Furthermore, the fact that constitutive expression of the TGFα did not substitute for DES as a reproductive tract carcinogen or act as a promoter of DES-induced uterine neoplasia suggest that DES carcinogenesis involves more than aberrant expression of this single growth factor.

INTRODUCTION

Studies have clearly shown that disturbances of reproductive tract growth and differentiation can be elicited by exposure to estrogens during critical developmental stages (Gray, et al., 1994; Iguchi, 1992; McLachlan, et al., 1982; Metzler and Mclachlan, 1977; Noller, et al. 1990). Although the mechanism(s) responsible for the carcinogenic and teratogenic effects of estrogens such as diethylstilbestrol (DES) remains unknown, the premature and permanent induction of genes normally under steroid hormone control has been proposed to contribute to the establishment of the ovarian-independent, "estrogenized" phenotype in the reproductive tracts of exposed animals (Gray, et al., 1994). Substantial evidence points to a critical role of the EGF pathway in the early development of the reproductive tract as well as in mediating steroid hormone effects in the adult (Bossert, et al. 1990; Gardner, et al., 1989; Gray, et al., 1994; Hall and Forsberg, 1993; Nelson-Gray, et al., 1989 and 1992; Pollard, 1990). In fact, exposure to DES during the critical neonatal developmental period in mouse has been shown to result in the persistent induction of EGF in the uterus and vagina. This evidence supports the hypothesis that the developmental toxicity of estrogens is due, in part, to the inappropriate expression of estrogen-regulated genes which may contribute to permanent disruptions in growth and differentiation. Dysregulation of autocrine/paracrine growth pathways due to either elevated expression of growth factors or synthesis, and/or mutation of their receptors is a consistent alteration observed in many neoplastic cells including in reproductive tissues (Gray, et a., 1994).

Studies of transgenic mice expressing growth factor genes have shown direct links between the overexpression or inappropriate expression of a single growth factor and the development of lesions marked by abnormal proliferation and differentiation, and neoplasia (Bockman, et al., 1995; Jhappan, et al., 1990 and 1994; Ma, et al., 1994p; Matsui, et al., 1990; Sharp, et al., 1995; Smith, et al., 1995; Takagi, et al., 1993; Vassar, et al., 1992). In various TGFα transgenic mouse strains, preneoplastic and neoplastic changes have been demonstrated in the mammary gland, salivary gland, stomach mucosa, coagulating gland, liver, and pancreas. Although, in most studies the aberrant expression of a growth factor transgene alone does not result in neoplastic lesions unless long latency periods are accommodated. For example, TGFα overexpression must be combined with wounding, or treatment with either a phorbol ester tumor promoter or an initiator in order to elicit papilloma formation in mouse skin (Jhappan, et al., 1994; Vassar, et al., 1992). Development of

mammary gland neoplasia in TGFα transgenic female mice requires exposure to the hormonal milieu of pregnancy (Matsui, et al., 1990; Smith, et al., 1995). Thus, often a proper hormonal or growth promoting environment must be provided before the neoplastic effects of TGFα can be manifested.

In addition to neoplastic lesions, tissues of TGFα transgenic animals exhibit changes that reflect disruption of the mechanisms that regulate tissue homeostasis, involving effects on cell proliferation, cell differentiation, and senescence (Bockman. et al., 1995; Jhappan, et al., 1990; Ma , et al., 1994; Sharp, et al., 1995). This is demonstrated by TGFα stimulating the redifferentiation of pancreatic acinar cells into simple ductal cells, mucin-producing cells, and occasionally to insulin-producing cells. Disorganization of the gastric mucosa with pathological accumulation of surface mucous cells and depletion in differentiated parietal and chief cells is another characteristic of TGFα mice. Effects of TGFα on the function and response of tissues in the hypothalamic-pituitary-ovarian axis results in alterations of sexual development in the TGFα transgenic animals.

Based on the evidence that TGFα affects reproductive function and that members of the EGF family mediate sex steroid hormone action, we questioned whether the EGF gene family also plays a critical role in pathological changes in the reproductive tract. Here we report the results of our study using TGFα transgenic mice to investigate the effects of constitutive TGFα expression on the induction of reproductive tract alterations by the potent estrogen, diethylstilbestrol (DES), and by the carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA). In comparison to the nontransgenic animals, the presence of the TGFα transgene was found to promote certain reproductive organ-specific pathological abnormalities, but not neoplasia. This supports the hypothesis that deregulation of a single peptide growth factor, like TGFα, can lead to growth and differentiation abnormalities; but, aberrant expression of TGFα alone is clearly not sufficient for carcinogenesis of reproductive tissues.

METHODS

This study was designed to determine whether TGFα expression could modulate DES- and DMBA-induced carcinogenesis of the reproductive tract. The animals were homozygous TGFα transgenic female mice from the MT42 line generated as described previously (Jhappan, et al., 1990) and the parental nontransgenic CD-1 outbred mouse strain. Study duration was 52 weeks with an interim sacrifice at 39 weeks. However, mice that became moribund were sacrificed. After evaluation of the data, there was not a difference between the two major sacrifices and the data were pooled for presentation. Mice were given NIH31 rodent chow (Ziegler Brothers) and water ad libitum. The experimental animal protocol that was followed was in accordance with the NIH approved procedures. At sacrifice, the reproductive tracts were removed, fixed in Bouins' Fixative for 24-48h, washed in water for at least 24h, and stored in 70% ethanol until paraffin embedded. Serial sections (5-7 μM) of the embedded tissues were stained with hematoxylin and eosin and evaluated for histopathological alterations. Unless designated, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO). Data was analyzed by CHI square and observed to significantly different at <0.05 with one degree of freedom.

There were eight experimental groups consisting of the parental CD-1 and TGFα transgenic mice treated with DES (2 ug/pup in sesame oil), DMBA (2mg/20g in peanut oil), both DES and DMBA, or with the vehicles alone using the regimen described below:
Group #1: CD-1 mice, vehicle control, received daily subcutaneous sesame seed oil injections on days 1-5 of age and a single intragastric peanut oil administration at 4 weeks of age.
Group #2: TGFα transgenic mice, vehicle control, treated as described for Group #1.
Group #3: CD-1 mice, neonatal DES treatment, received daily DES treatment by subcutaneous injections from days 1 to 5 of age, and then administered by gavage a single peanut oil vehicle dose at 4 weeks of age.
Group #4: TGFα transgenic mice, neonatal DES treatment, treated as described for Group #4.
Group #5: CD-1 mice, neonatal DES and DMBA treatment, received daily subcutaneous DES treatments on days 1 to 5 of life, then at 4 weeks of age a single intragastric dose of DMBA was administered.
Group #6: TGFα transgenic mice, neonatal DES and DMBA treatment, following the same strategy as performed for Group #5.
Group #7: CD-1 mice, DMBA treatment, received sesame seed oil vehicle subcutaneous injections on days 1 to 5 of age and a single intragastric DMBA treatment at 4 weeks of age.
Group #8: TGFα transgenic mice, DMBA treatment, following the same regimen as Group #7.

RESULTS

Uterine Alterations: A summary of the most distinctive histological changes observed in the uterus of CD-1 and TGFα transgenic mice is shown in Figure 1. Of special note is that the vehicle control TGFα transgenic animals (Group #2) exhibited a significant higher incidence of endometrial hyperplasia (ranging from microcytic to atypical) and of uterine polyps than observed in the vehicle treated nontransgenic CD-1 parental mice.

Similarly, neonatal DES treatment of TGFα transgenic mice (Group #4) resulted in a significantly greater incidence of endometrial hyperplasia than observed in the nontransgenic animals (Group #3), further indicating a propensity of TGFα transgenic mice for the development of hyperplasia. In contrast, the presence of the TGFα transgene did not potentiate the incidence of the uterine adenocarcinomas from that induced by DES in the CD-1 parental strain. Also, DES treatment suppressed the development of uterine polyps in the TGFα animals.

A single DMBA treatment given at four weeks of age did not dramatically modify DES induction of uterine adenocarcinomas in the CD-1 (Group #5) and only slightly decreased the number of tumors in the transgenic (Group #6). In contrast, DMBA exposure significantly reduced cystic endometrial hyperplasia in the CD-1 animals compared to the mice receiving DES treatment only (Group #3). Although not statistically significant, the transgenics also demonstrated a decrease in the incidence of endometrial hyperplasia upon

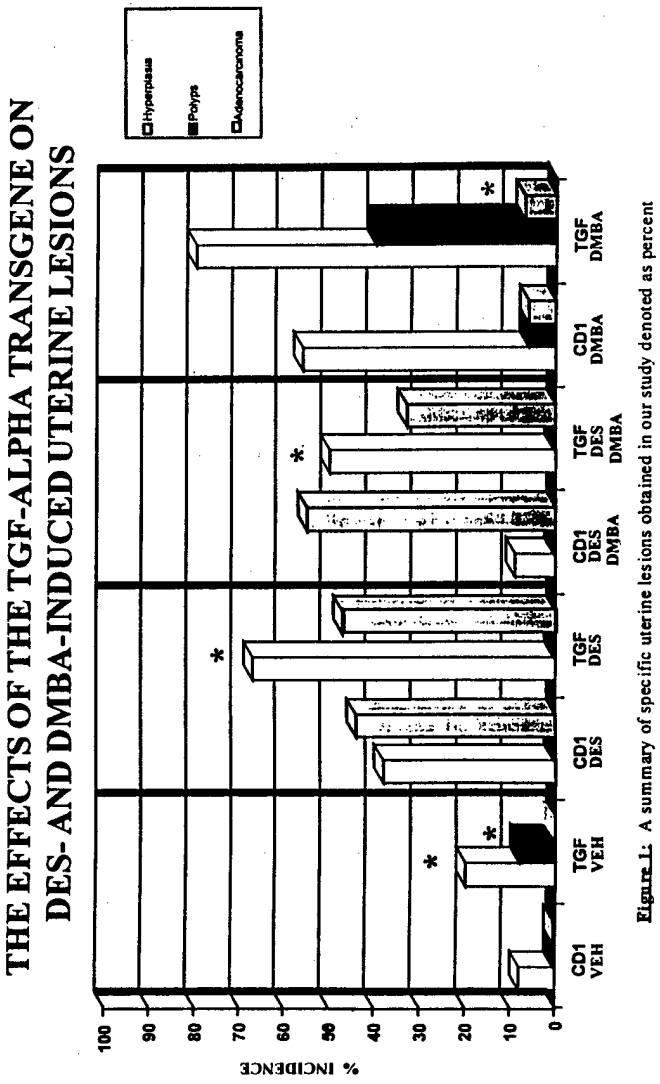

Figure 1: A summary of specific uterine lesions obtained in our study denoted as percent incidence obtained by dividing the number of animals with a lesion by the total number of animals in the treatment group. The asterisk (*) indicates that the TGFα animals exhibit significantly greater number of lesions than observed in the parental CD-1 mice, as determined by CHI square analysis. Note the clear differences in uterine histology between the CD-1 mice and the TGFα transgenic animals.
Abbreviations: CD1=parental mouse strain; TGF=TGFα transgenic mice; VEH=vehicle treated; DES=diethylstilbestrol treated; DES & DMBA=treated with both DES and 7,12-dimethybenz[a]anthracene; DMBA=DMBA treated.

DMBA exposure. Only one animal in Groups #5 and #6, a CD-1 mouse, exhibited a uterine polyp.

Treatment with DMBA alone induced only a low incidence of uterine adenocarcinomas in the CD-1 (Group #7) and the TGFα transgenic (Group #8) animals. In addition, rare tumors, a uterine leiomyosarcoma, a stromal cell sarcoma, and a hemangiosarcoma were found in the DMBA treated CD-1 animals and a single benign leiomyoma was found in a uterus of a TGFα transgenic mouse. Angioecstasis and hemangiomas were commonly found in the uterus of both CD-1 and transgenic mice which are characteristic DMBA-induced lesions. In contrast to the low incidence of adenocarcinomas, a single treatment with DMBA resulted in the greatest induction of endometrial hyperplasia over vehicle controls in both the transgenic and parental mice with the TGFα transgenics demonstrating the greatest response (CD-1 56%; TGFα 80%). Also, DMBA significantly promoted the development of uterine polyps in the TGFα transgenic mice (40%) in comparison to the CD-1 animals (6%).

Of special note is that there did not seem to be a correlation between the incidence of endometrial hyperplasia and the development of adenocarcinoma in animals exposed to the various treatments.

Alterations of Vaginal Histology: A summary of the vaginal abnormalities observed in the CD-1 parental and transgenic animals following DES and/or DMBA treatment in relation to vehicle controls is shown in Figure 2. A striking abnormality exhibited by the TGFα transgenic animals was the retention of the Wolffian duct, also known as Gardner's duct. Wolffian duct remnants, which normally regress during development in females, were consistently found in the TGFα transgenic mice regardless of treatment, but only in the CD-1 parental animals exposed to DMBA with or without DES. In all cases, the presence of the TGFα increased the incidence of Wolffian duct retention over that found in the control animals. Interestingly, retention of the Wolffian duct is documented to be one of the pathological alterations induced by neonatal DES exposure of nontransgenic CD-1, albeit at a very low incidence. This data links abnormal TGFα expression to the pathological occurrence of Wolffian ducts in the female reproductive tract.

Another classical DES-induced vaginal lesions is adenosis which refers to the abnormal appearance of columnar and glandular epithelium within a region of the vagina which is normally lined with squamous epithelium. Vaginal adenosis was consistently observed following neonatal DES exposure in both the CD-1 and TGFα transgenics; however, the presence of the TGFα transgene significantly potentiated the appearance of vaginal adenosis upon exposure to DES with or without subsequent DMBA treatment.

Besides adenosis, TGFα mice also exhibited enhanced sensitivity to DES exposure for the development of vaginal concretions, which are thought to be caused by abnormal urethral development. No concretions were found in the CD-1 mice of this study, but this lesion has been reported to occur in CD-1 mice at a very low incidence as a result of DES exposure. This evidence is the first to suggest that inappropriate expression of TGFα interferes with normal urethral development.

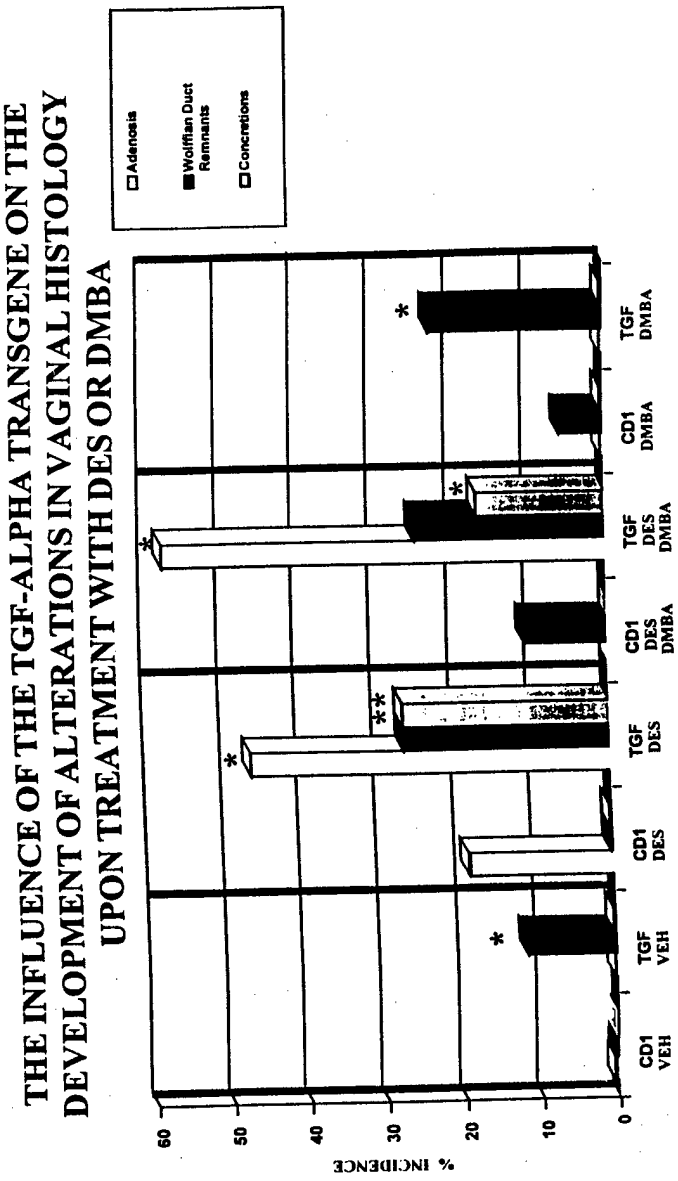

Figure 2: A summary of specific vagina lesions obtained in our study denoted as percent incidence and the asterisk (*) indicates that the TGFα animals exhibit significantly greater number of lesions than observed in the parental CD-1 mice, as determined by CHI square analysis. The presence of the TGFα transgene potentiated DES induction of vaginal adenosis, vaginal concretions caused by abnormal urethral development (hypospadia), and retention of the Wolffian duct.
Abbreviations: CD1=parental mouse strain; TGF=TGFα transgenic mice; VEH=vehicle treated; DES=diethylstilbestrol treated; DES & DMBA=treated with both DES and 7,12-dimethylbenz[a]anthracene; DMBA=DMBA treated.

Growth abnormalities in the Oviduct: Exposure to neonatal estrogen induces histological alterations in the oviduct associated with hyperplasia and gland formation (diverticuli) of the oviductal mucosa. The diverticuli extend into the muscle wall, resembling the clinical lesion termed salpingitis isthmica nodosa (SIN). Although there was an elevating trend, the presence of the TGFα transgene did not significantly increase the incidence of SIN-like lesions induced by DES over that observed in the parental CD-1 mice (Figure 3). DMBA treatment did enhance DES-induction of SIN-like oviductal lesions in both transgenic and nontransgenic mice.

Abnormalities of the Ovary: The most notable effect of the TGFα transgene on the ovary was that the transgenic mice treated with vehicle, DES, or DMBA demonstrated significant increases in the incidence of benign ovarian cysts over that found in the parental CD-1 mice (Figure 4). In addition, neonatal exposure to DES was found to significantly promote the development of ovarian granulosa cell tumors upon exposure of both transgenic and nontransgenic animals to DMBA. Although not significant, the presence of TGFα transgene potentiated the development of these ovarian tumors in response to DMBA.

Pathological findings in the Pituitaries: The pituitaries of transgenic animals resembled adenomas with malformed Rathke pouch remnants that formed ductular structures. In contrast, none of the pituitaries obtained from the CD-1 mice exhibited these alterations. Treatment did not influence the histology of the pituitaries (data not shown).

DISCUSSION

Since estrogens are known to regulate the production of members of the EGF family, we have used TGFα transgenic mice as a model system to evaluate the role of TGFα in carcinogenesis of the reproductive tract. Specifically, we wanted to investigate the effects of constitutive expression of this growth factor in the reproductive tract and to determine whether TGFα would potentiate carcinogenesis induced by DES and DMBA. We show here that deregulated TGFα expression augmented the effects of DES and DMBA in eliciting hyperplastic and differentiation changes in the reproductive tract. The presence of the TGFα transgene significantly increased the incidence of DES-induced vaginal adenosis, uterine hyperplasia, uterine polyps, hypospadia, and benign ovarian cysts; however, the incidence of reproductive tract neoplasia was not promoted.

The significant potentiation of DES-induced vaginal adenosis by the TGFα transgene strongly implicates TGFα in pathogenesis of abnormal gland formation that is proposed to be a precursor lesion for the generation of vaginal adenocarcinoma in women (Noller, et al., 1990). Furthermore, the fact that TGFα transgenic animals exhibited a greater incidence of vaginal concretions due to malformation of the urethra and retention of Wolffian ducts suggests that TGFα plays an important role in the regulation of developmental and morphogenic events in both the Mullerian duct and the urogenital sinus of the female animal. Our results are supported by other studies which document that TGFα overexpression alters the growth and differentiation of both glandular and squamous epithelia in several different

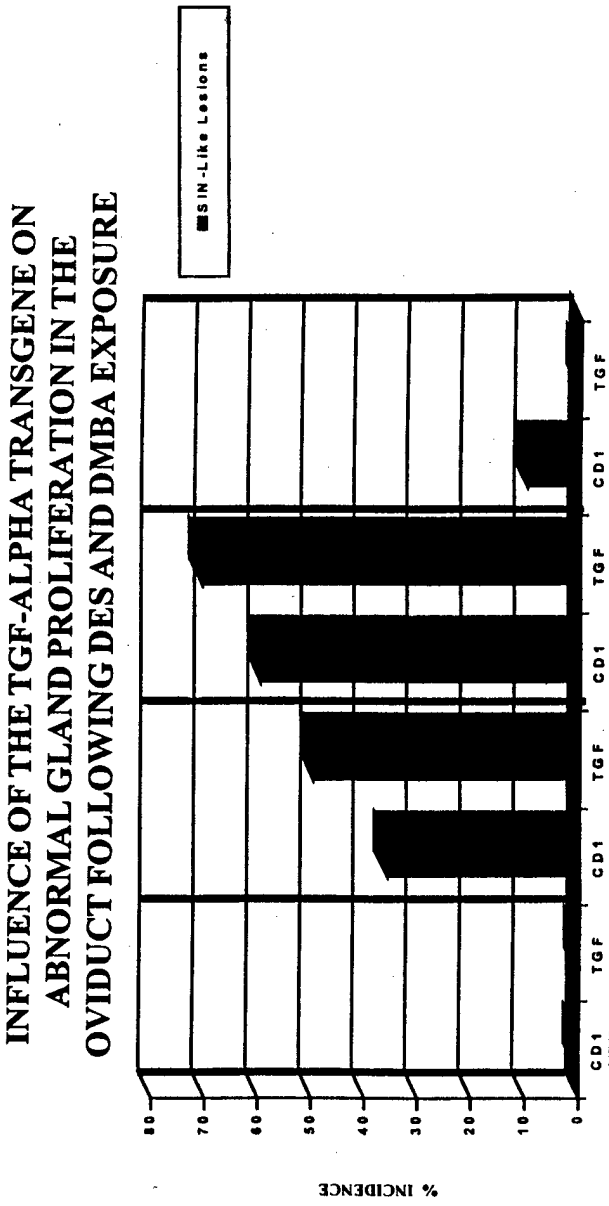

Figure 3: A summary of the incidence of oviductal lesions. Exposure to neonatal estrogen induced histological alterations in the oviduct associated with hyperplasia and gland formation (diverticuli) of the oviductal mucosa which extend into the muscle wall, resembling the clincial lesion, salpingitis isthmica nodosa (SIN). Both CD-1 and TGF α exhibited SIN-like lesions following DES exposure which appeared to be slightly amplified by DMBA treatment. Although, not significant, the percentage of animals with oviductal lesions was consistently higher in the transgenic animals.
Abbreviations: CD1=parental mouse strain; TGF=TGF α transgenic mice; VEH=vehicle treated; DES=diethylstilbestrol treated; DES & DMBA=treated with both DES and 7,12-dimethylbenz[a]anthracene; DMBA=DMBA treated.

organs. As an example, TGFα has been shown to disrupt the normal program of cellular differentiation in the gastric mucosa by interfering with the terminal differentiation of parietal and chief cells while promoting the growth of surface mucous cell progenitors (Bockman, et al., 1995; Sharp, et al., 1995). Zymogen granule-containing cells in the parotid gland have been shown to undergo redifferentiation to form ductular-like structures in transgenic animals. These studies support a morphogenic role for TGFα in that inappropriate expression can redirect epithelia along alternative differentiation pathways.

Interesting, DES- and vehicle-treated TGFα transgenic animals exhibited a significantly greater incidence of benign ovarian cysts than the corresponding nontransgenic mice. Unopposed estrogen in humans is associated with follicular polycystic degeneration of the ovary (Futterweit, 1984). In light of the fact that TGFα is regulated by estrogen in hormonally sensitive tissues and that experimental studies support a role for TGFα in ovarian function, our study provides further support that TGFα has an important role in the development of polycystic ovaries. Also, our data show that neonatal DES treatment promoted the development of ovarian granulosa cell tumors following DMBA treatment which has also been documented by a previous study (Manohara and Rao, 1980). The mechanism of DES action is unknown but it has been proposed that DMBA mediated deletion of oocytes is associated with the development of ovarian tumors in certain strains of mice. The fact that DES also reduces ovarian components (oocytes, follicles, and corpora lutea) and increases the interstitial tissue of the ovary may provide an environment that promotes the development of tumors following DMBA exposure.

Uterine polyps were also another growth abnormality identified in the TGFα transgenic mice. This finding leads to the question of whether up-regulation of TGFα expression may account for polyp development in women, which recently has been found to be a common side-effect of tamoxifen therapy in breast cancer patients (Cohen, 1994).

There is an association between endometrial carcinoma and unopposed estrogen exposure in women. It is proposed that estrogen-related endometrial lesions undergo progression from cystic to adenomatous to atypical hyperplasia to carcinoma (Cohen, 1994; Reinartz, et al., 1994). The observation that TGFα increases the incidence of both cystic endometrial hyperplasia and atypical hyperplasia in mice is of particular importance because there is a strong association between the presence of adenomatous hyperplasia with atypia and the subsequent development of adenocarcinoma. In fact, a role of aberrant growth factor expression such as TGFα has been suggested to be involved in the development of uterine adenocarcinomas in women (Reinartz, et al., 1994). Our results support a role for TGFα as a mediator of events that accompany the development of hyperplastic endometrial lesions; however, whether deregulation of TGFα is involved in the pathogenesis of endometrial cancer is still not clear.

DMBA treatment was found to reduce the incidence of cystic endometrial hyperplasia in both transgenic and nontransgenic although the effect was most significant in the CD1 mice. DMBA treatment has been shown to reduce the mean uterine weight in female rodents which is associated with significant depletion of oocytes, follicles, and corpus lutea (Manoharan and Rao, 1980). The reduction in cystic hyperplasia seen in the uteri of both CD-1 and TGFα transgenic animals upon DMBA treatment may be explained by the depletion of

INFLUENCE OF THE TGF-ALPHA TRANSGENE ON OVARIAN HISTOLOGY FOLLOWING DES AND DMBA EXPOSURE

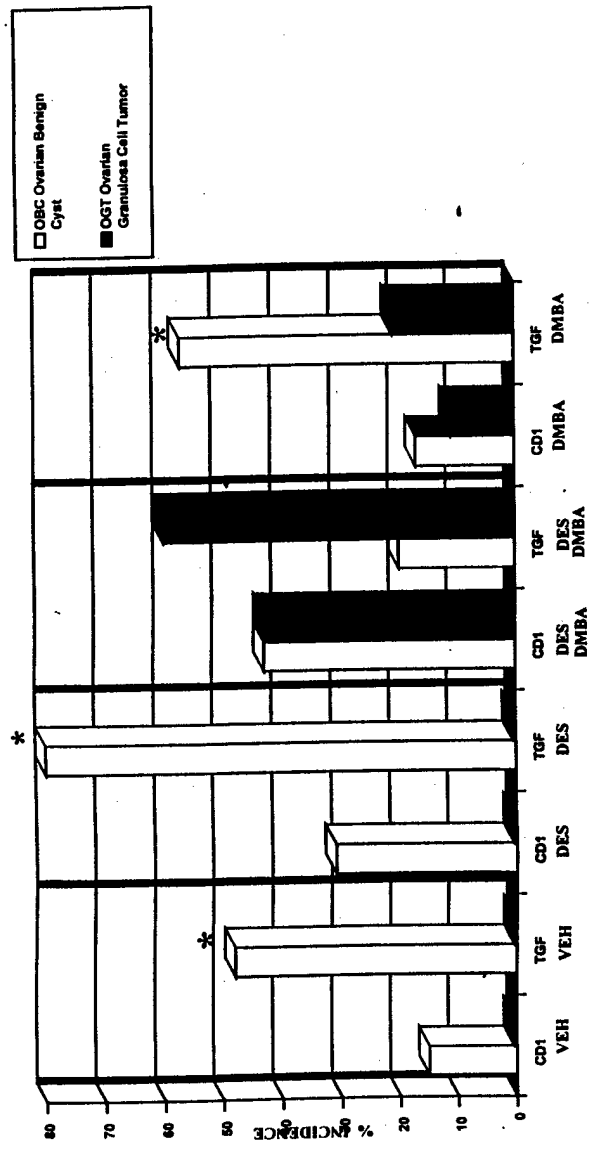

Figure 4: A summary of specific ovarian lesions obtained in our study represented by percent incidence with the asterisk (*) denoting significant differences in the response of the TGFα animals, as determined by CHI square analysis. The presence of the transgene was associated with increased incidence of benign ovarian cysts (OBC) over that found in the CD-1 mice following treatment with DES or the vehicle.
Abbreviations: CD1=parental mouse strain; TGF=TGFα transgenic mice; VEH=vehicle treated; DES=diethylstilbestrol treated; DES & DMBA=treated with both DES and 7,12-dimethybenz[a]anthracene; DMBA=DMBA treated.

ovarian follicles resulting in decreased ovarian function and less production of the sex steroids in these animals. In fact, it has been shown that combined neonatal estrogen plus DMBA results in a greater depletion of oocytes than in neonatally estrogen treated only. Regardless of the mechanism of DMBA effects, the presence of the TGFα transgene maintained higher levels of cystic endometrial hyperplasia under the influence of DMBA than was seen in the CD-1 nontransgenic mice suggesting that the presence of TGFα may compensates somewhat for reduced ovarian function possibly by mimicking estrogen. However, it is notably that DMBA effects on endometrial hyperplasia was not concordant with the development of uterine adenocarcinomas.

Transgenic studies have clearly demonstrated that the carcinogenic, co-carcinogenic, or promoting effects of TGFα varies depending on the organ, carcinogen, promoter, and hormonal environment. The most impressive characteristic of mammary carcinogenesis in TGFα transgenic mice is that tumor development is clearly dependent on exposure of the mammary gland to multiple rounds of pregnancy involving repeated cycles of lobular-alveolar growth, differentiation, lactation, and involution (Matsui, et al., 1990; Smith, et al., 1995). Inappropriate TGFα expression appears to potentiate the development of preneoplastic hyperplastic alveolar nodules by increasing the proliferative index and enhancing survival during postlactationally-induced apoptosis. These events are proposed to result in the creation of a population of cells that may be more susceptible to additional transforming mutations which ultimately leads to malignant transformation.

We can only provide speculation concerning the mechanism by which TGFα potentiates DES-induced lesions in the reproductive tract. Carcinogenesis of the reproductive tract has several features in common with mammary gland tumorigenesis in that genital tract neoplasia is clearly a multistage process involving pathologically distinct intermediate preneoplastic stages consisting of hyper-proliferative premalignant lesions. Also, long term exposure to endogenous hormones is instrumental to the DES oncogenic process in the reproductive tract. Thus, as found in the mammary gland, we speculate that inappropriate expression of TGFα may contribute to the development of pathological lesions in the reproductive tract by conferring a selective growth advantage and enabling cells to escape from apoptosis.

However, it is clear that the role TGFα plays in hormonal carcinogenesis of the mammary gland is different from that played in the uterus and vagina. Unlike in the mammary gland, TGFα did not significantly increase the development of uterine carcinomas in the transgenic animals compared to the parental mice in the investigated time-frame of 12 months, even though the incidence of various proliferative and differentiation lesions were increased. We raise the question as to why the increase in endometrial hyperplasia seen in the transgenics does not translate into increased tumorigenesis? It is also possible that hyperplastic endometrial lesions may not be precursors to uterine adenocarcinomas so that an increase in hyperplasia will not result in more neoplasias. The role of estrogen in mediating the transition between excessive growth and neoplastic transformation of the endometrium is still unclear. In addition to estrogen-dependent endometrial tumors that progress through distinct precursor stages, it is also proposed that endometrial cancer can arise independent of estrogen without evidence of hyperplastic preneoplastic stages. Consequently, the etiology of these tumors may

not involve persistent induction of peptide growth factors such as TGFα. Our study did not show a correlation between the incidence of endometrial hyperplasia and the development of adenocarcinomas which indicates that the mechanism of DES-induced carcinogenesis of the uterus may involve events other than hyperplasia and the overexpression of a single growth factor.

It cannot be ruled out that a general effect of the TGFα transgene on the hypothalamic-pituitary-ovarian axis may be contributing to the development of uterine and vaginal lesions by altering the endocrine environment of the animal. In fact, a recent study by Ma et al (1994) clearly demonstrates that overexpression of the TGFα transgene has disrupting effects on hypothalamic and ovarian function, and that chronic TGFα expression is deleterious to female reproduction. Furthermore, our finding of pituitary adenomas in TGFα female transgenics implicates TGFα in growth regulation of the pituitary (Kudlow, et al., 1989). Clearly further studies are needed to understand the relative contributions of TGFα-induced alterations in the endocrine environment versus direct autocrine/paracrine tissue effects in reproductive tissues; but nonetheless, our study presented here provides further evidence of the importance of TGFα in many aspects of reproductive physiology.

In conclusion, the potentiation of DES and DMBA lesions in TGFα transgenic mice further supports the hypothesis that deregulation of peptide growth factors is involved in the pathogenesis of some reproductive tract diseases. However, the TGFα transgene did not promote DES-induced reproductive tract neoplasia indicating that estrogen carcinogenesis involves more than aberrant expression of this single peptide growth factor.

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Transgenic mouse models of breast cancer

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Summary

Although valuable initial information can be gathered about transformation from *in vitro* studies, human cancer occurs in the context of a complex interaction with its environment and must ultimately be studied in living animals. Transgenic animal models have been used to study breast transformation for a number of years and have yielded valuable information on the subject. In this paper, we will summarize results from our laboratories, and others, regarding the use of transgenic mice to study breast tumorigenesis. We will also suggest future directions for the use of transgenic models to understand, and hopefully, one day to cure the disease.

Introduction

Breast cancer is one of the most common malignancies in women today, striking almost one in every eight. A number of genetic abnormalities have been associated with the disease, such as gene amplifications, overexpressions, rearrangements, point mutations, and deletions. The genes thought to be involved in the genesis of human breast cancer encode growth factors, growth factor receptors, nuclear transcription factors, guanidine nucleotide binding proteins, cell cycle regulatory proteins, tumor suppressor proteins, and others.

Although valuable initial information can be gathered about transformation from *in vitro* studies, the oversimplicity of such studies calls for examining transformation in living animals. Human cancer occurs in the context of a complex interaction with its environment, i.e. neighboring tissues, the immune system, hormones, and dietary factors. Transgenic animal models have been used to study breast transformation for a number of years and have yielded valuable information on the subject. In this paper, we will summarize results from our laboratories, and others, regarding the use of transgenic mice to study breast tumorigenesis. We will also suggest future directions for their use to understand, and hopefully, one day cure the disease. For a brief summary on the different studies see Tables 1 and 2.

Promoters that have been used to target oncogenes to the mammary gland in transgenic mouse models include mammary gland-specific promo-

Note: genes are referred to as lowercase names in italics (e.g. *myc*) and their protein products as uppercase (e.g. Myc).
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Table 1. Single transgenic strains

Transgene	Promoter	Phenotype	Age of onset	Other aspects	Refs.
Growth factors and receptors					
tgfα	MT	Adenocarcinoma	7-14 months		[16,17]
tgfα	MMTV-LTR	Adenocarcinoma	7-9 months		[18]
erbB2/neu	MMTV-LTR	Adenocarcinoma	5-10 months	metastases frequent	[44,45]
neu	MMTV-LTR	Adenocarcinoma	3 months	synchronous tumor formation metastases frequent	[47,48]
Nuclear oncogenes					
c-*myc*	MMTV-LTR	Adenocarcinoma	7-14 months		[63,64]
c-*myc*	WAP	Adenocarcinoma	4+ months		[65]
Viral oncogenes					
Polyoma middle T	MMTV-LTR	Adenocarcinoma	5 weeks	synchronous tumor formation metastases frequent	[75]
SV40 large T	WAP	Adenocarcinoma	4-6 months		[78]
***Ras* genes**					
v-Ha-*ras*	MMTV-LTR	Adenocarcinoma	4-10 months	metastases seen	[85,86]
Ha-*ras* (activated)	WAP	Adenocarcinoma	10-12 months	low frequency	[87]
***Int* genes**					
wnt-1	MMTV-LTR	Adenocarcinoma	4-7 months		[91]
int-2	MMTV-LTR	Adenocarcinoma	10+ months	low frequency	[92,93]
int-3 (activated)	MMTV-LTR	Adenocarcinoma	2-7 months	developmental arrest	[95]
Growth suppressing genes					
p53 (mutated)	WAP	No tumors		developmental arrest	[102]
tgfβ	WAP	No tumors		developmental arrest	[105]
tgfβ	MMTV-LTR	No tumors		developmental arrest	[106]
Cell cycle genes					
cyclin-D1	MMTV-LTR	Adenocarcinoma	18 months		[126]

ters, such as the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter/enhancer, the whey acidic protein promoter (WAP), and the ß-lactoglobulin promoter. The latter two are from milk-specific genes, causing expression of transgenes in later stages of pregnancy and during lactation. The MMTV promoter/enhancer is active in the virgin mammary gland, with augmented expression during pregnancies. It also causes expression of transgenes in other tissues, such as the salivary glands and some reproductive organs. The mouse metallothionein 1 (MT) promoter has also been used in these studies, directing expression to most epithelial tissues, including the mammary gland. We direct readers interested in the technology of how to make transgenic animals or knockout mice to excellent reviews on those subjects [1-3].

I. Growth factors and their receptors

Transforming growth factor α (TGFα) is a 50 amino acid secreted polypeptide, originally detected in the culture media of certain retrovirus-transformed fibroblasts [4]. It is a member of the epidermal growth factor (EGF) family of mitogens; human TGFα shares 42% homology with human EGF [5]. Both TGFα and EGF bind to and activate the EGF receptor, the protein product of the protooncogene c-*erb*B [6].

Although the *tgfα* gene has not been found to be amplified in human breast cancer, its expression is frequently increased compared to the normal gland. Numerous studies have shown that TGFα mRNA and protein are expressed by many human breast cancer cell lines and about 40-70% of primary human breast tumors, compared to

only 20% of normal/benign breast samples [7-12]. The same applies for at least some other members of this family of ligands: both amphiregulin and cripto-1 are preferentially expressed in human breast cancer cases compared to normal breast tissues [13,14]. The epidermal growth factor receptor (EGFR) itself is also expressed in about 30-50% of human breast cancers, with elevated expression associated with a poor prognosis of the disease and a high degree of invasiveness [15].

Overexpression of TGFα in the mammary gland of transgenic mice has been achieved by fusing the coding sequences of its gene with either the mouse metallothionein-1 (MT) promoter or the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter/enhancer [16-18]. In these models, TGFα caused hyperplastic growth of the mammary gland and the appearance of carcinomas after a long latency period of about 8-10 months. The tumors were stochastic and arose predominantly in mice that had undergone multiple pregnancies [16-18]. These studies confirmed previous *in vitro* data that had suggested an oncogenic role for TGFα, and established its importance for tumorigenesis in the mouse mammary gland *in vivo*. The monoclonal nature of tumors and long latency times imply that other genetic events are necessary for progression to frank neoplasia. It is reasonable to believe that TGFα induces tumorigenesis by acting through the EGFR, since it has not been shown to bind other known growth factor receptors. Upon stimulation with ligand, the receptor binds to a number of intracellular molecules that convey signals from the cell surface into the cytoplasm on their way to the nucleus. Five known molecules that bind the activated (i.e. phosphorylated) EGFR are Grb2, Shc, GAP, PLCγ, and PI-3 kinase [19-24]. The EGFR therefore probably activates a number of intracellular signalling pathways, supporting the importance of the receptor and its ligands in normal and abnormal growth control.

The c-*erbB-2/neu* growth factor receptor gene was initially detected as the transforming gene in rat neuroglioblastomas induced by a chemical mutagen [25]. The human gene was cloned by virtue of its similarity to v-*erbB* and named c-*erbB-2*, *HER2*, or *NGL* [26,27]. It encodes a 185-190 kDa protein ($p185^{erbB2}$) with a strong sequence homology to the EGF-receptor, especially in the kinase domain [28,29]. The rat *neu* oncogene gains its transforming ability *via* a point mutation in the transmembrane domain of the receptor, converting amino acid 664 from valine to glutamic acid [30]. The mutation stabilizes dimerization of the receptor, and constitutive tyrosine kinase activity is seen. Therefore, it appears that a single mutation in $p185^{erbB2}$ mimics the effects of ligand binding preceding receptor dimerization and internalization [31-33].

No case of activation of the human c-*erbB-2* gene by point mutation in the transmembrane domain has been reported in human breast cancers to date [34]. On the other hand, amplification of the non-mutated c-*erbB-2* gene (15-30%) and/or overexpression of the protein (40-50%) are predominant findings in human breast cancer. Furthermore, clinical studies have indicated that $p185^{erbB2}$ overexpression in breast cancer is correlated with reduced survival of the patient [35-43].

A number of studies have been performed using transgenic mice that overexpress *erb*B-2 or *neu* in the mouse mammary gland [44-48]. It caused the appearance of stochastic tumors in the mammary gland of a high number of female mice after 5–10 months, and in some cases tumorigenesis was pregnancy-independent. However, transgenic male animals were not affected. The tumors metastasized with high frequency, indicating that elevated expression of Neu can induce metastatic mammary gland tumors [44,45]. One study described the synchronous development of mammary tumors involving the entire epithelium of the gland in both female and male animals, indicating that the expression of the activated *neu* gene induced transformation in a single step [47]. The reason for the inconsistency between studies is not clear. It has been suggested that the site of integration in this particular study might play a role in the phenotype. However, the study has

been repeated using the same transgene construct with similar results, and it is not likely that a tumor suppressor gene would be disrupted in two independent studies [48]. Another explanation could be the level of transgene expression in the different transgenic strains and/or the presence of transformation-inducing sequences in the construct used.

An increased Src tyrosine kinase activity was found in mammary gland tumors from mice overexpressing the *neu* protooncogene, implying that Src may be activated by Neu [49]. Src was preferentially complexed to tyrosine-phosphorylated Neu, indicating that activated Neu might phosphorylate and activate Src through an immediate protein-protein interaction. Although the data point to the possibility that Src is important for Neu-mediated tumorigenesis, direct evidence has yet to be presented. This could be done *in vitro* by antisense strategies to eliminate Src expression in Neu-overexpressing cells, or *in vivo* by mating Neu transgenic mice to Src-deficient mice. If transformation is abolished or decreased, it would point to Src being an important mediator in transformation by Neu.

II. Nuclear oncogenes

The c-*myc* proto-oncogene encodes a short-lived nuclear phosphoprotein which is highly conserved throughout evolution. Its mechanism of action seems to be quite complex and is still not completely resolved despite avid investigation for a number of years [50]. Expression of c-*myc* is closely related to cell proliferation; quiescent cells express very low levels of the RNA and protein. However, upon mitogen stimulation and entry into the cell cycle, a significant increase in c-*myc* expression is seen [51]. The discovery of three conserved structural motifs in the carboxy-terminal region of c-Myc placed it in a family of sequence specific DNA binding proteins. The leucine zipper, helix-loop-helix, and basic regions are involved in binding DNA and other proteins. The amino terminus of the protein contains a region involved in activation of gene transcription [52-54]. A dimerization partner, Max (Myn is the mouse homologue), has been discovered that cooperates with Myc to bind a specific DNA sequence [55-57].

Abnormalities in the c-*myc* protooncogene locus are strongly associated with a number of human malignancies. The gene is amplified in 25 to 30% of breast cancer cases and, in addition, is rearranged and overexpressed (without gene amplification) in many more cases [58-61]. Furthermore, amplification of the c-*myc* gene has been shown to correlate with poor prognosis of the disease [60,62].

MMTV-*myc* transgenic mice (with normal or activated *myc* constructs) have been reported to develop solitary mammary gland tumors at about 7–14 months of age, after multiple pregnancies, suggesting that additional genetic events are necessary for tumor onset [63,64]. Expression of the gene from the milk protein promoter WAP (WAP-c-*myc*) resulted in an 80% incidence of mammary tumors, some occurring as early as two months after the onset of transgene expression. Pregnancies were required for tumor onset, which is not surprising since the WAP promoter is active during late pregnancy and lactation. Tumors were often observed in multiple mammary glands and sometimes more than one tumor appeared in the same gland [65]. These studies indicate that Myc contributes to mammary gland tumorigenesis, although it is not able to induce transformation on its own.

Additional studies have been performed using *in vitro* infection of primary mouse mammary epithelial cells with a recombinant retrovirus containing the v-*myc* oncogene. When these cells were reintroduced into cleared mammary fat pads of syngeneic mice, they produced abnormal hyperplastic glands, although tumors were not seen [66]. This method results in a transgenic mammary gland although the remainder of the animal is nontransgenic. The technique is possible because the development of the mammary gland, unlike most other organs, occurs mainly postnatally. In mice, the mammary gland

epithelium starts to penetrate the fat pad from the nipple at three weeks of age. This coincides with the onset of ovarian function and requires the presence of female hormones [67]. If the epithelium is removed surgically before it grows into the fat pad, the cleared fat pad can serve as a host for foreign mammary epithelia capable of repopulating the gland [68]. Use of inbred strains is vital, so that animals do not reject the foreign tissue. Therefore, primary mammary epithelial cells from one animal (donor) are grown *in vitro*, where the gene of interest is introduced by transfection or infection. After selection of cells that harbor the transgene, they are transplanted into the cleared fat pad of another mouse (acceptor) where they grow and form a transgenic mammary gland epithelial network. The advantages of this method are that there are no complications from expression of the transgene in other organs of the animal. However, since the transgene is not transmitted to the offspring, it hinders the propagation of transgenic animals so that each animal must be made independently.

In summary, Myc overexpression in the mammary gland predisposes transgenic animals to mammary gland cancer, although this oncogene is not sufficient to induce tumors on its own.

III. Viral oncogenes

Although viruses have not been strongly implicated in the genesis of human breast cancer, they can serve as valuable tools to study the disease. Viral oncoproteins are known to bind to, and interfere with, the function of cellular proteins in order to cause propagation of viral DNA. As a consequence, cell proliferation is induced, often resulting in transformation. One can therefore draw conclusions about the function of these cellular proteins in mammary gland transformation by studying transgenic mice that overexpress viral oncoproteins in the mammary gland.

Polyomavirus probably transforms cells by activating a number of intracellular signal transduction pathways. The signalling molecules known to be bound by polyomavirus middle T include the Src, Yes, and Fyn tyrosine kinases, the 85 kDa subunit of phosphatidylinositol 3'-kinase, and protein phosphatase 2A [69]. In the case of Src and Yes, binding has been shown to increase their tyrosine kinase activity [70,71].

Increased Src tyrosine kinase activity has been seen in breast cancer samples compared to normal breast tissues, although protein levels are similar. An association has also been found between high cytosolic Src activity and an early relapse [72-74]. Tyrosine kinase activities for Yes, Fyn, PI 3'kinase, and phosphatase 2A have not yet been compared between breast tumors and normal tissues. Since the polyomavirus middle T viral antigen interacts with all these signalling molecules, it is quite pertinent to study mammary gland transformation by overexpressing it in transgenic mice.

Both female and male mice expressing middle T from the MMTV-LTR promoter/enhancer developed multifocal mammary adenocarcinomas that were palpable by 5 weeks of age and involved the entire mammary fat pad [75]. Tumor onset was not dependent upon pregnancies since virgin females also developed mammary tumors. However, onset in transgenic male animals was somewhat delayed compared to females. A high number of transgenic animals of both sexes also developed lung metastases by about 3 months of age, implying that one or more of the signal transduction pathways perturbed by middle T could be important in the generation of metastatic disease. Increased activation of Src was seen in mammary gland tumors from transgenic animals, indicating that it could be important for tumorigenesis in this model [75]. To gain insight into how the middle T antigen causes transformation, polyomavirus middle T expressing mice were mated to mice lacking either the Src or Yes tyrosine kinases. In the resulting offspring, tumor formation by middle T was severely inhibited in mice lacking Src, whereas no change at all was seen in the absence of Yes [76]. This indicates that Src is an important mediator of transformation by polyomavirus middle T antigen. Yes

either is not involved in tumorigenesis by middle T, or at least is involved to a lesser degree than Src.

The SV40 virus large T antigen is another viral oncogene whose expression has been targeted to the mammary gland. Its protein product binds to the tumor suppressor protein p53, and thereby relieves its restraining effects on passage through the cell cycle [77]. The construct used includes the SV40 early region and contains coding sequences for both the large and small T proteins, expressed from the WAP promoter. Females from 3 out of 8 WAP-SV40 T antigen lines developed stochastic, but often multiple mammary tumors, with high frequency at ages between 4–6 months [78]. The study implies that a viral protein, which binds to and inhibits normal p53 function, is important in mouse mammary gland tumor formation. However, this viral protein is not sufficient for tumorigenesis on its own, and consistent with the multi-hit hypothesis of cancer, requires other events to occur before cancer can arise.

IV. Ras

The Ras proteins are small, guanine nucleotide binding proteins located on the inside of the plasma membrane of cells. Stimulation of cells by a number of extracellular ligands results in an exchange of guanine nucleotides on Ras, leading to transfers of signals from the cell surface into the cytoplasm and nucleus [79]. The Ras proteins mediate cellular proliferation signals from a number of stimulated growth factor receptors, including the EGF-receptor. At least two adaptor proteins, Grb2 and Sos, convey signals from the EGFR to Ras, thereby transmitting signals of growth and differentiation to the cell nucleus [20,80].

Augmented expression of *ras* mRNA and protein has been demonstrated in a very large number of human breast cancer cases [81-83]. Expression was not due to amplification or rearrangement of *ras* genes, nor were point mutations found [84]. In addition, a high frequency of rare *ras* alleles has been found in breast cancer patients compared to normal individuals [84]. Transgenic animals have been made which overexpress the *ras* oncogene in the mammary gland from either the MMTV-LTR promoter/ enhancer or the WAP milk protein promoter. MMTV-v-Ha-*ras* female and male animals developed stochastic, but sometimes multiple, mammary gland tumors, some as early as 5 weeks of age. Half of all female animals developed tumors by 168 days, while male animals had a delayed onset and frequency of tumor onset (>300 days). Metastases were in some cases found in liver and lungs [85]. Another study of MMTV-v-Ha-*ras* transgenic mice described similar results with mammary tumors in animals of four transgenic lines arising between 4 and 10 months of age [86]. An activated human Ha-*ras* oncogene expressed from the WAP promoter was less potent than in the two previously described studies: a small number of females developed mammary tumors after a long latency and multiple pregnancies (10-12 months) [87]. Thus Ras, like most of the other oncogenes that have been overexpressed in transgenic mice, predisposes to mammary gland cancer. However, contrary to suggestions from some *in vitro* studies, Ras is not capable on its own of inducing cancer in a single step in living animals.

V. The *int* genes

The *int* genes were first discovered because of adjacent integration of the mouse mammary tumor virus (MMTV) proviral DNA. The virus does not carry a transduced cellular oncogene like many of the well known oncogenic retroviruses, but rather acts as an insertional mutagen. Integration of the virus into the host cell DNA activates expression of adjacent cellular genes that can lead to tumorigenesis with a latency time of up to about one year [88]. The *int* genes fall into two groups: the *wnt*-1/*int*-4 family and the *int*-2/*hst* family. Although the two groups show no sequence similarity to each other, they may have similar functions:

besides being implicated in mouse mammary gland tumorigenesis, both are thought to have essential functions in pattern formation in early embryogenesis [88,89]. The *int*-2 and *hst* gene products are related to the fibroblast growth factor family of mitogens, whereas *wnt*-1, *int*-3, and *int*-4 have sequence similarities to *Drosophila* embryogenesis genes [88,90].

The *wnt*-1 gene is related to the *Drosophila* gene wingless, and probably encodes a growth factor. Overexpression of the *wnt*-1 (same as *int*-1) gene from the MMTV-LTR promoter/enhancer resulted in a striking proliferation of mammary gland epithelium of both female and male transgenic mice. Mean tumor onset time was around 5 months for transgenic females, and by 7 months of age more than 80% had developed tumors. Transgenic male animals developed tumors less frequently and later in life [91].

The Int-2 protein is a member of the fibroblast growth factor (FGF) family of proteins [90]. When the *int*-2 gene was expressed in transgenic mice from the MMTV promoter, hyperplasia was seen after one or more pregnancies. About one half of transgenic females developed tumors before the age of one year. Some virgin females also developed tumors, but at a lower frequency than multiparous females [92]. Another study described the occurrence of well differentiated hyperplasias in multiparous females with a very low frequency of tumors after more than 14 months and multiple pregnancies [93].

The *int*-3 gene is related to the *notch* family of genes from *Drosophila* and encodes a transmembrane protein that is probably a receptor [94]. The MMTV virus integrates in the middle of the gene and thereby separates the extracellular and intracellular domains. This may alleviate negative regulation of the function of the receptor by its ligand binding domain, and thereby potentiate its oncogenic function [94,95]. When the activated *int*-3 gene was expressed from the MMTV promoter in transgenic mice, focal but often multiple mammary adenocarcinomas occurred between 2 and 7 months of age in most male and female animals. In contrast to the hyperplastic growth observed in *wnt*-1 and *int*-2 transgenic mice, mammary glands of *int*-3 mice were arrested in development; the mammary epithelia did not penetrate the fat pad in a normal fashion and *int*-3 females were incapable of nursing their young [95].

Although the *int*-genes are obviously important for mouse mammary gland cancer, they have not been strongly associated with the human disease. Human breast cancer, unlike the mouse disease, is not associated with etiologic viral infections. *int*-2 and *hst* were long thought to play a role in the human disease, since the chromosome region where they reside is amplified in a number of breast cancers. However, expression of *int*-2 and *hst* was rarely seen in those tumors, implicating the presence of another transforming gene(s) on the amplicon. A newly discovered gene on this chromosome region, *cyclinD1*, is usually expressed in breast cancers with the amplification, suggesting that it is the long sought oncogene on this amplicon (see discussion on cell cycle regulating genes).

VI. Growth suppressing genes

The p53 protein is now a well characterized tumor suppressor protein, with mutations in its gene being the most prevalent genetic change found in human cancers [96]. p53 has been shown to be a DNA binding protein, regulating gene transcription *via* a specific sequence element. Mutations result in a changed conformation and augmented stability of the protein, leading to its inability to bind DNA and repress cellular proliferation [77,97]. The p53 protein is also inactivated by the binding of several viral oncoproteins which block its normal function. Those are the SV40 large T, adenovirus E1B, and papillomavirus E6 proteins. Inactivation of p53 results in a stimulation of cell proliferation and can lead to transformation [77].

The p53 gene is mutated in about 50% of breast cancer cases, and its expression is associated with poor prognosis [97-99]. This supports

the idea that loss of normal p53 function is probably a very important genetic change in breast cancer. Mice deficient for one or both alleles of p53 develop lymphomas and sarcomas at a high rate, but mammary gland tumors occur infrequently [100]. It is possible that mammary gland tumors would arise at a higher frequency in these mice if they lived longer. It is therefore important to look at the effects of the selective absence of a functional p53 protein in the mammary gland. This might be accomplished with a transgenic mammary gland study, where p53 is knocked out in mouse mammary epithelial cells that would subsequently be introduced into a cleared fat pad. Such studies could eliminate complications from the absence of p53 in other organs.

A mutant p53 gene, in which amino acid 172 in the protein is converted from arginine to leucine, has been expressed in the mammary gland of transgenic mice. This amino acid is equivalent to residue 175 in human p53, which is the most frequently mutated amino acid in human breast cancer [101]. Expression of the mutated transgene from the WAP promoter caused an inhibition of both lobuloalveolar development in late pregnancy and gene expression of milk proteins (ß-casein and WAP), leading to an inability of the transgenic females to nurse their young [102]. No mammary gland tumors were observed in these mice, but there appeared to be an increase in radiation-induced apoptosis in the gland indicating that the mutant p53 protein was behaving like the wild type protein. Furthermore, the mutant p53 was shown to transactivate and trans-repress a number of genes in the same way as the wild type p53. Although these two studies have not yielded the anticipated insight on the role of this well known tumor suppressor molecule in mammary gland tumorigenesis, it is critical that more studies with p53 null mice be done in order to specifically test its role in breast cancer *in vivo*. These might require specifically targeting p53 gene disruption in the mammary gland or mating p53 knockout mice to other oncogene-bearing mice described here.

Transforming growth factor β (TGFβ) is a member of a multigene family which includes, among others, activins, inhibins, and Mullerian inhibiting substance [103]. TGFβ is growth inhibitory and/or differentiation-promoting for most epithelial cells *in vitro*. It is also extremely potent in inhibiting ductal development of the mouse mammary gland when it is released from implant pellets inserted in the mammary gland [104]. In accord with these studies, TGFβ1 caused an inhibition of lobuloalveolar development during the later stages of pregnancy and an inhibition in milk protein expression when expressed from the WAP promoter in the mammary gland of transgenic mice [105]. Another study showed that TGFβ1 expressed from the MMTV-LTR in the mammary gland caused an inhibition in ductal development, although alveolar structures developed during pregnancies and lactation did ensue [106]. These two studies, therefore, show similar results although milk protein expression is inhibited in one study but not the other. The reason for this difference is not known, but it could be a result of different levels of transgene expression or different timing of expression. Since TGFβ inhibits growth of the mammary gland, its expression could reverse or delay the transforming effects of oncogenes. This could be easily studied by mating the TGFβ strains to some of the other oncogene-carrying strains described here.

VII. Cell cycle regulatory genes

The recent surge of knowledge about the cell cycle has emphasized the importance of cell cycle regulators in transformation. In this regard, cyclins and cyclin-dependent protein kinases (CDKs) have been identified as key regulators of cell cycle progression in eukaryotic cells [107-109]. At various stages in the cell cycle, cyclins associate with and activate a family of cyclin-dependent protein kinases (CDKs). The activated kinases then engage in a cascade that directs the cell into DNA synthesis and/or mitosis [110-112].

Cyclins were first identified because of their spectacular periodicity during cell divisions. They accumulate at particular stages in the cell cycle, and are then abruptly degraded. Based on sequence homology and pattern of appearance during various stages of the cell cycle, cyclins are divided into five categories: A, B, C, D, and E-type cyclins. Cyclins C, D, and E function during the G_1 stage of the cell cycle and are accordingly called G_1-cyclins. They are thought to be involved in the decision of whether a cell goes into a resting phase (G_0) or continues another round of cell division. Cyclins A and B play a role during the later stages of the cell cycle (S, G_2, and mitosis) [113].

Consistent with their roles in cell cycle progression, it is becoming apparent that cyclins are involved in many types of human cancer. Their genes have been found amplified, translocated, overexpressed, or expressed at inappropriate stages in the cell cycle in many different types of tumors. The cyclins that are currently thought to be involved in the generation of human breast tumorigenesis are cyclins D and E. The cyclin E gene is amplified, its expression deranged, and/or the stability of the mRNA enhanced in a number of breast cancer cell lines [114]. The cyclin E protein is also overexpressed in human breast cancer samples compared to noninvolved breast tissues, and new protein species are present in addition to the normal 50 kDa protein [115]. Human cyclin D1 was first isolated as a gene rearranged in parathyroid tumors (*PRAD-1*). It was translocated to the promoter/enhancer region of the parathyroid hormone gene and thereby grossly overexpressed [116]. The mouse homologue was isolated as a CSF-1 stimulated gene expressed in the G_1 phase of the cell cycle [117]. In the T47D breast cancer cell line, cyclin D1 is the first of the G_1 cyclins to be expressed after insulin treatment. Its expression is also induced by estrogen and progesterone [118]. It is now widely believed that cyclin D1 is involved in mediating the mitogenic response to growth factors and hormones, although this has not yet been tested by inhibition studies such as antisense methods.

About 15–20% of primary breast tumors show amplification of a region on chromosome 11 band q13 [119-122]. Of the known genes on the amplicon (*int-2*, *hst-1*, *ems*1, and *PRAD-1*/cyclin-D1), only *PRAD-1*/cyclin-D1 and *ems*-1 are overexpressed in tumors with the 11q13 amplification, while the other two are rarely seen expressed [123,124]. Overexpression of cyclin-D1 mRNA is seen in about 45% of breast cancer samples [121]. It is also found amplified and overexpressed in other types of cancers; for example, about 25% of esophageal cancers show an amplification of the gene [125].

Transgenic mice with the MMTV-LTR promoter/enhancer directing expression of cyclin D1 to the mammary gland developed hyperplasias at young ages, and adenocarcinomas after about 18 months, which is considerably longer than for most of the transgenic mice described above. Multiple independent adenocarcinomas were observed in a number of transgenic mice [126]. This first study on the importance of cyclin gene expression for mammary gland tumorigenesis shows that Cyclin D1 is indeed involved. It also calls for transgenic mouse studies of how other cyclin genes might play a role in the disease.

VII. Studying breast tumorigenesis as a multi-step process

In agreement with the multi-hit hypothesis of cancer, most single transgenic mice require additional genetic events to occur over a long period of latency before mammary gland cancer can arise. For that reason, it is critical to look at the effects of combinations of oncogenes rather than single genes. The simplest way to achieve this goal is to mate different single transgenic strains and thereby yield double or triple transgenic offspring (see Table 2 for summary). Another method is to introduce genes into mammary epithelial cells from single transgenic strains *in vitro* and then use them to repopulate a cleared mammary fat pad of a recipient mouse of the same

Table 2. Double and triple transgenic strains

Transgenes	Tumor onset in single transgenic strains	Tumor onset in double/triple transgenic strains	References
ras and *myc*	5-11 months	46-113 days	[46,85]
tgfα and *myc*	10-14 months	66 days	[133]
wnt-1 and *int*-2	6 months or no tumors	4 months	[131]
neu and *ras*	5-9 months	6 months	[46]
neu and *myc*	7-10 months	5 months	[46]
neu, *ras*, *myc*	5-10 months	72 days	[46]

strain. This yields a double transgenic mammary gland, whereas the remainder of the animal is still single transgenic. The latter requires the single transgenic strain to be inbred, in order to transplant epithelial cells between animals without tissue rejection. The third method is to infect transgenic strains with slowly transforming retroviruses, and look for genes activated in tumors as a result of proviral integration. This method should uncover genes that synergize with transgenes in tumorigenesis. It was initiated by using Moloney Murine Leukemia virus in transgenic mice studying oncogene cooperation in lymphomagenesis. Viral infection expedited tumor formation and specific genes were activated in lymphomas, indicating that they synergize with the transgenes in forming lymphomas [127-129]. These genes were both known oncogenes, such as c-*myc*, N-*myc*, and others, and new genes that had not been described before. The Mouse Mammary Tumor Virus (MMTV) has been used in the same way to study oncogene cooperation in mammary gland tumor formation. Its drawback is that only a subset of known oncogenes seem to be activated by MMTV, since it usually identifies the *int* genes but not other well known oncogenes such as *myc*, *erb*B2, and *ras*.

"Cooperative interaction" between oncogenes can be defined in the following way: if expression of each gene singly does not induce tumor formation on its own, but simultaneous expression of two or more genes does [130]. An "additive interaction" is when tumor formation in double transgenic animals is additive to the effects in the single transgenic strains but not greater. Finally, the term "synergistic interaction" can be used when the effect of two genes is greater than the combined effects of the individual genes.

The first two transgenic breast tumor model strains to be crossed were MMTV/v-Ha-*ras* and MMTV/c-*myc* transgenic mice. Offspring carrying both genes developed mammary gland tumors at an accelerated rate (50% of female animals developed tumor by 46 days, versus 168 and 325 days for the single transgenic *ras* and *myc* strains respectively) indicating a synergistic interaction, although tumor formation was still stochastic and seemed to require additional genetic events. Double transgenic male animals developed tumors with a slight delay compared to females (100 days versus 47 days) [85].

In this regard, MMTV-*wnt*-1 and MMTV-*int*-2 transgenic strains were also crossed to yield double transgenic *wnt*-1/*int*-2 mice. Single transgenic virgin *int*-2 females did not develop mammary gland tumors at all, whereas *wnt*-1 females developed tumors at about 6 months of age. Double transgenic *wnt*-1/*int*-2 virgin females, however, developed mammary gland tumors after about 4 months on average. In the case of transgenic male animals, *int*-2 males did not develop mammary gland tumors, and only about 15% *wnt*-1 males developed tumors after a long latency period. When both genes were present, male mice developed tumors after about 6 months [131]. This indicates that *wnt*-1 and *int*-2 synergize in causing mammary gland tumors in transgenic mice. An independent line of evidence suggesting interaction between the *int*-genes comes from studies where transgenic *wnt*-1 mice

were infected with the MMTV. Infection resulted in an increased formation of mammary tumors that appeared earlier in life than without the virus. When tumors from these animals were examined, they had the virus integrated around the *int*-2 and *hst* genes with a high frequency, causing activation of their expression [132].

In the case of MMTV-*neu*/MMTV-*ras* double transgenic animals, tumor onset was accelerated to 180 days (from 228 and 263 days for the single transgenic strains). For MMTV-*neu*/MMTV-*myc* double transgenic animals, mammary gland tumor onset was accelerated to 141 days (from 228 and 299 days for the single transgenic strains). Triple transgenic MMTV-*neu*/MMTV-*ras*/MMTV-*myc* animals tumor onset was accelerated to 72 days, which does not appear too dissimilar from the MMTV-*myc*/MMTV-*ras* double transgenic animals (89 days) in the same study [46].

Double transgenic mice for MT-TGFα and MMTV-c-*myc* have recently been generated in our laboratory. Mean tumor onset in double transgenic MT-TGFα/MMTV-c-*myc* virgin mice was 66 days, which is remarkably accelerated compared to single transgenic animals. About one half of single transgenic c-*myc* virgin females developed tumors around 7–14 months, whereas virgin TGFα females did not develop any tumors over the same time period. Furthermore, normal tissue was not found in any mammary gland from double transgenic animals at the time of tumor necropsy. This implies that overexpression of TGFα and c-*myc* is sufficient to cause transformation of the mammary gland in a single step, or that subsequent genetic changes rapidly occur at an extremely early stage of transformation. An interesting result from the experiment was that double transgenic male animals developed mammary gland cancers in an indistinguishable manner from females, regarding both tumor onset and frequency [133]. The results indicate that TGFα and Myc are powerful, synergistic genes in mouse mammary gland tumorigenesis.

The conclusion we can draw from the various studies with double and triple transgenic animals is that mammary gland tumorigenesis is dramatically accelerated compared to single transgenic strains. Nevertheless, it is evident that tumor onset requires two, three, or even more genetic events to occur in most cases. The genes that synergize in the strongest manner are *ras* and *myc* and *tgfα* and *myc*. Double transgenic animals of the former genotype develop tumors at 46-113 days of age, depending on the study, and in the latter tumors arise at 66 days of age. This strongly suggests that the EGFR pathway, acting through Ras, interacts in a powerful way with overexpressed c-Myc, to induce proliferation and malignant changes in the mammary gland.

VIII. Future directions

Transgenic mouse studies have given insights into the effects of various oncogenes thought to be important in human breast cancer and in mouse mammary gland tumorigenesis. Genes such as c-*myc*, c-*erb*B2, *tgfα* and *cyclinD1* have all been found amplified and/or overexpressed in the human disease. They also can cause the formation of mammary gland tumors in transgenie mice, usually with a long latency time and stochastic appearance. Furthermore, the generation of bi- and tri-transgenic animals usually results in a marked acceleration of tumor onset and frequency. Knowledge about how the various genes interact in transgenic mice should direct us to examining the effects of such combinations in human breast cancer patients. It could be of great importance in predicting outcome for patients with the disease and will hopefully enable us in the future to target a combination of oncogenes to prevent or cure the disease.

By studying tumor onset in transgenic mouse offspring from the matings of strains transgenic or deleted for various oncogenes, suppressor genes, and potential downstream effectors, we should get closer to determining which intracellular effector pathways are important for tumorigenesis by the specific oncogene products. This approach has recently been highlighted by generation of trans-

genic mice expressing the polyomavirus middle T antigen in the mammary gland without either a Src or Yes protein. The results suggest that Src is an important mediator of tumorigenesis by middle T, whereas the presence of Yes was not required [76]. More studies along this line are needed to establish which intracellular signalling pathways are important for mediating tumorigenesis by specific oncogenes. They could then also enable us to target different specific signalling pathways, first in transgenic mice and then hopefully in humans with the disease. In this regard it is evident that Ras+Myc and Tgfα+Myc are extremely powerful combinations of gene products that cause mammary gland tumors in transgenic mice. It would be worthwhile to study whether these combinations are associated with a worsened prognosis in the human disease, and could help in deciding its treatment.

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Growth Factor and Sex Steroid Interactions in Breast Cancer

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Mitogenic and inhibitory growth factors and steroid ovarian hormones play important roles as selective modulators of normal mammary development and in the onset and the progression of human breast cancer. The focus of this article is to review past and current research on the interactions of these two classes of effectors in mammary gland development and neoplasia. Steroid hormones regulate synthesis of growth stimulatory and inhibitory growth factors, growth factor receptors, and growth factor binding proteins. In turn, growth factor pathways may modulate phosphorylation and function of steroid receptors and potentiate or inhibit the mitogenic actions of steroids. Ultimately, during the progression of the malignant mammary epithelial cell to hormonal autonomy, overexpression, mutation, or disregulation of key elements of growth factor signal transduction pathways all may play critical roles.

KEY WORDS: Autocrine; paracrine; endocrine; morphogenesis.

BIOLOGICAL ACTIVITY OF GROWTH FACTORS AND SEX STEROIDS

Growth Factors

Mitogens. The growth promoting effects of polypeptide growth factors in the development of the mammary gland were initially described as early as three decades ago (1, 2). We now know that there are several families of these potent mitogens which may collaborate with sex steroids to regulate mammary epithelium and the stroma. These factors function as self-acting (autocrine), locally-acting (paracrine), and systemically acting (endocrine) hormones that elicit a wide variety of biological functions. The most well-studied are the epidermal growth factor (EGF)[3], insulin-like growth factor (IGF), and transforming growth factor beta (TGFβ) families of regulatory molecules. While other locally-acting growth factor families are probably important (such as fibroblast growth factor, hepatocyte growth factor/scatter factor, mammary derived growth factor 1 and more recently prolactin) they are not discussed in this review due to a lack of information on their interactions with steroid hormones.

Members of the EGF family are single chain polypeptides, containing a conserved cysteine motif that forms three intrachain disulfide bonds. The members of this family include EGF, transforming growth factor alpha (TGFα), amphiregulin, heparin binding EGF (HB-EGF), and betacellulin, all of which bind to and activate the EGF receptor (EGFR), the prototypic Type I receptor tyrosine kinase (3). Newly described family members, such cripto-1 and the heregulins apparently do not bind to the EGFR, but directly activate different Type I tyrosine kinases. Heregulins bind erbB3 and erbB4 which are other members of the EGFR family (3). A fourth Type I receptor kinase family member, known as erbB2, has no known ligand at the present time. See Hynes (4) for more extensive information on these points.

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Abbreviations: Estrogen receptor (ER); 17β estradiol (E2); epidermal growth factor (EGF); transforming growth factor α (TGFα); transforming growth factor β (TGFβ); progesterone receptor (PgR); epidemal growth factor (EGF); epidermal growth factor receptor (EGFR).

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In the developing and pregnant rodent mammary epithelium, EGF is expressed by the ductal (luminal) epithelial cells and is also found in milk, where it is thought to be the major growth factor (5, 6). In mouse mammary explants *in vitro* and after mammary implantation of slow release pellets containing recombinant EGF *in vivo*, the growth factor appears to stimulate both proliferation and ductal growth of the mammary epithelium (5). EGF (or a related family member) is required for clonal growth *in vitro* of human and mouse primary cultures and nontransformed immortalized cell lines (6). EGF can also partially replace estrogen to promote the growth of MCF-7 breast cancer cells in nude mice and can function as an oncogene when its gene is overexpressed in certain rodent fibroblast cell lines (6).

TGFα is also a potent mitogen for nontransformed fibroblasts and mammary epithelial cells as well as a variety of human and rodent breast cell lines (6). In contrast to EGF, TGFα can be detected in the cap cells of the terminal end bud and in the luminal cells of the virgin juvenile female mouse mammary gland (7). TGFα has also been detected in human mammary tissue by *in situ* hybridization and immunohistochemistry (8, 9). Interestingly, levels of TGFα rise dramatically rise in mammary epithelial lesions such as ductal hyperplasia, atypical ductal hyperplasia, and carcinoma (9). Similar to EGF, recombinant TGFα has been shown to stimulate ductal morphogenesis in the mammary gland of ovariectomized mice (7, 10). Moreover, overexpression of human and rat TGFα cDNAs can lead to the *in vitro* transformation of mouse mammary epithelial cell line NOG-8 and the human mammary epithelial cell line MCF-10A . However, neither of these TGFα-transfected cell lines was fully tumorigenic when implanted into nude mice, suggesting that TGFα may function as an autocrine progression factor most prominently during the early stages of malignant transformation. Consistent with this hypothesis are observations that when human TGFα is overexpressed in transgenic mice under promoters permissive for expression in the mammary gland, several pathological aberrations within the mammary gland are observed. Abnormalities in the TGFα transgenic mouse included hyperplasia, loss of ductal morphogenesis and eventually the appearance of adenocarcinomas after multiple pregnancies (11, 12). The data to date (including hormonal regulation and antisense mRNA studies described later) are consistent with a role for TGFα as an autocrine factor in early development, pregnancy and at least some hormone dependent breast cancers (8). However, the role of TGFα in hormone dependent cancer appears to be somewhat limited; when hormone dependent MCF-7 human breast cancer cells were transfected with TGFα cDNA expression construct, they did not demonstrate a significant growth advantage *in vitro* or *in vivo* (4). Together, these data implicate TGFα overexpression most strongly in the development of the mammary gland and in early stages of mammary carcinoma under the influence of sex steroids.

Amphiregulin, an additional EGF family member, was initially purified from the conditioned medium of tumor promoter (12-O-tetradecanoylphorbol-13-acetate)-treated MCF-7 cells (13). It appears to have even more pervasive mammary expression than EGF and TGFα. In the developing mouse mammary gland, amphiregulin is expressed by the luminal epithelium as well as cap cells within the terminal end bud. In addition, expression of amphiregulin protein is increased up to two-fold following pregnancy and lactation (14). Like EGF and TGFα, amphiregulin stimulates the growth of nontransformed mouse mammary epithelial cells *in vitro* and induces ductal morphogenesis of mammary epithelium *in vivo* (15, 16).

A number of tumorigenic pathways appear to converge on expression of certain EGF family members, particularly amphiregulin. Oncogenic expression in mouse or human mammary cells of c-*Ha-ras*, c-*erbB2*, TGFα, *neu*, *int-3*, SV 40 large T antigen and polyoma virus middle T antigen, results in induction of amphiregulin mRNA and its protein (17). Interestingly, there are a diversity of patterns of processing of the amphiregulin protein in these model systems. Because these studies suggested that amphiregulin is quite commonly expressed in mammary tumorigensis, we tested a possible causal role in this regard by transducing the human amphiregulin cDNA vector into mouse mammary epithelial cells. When these transduced cells were transplanted into the cleared stroma of the mouse mammary gland, atypical lobular and ductal structures as well as tumors were observed (16). This observation may indicate that, in analogy to TGFα, disregulated expression of amphiregulin acts to promote malignant transformation in the mammary epithelium *in vivo*.

Cripto-1 is an 188 amino acid peptide, initially identified in the human teterocarcinoma cell line NTERA 2/D1 (18). Cripto-1 protein, like TGFα and amphiregulin, has also been immunolocalized within the cap cells of the terminal end bud and luminal epithelial cells of the juvenile and adult mammary ducts (14). Cripto-1 protein levels also increase follow-

g pregnancy and lactation. *In vitro* data suggest that hough recombinant, refolded cripto-1 stimulates the oliferation of MDA-MB-453 human breast cancer lls, the biological activity of CR-1 does not involve e EGFR tyrosine kinase pathway (19). In spite of is difference, like other EGF family members, cripto- can also function as a dominant transforming gene NOG-8 mouse mammary epithelial cells and NIH 3 fibroblasts (8). In addition, cripto-1 protein can mulate ductal morphogenesis and induce hyperproli- rative ductal mammary epithelium *in vivo* (20).

HB-EGF, betacellulin, and the heregulins are wly described members of EGF-family that can also t as potent mitogens for mammary epithelial cells. ne exact expression patterns of these peptides within e rodent and human mammary gland and their steroid gulation have not been fully addressed. Insulin-like owth factors, their receptors and their secreted bind- g proteins are also important mitogens that interact ith steroid hormones, but studies of their localization the normal gland are not yet complete; they will be rther discussed later in this review.

While it is clear that the EGF family of growth ctors stimulates mammary epithelial cell differentia- on and proliferation, there are also data to suggest at EGF and TGFα are required for mammary epithe- al cell survival, since both act to suppress pro- ammed cell death (apoptosis) *in vitro* and *in vivo* 1, 22). It is of interest that estrogen and progesterone so function as mammary epithelial cell survival fac- rs (23). These survival-promoting functions of owth factors and sex steroids may be important in e context of both the normal and the malignant gland.

Inhibitory Growth Factors. It is likely that mam- ary proliferative and survival controls reflects a bal- ice between stimulatory and inhibitory influences. he major inhibitory family of growth factors in the ammary gland is the TGFβ family; 25 KDa homo- eterodimeric molecules that interact with several dif- rent and highly specific binding receptors and pro- ins (23). The major biological actions of this family e to modulate the growth, function and epithelial ganization of the gland (24–26). *In vitro,* TGFβs are own to inhibit the growth of mouse and human east cancer lines *via* autocrine signaling (27–29). hree isoforms of the TGFβ family exist: TGFβ1, GFβ2, TGFβ3. The structure, actions, and receptors or TGFβ are related to those of the activin family 30–32).

TGFβ1 has been localized within various cellular lineages of the developing mammary tree and can be detected in the stroma at the leading edge of the end bud or lateral end bud (24). When recombinant TGFβ is surgically implanted in the mammary glands of 4-week-old mice, TGFβ1 protein prevents ductal morphogenesis and migration within the fatty stroma (25).

TGFβ overexpression in the developing mammary gland dramatically compromises functional differentiation of the gland. A likely mechanism of this effect is promotion of epithelial apoptosis. In virgin transgenic mice that overexpress human TGFβ1, mammary ducts appeared to be hypoplastic. In two separate studies of pregnant transgenic mammary glands, TGFβ1 disrupted alveolar development such that mothers were unable to suckle their pups. Furthermore, in a more detailed analysis (26), TGFβ induced premature senescence or apoptosis in ductal and lobular mammary epithelial stem cell progenitors. In striking contrast to these studies, TGFβ1 appears to be elevated in more aggressive human breast cancers where it may function as a immunosuppressor and an angiogenesis-inducing agent (33). Moreover, when TGFβ1 is overexpressed in MCF-7 cells, the growth of these cells is converted into estrogen-independent tumors when transplanted into nude mice (34). Thus while TGFβ1 suppresses normal gland function and perhaps early stage cancers, its expression clearly promotes later stage malignancy. The mechanisms at work in this functional switch probably include desensitization of the cancer to the growth factor and its overexpression to allow for more profound tumor–host interactions.

Estrogens and Progesterone. The sex steroids estrogen and progesterone are strong regulators of mammary epithelial cell proliferation and malignancy. Seminal observations by Daniel and co-workers (35) established that localized action of 17β-estradiol (E2) stimulates ductal morphogenesis in the mouse mammary gland, an effect that can be blocked by the addition of an antiestrogen, such as keoxifene. In the developing mouse mammary gland, estrogen receptors (ER) appear to be localized in areas adjacent to the nipple region and expressed by the luminal cells of the end bud and the subtending duct (35,36). Although the ER is primarily expressed in epithelial cells, coculture studies *in vitro* have shown that stromal fibroblasts probably secrete growth factors to allow a full epithelial proliferative reponse to estrogen (37–40).

Several ER positive (ER+) human breast cell lines, but not ER negative (ER−) human breast cell lines, are responsive *in vitro* and *in vivo* to the growth promoting effects of physiological concentrations of E2 (23). The proliferative response of breast cancer cells to estrogen clearly depends upon culture conditions. In particular, the EGF and IGF pathways play predominant, permissive roles, depending upon the individual cell type (37–39). The potential mechanism of these effects will be addressed later in this review.

Progesterone is complimentary to estrogen in its ability to promote the second phase of mammary development, lobular alveolar growth and differentiation (41). The stimulatory actions of progesterone appears during, as well as after ovulation. It is of interest that progesterone stimulates breast epithelial proliferation but inhibits uterine growth. *In vitro* and *in vivo* analysis suggests the progestins may have complex effects on breast cancer growth. *In vivo,* progesterone stimulates the proliferation of DMBA-induced mammary tumors. However, in human breast cancer cells *in vitro,* progestins not only shorten early G_1 of the cell cycle but also block the cycle later in G_1 prior to entry into the S phase (6, 42). The reader should refer to Kiley *et al.* (43) for further discussion of this topic.

MECHANISMS OF SEX STEROID-GROWTH FACTOR INTERACTIONS

Introduction. Several levels of steroid-growth factor interaction are depicted in Fig. 1. Probably the most extensive evidence supports their role in transcriptional regulation of growth factor genes. Additional recent data support steroid regulation, possibly at the transcriptional level of growth factor receptors and secreted binding proteins. The steroid effects on growth factor systems appear to interact with other steroid effects on cell cycle proteins to regulate multiple cellular events. Conversely, growth factors acting through their receptors catalyze phosphorylation and dephosphorylation of steroid receptors to modulate their functional status.

Regulation of Growth Factor Transcription. Several lines of evidence strongly suggest that estrogens and progestins stimulate the mRNA transcription of growth factors and their receptors and in this manner contribute to normal and abnormal growth patterns (44–48). At the transcription level, TGFα, amphiregulin, the EGFR, IGF-II, the IGFR, and TGFβ2 mRNAs are modulated by E2 and/or by the antiestrogens tamoxifen, droloxifene, and ICI 164, 384 (27, 47–51). The regulation of TGFα transcription appears to involve a poorly defined promoter region (a 13bp imperfect palindromic ERE) 5′ to the coding region of the gene. Physiological concentrations of E2 increase TGFα mRNA and protein levels 2- to 10-fold in MCF-7, ZR-75-1 and T47D cells (49, 50, 52). Amphiregulin transcription has been shown to be enhanced to an even greater extent following estrogen treatment of MCF-7 cells (53). Both transcriptional and posttranscriptional regulation of TGFβ2 expression has also been observed. TGFβ2 can be downregulated by estrogen and progestins and upregulated by antiprogestins and antiestrogens (54–57), but the effects of steroids on receptors and binding proteins for the TGFβ system are unknown. Interestingly, induction of TGFβ2 by antiestrogens has been observed in the blood in patients undergoing a favorable clinical response to tamoxifen (58). Further studies are required to compare and contrast detailed regulatory regions of growth factor genes with which steroid receptors interact and the detailed molecular mechanisms involved.

Posttranscriptional Regulation of Steroid Receptors. While the sex steroids clearly regulate production of growth factors and their receptors in breast tumors, it has also become clear that growth factors modulate steroid receptor function. Growth factors, acting through both tyrosine kinases and serine/threonine kinases such as protein kinase C can activate or inhibit sex steroid receptor function by multiple mechanisms involving direct phosphorylation of the receptor and modulation of the stability of the steroid receptor-encoding mRNA (59–62). For example, in experiments involving either growth factor gene transfection or simple addition of growth factors to target cells, heregulin and IGF-I induced ER phosphorylation and allowed activation of ER function to promote gene transcription (61, 63). In contrast, PKC is thought to inhibit both ER function and expression by different phosphorylation events and by decreased mRNA stability, respectively (59, 60). Future studies will undoubtably use site-directed mutagenesis to test the functionality of phosphorylated amino acid residues in the ER and PgR. Another theoretical possibilty for growth factor modulation of steroid receptor function or action is by the regulation of phosphatase activity

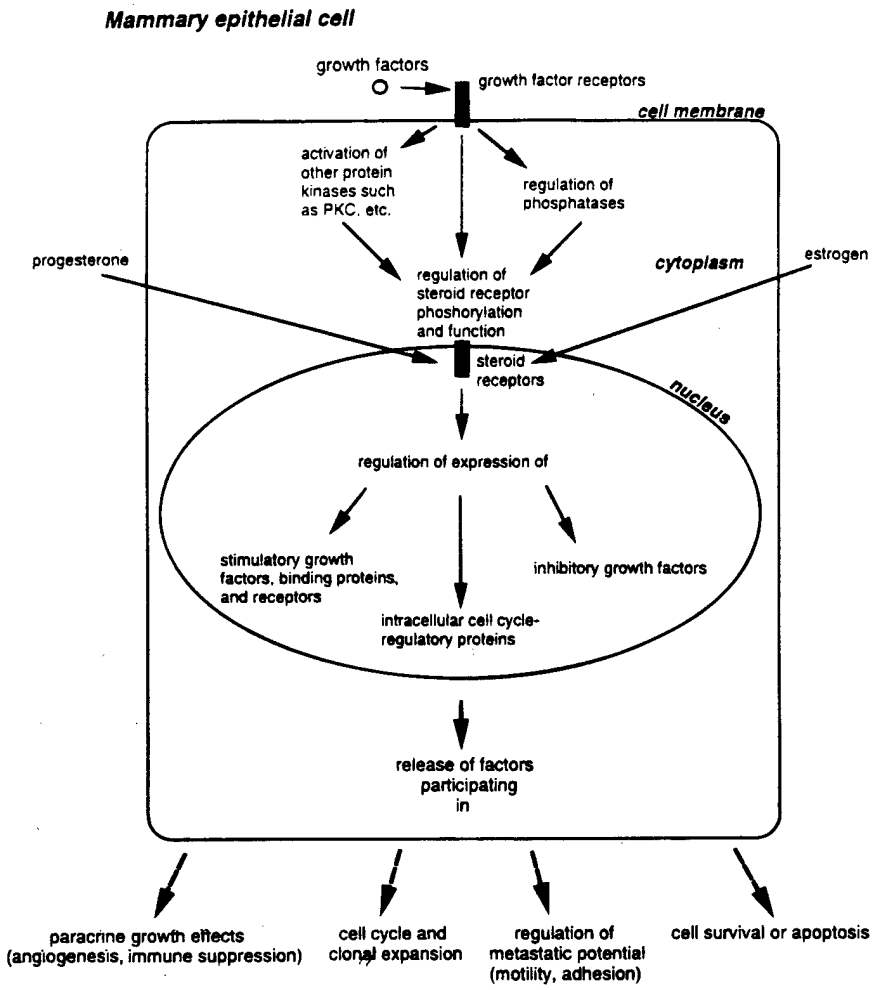

Fig. 1. Biochemical basis for steroid-growth factor interactions.

dephosphorylate steroid receptors. This is currently extremely active area of investigation.

Growth Factor Modulation of Estrogen and Progesterone Induced Growth (Table I). As noted earlier, e growth regulation of several ER+ and PgR human east cancer cell lines in the presence of E2 is accomnied by the production and secretion of both autoine and paracrine growth factors (6, 23). Several egant studies have supported different modes of owth factor action to mediate or modulate steroid fects on cellular proliferation and survival.

In two independent studies, TGFα was demonrated to be a growth mediator/modulator of E2-duced growth by introduction of a replication defeve, antisense TGFα mRNA retroviral expression vector into MCF-7 and ZR-75-1 cells. In these studies the antisense TGFα mRNA significantly reduced TGFα production and suppressed both the anchorage-independent and anchorage-dependent growth of these cells in response to estrogen (64, 65).

IGF-I- and IGF-II-mediated growth in ER+ human breast cancer cell lines may also be modulated by E2. This growth factor response appeared to be mediated in part by E2 induction of the IGF-IR and the IGF binding protein 3 and by the inhibition of the expression of binding protein 4 (66, 67).

Another type of hypothesis addressed in the breast cancer literature is that when appropriately expressed, growth factors may be able to supplant the growth promoting effects of estrogen. Experiments with TGFα and IGF-II have only partially confirmed this hypothesis. Transfection of MCF-7 human breast cancer cells

Table I. Growth Factor-Sex Steroid Regulation During Breast Cancer Cell Growth[a]

Growth factor/ receptor	Estrogenic effect	Progesterone effect
EGF	+	+
TGFα#	+	+
Amphiregulin#	+	+
Cripto-1	0	−
Heparin binding-EGF	+	nd
Betacellulin	nd	nd
Heregulin α and β	nd	nd
TGFβ	−	−
TGFβ2#	−	nd
TGFβ3	−	nd
IGF-I	+	nd
IGF-II	+	nd
EGFR	+	+
ErbB2	−	−
ErbB3	nd	nd
ErbB4	nd	nd
IGF-IR	+	+
IGF-IIR	+	nd
ER#	−	0
PgR#	+	−

[a] # = regulated at the level of transcription; nd = not determined; 0 = no effect; − = negative effect; + = positive effect.

with either these growth factors resulted in only minor alterations in cellular proliferation and morphology *in vitro*. No significant effects were observed *in vivo* in nude mouse models of tumor growth. Thus, although the EGF and IGF growth factor family and steroid receptor effects are clearly interdependent, neither the steroid nor the growth factor effect may fully substitute for the other (50, 68, 69).

In striking contrast to results with IGF-II and TGFα, when heregulin β1, TGFβ, or an FGF family member are transfected into MCF-7 cells, the cells clearly progress to hormone independent malignancy *in vivo*. Interestingly, the effects of heregulin β1 were attributed to autocrine activational phosphorylation of the ER, FGF-4 effects were attributed to autocrine tumor cell proliferation and paracrine induction of angiogenesis and TGFβ effects were attributed to paracrine stimulation of angiogenesis and immune suppression (34, 63, 70). While none of these factors are known to be under positive hormonal regulation in breast cancer, at least in the case of TGFβ, the factor are known to be upregulated during progression of the disease (33).

Collectively, these data support an interactive role of growth factors and estrogens in tumorigenesis. An important question for the future is to determine the extent to which growth factors mediate survival-promoting effects of steroids. With regard to progestins, growth factor mediation of proliferative and survival effects has been less well-characterized. *In vitro*, as noted earlier, progesterone inhibits the anchorage-dependent growth of ER+ human breast cancer cell lines, while under anchorage-independent conditions, progesterone appears to be a stimulatory factor (71). Resolution of this paradox will probably require much more study of regulation of the cell cycle by signaling through cell adhesion mechanisms.

ASSOCIATION OF SEX STEROIDS, GROWTH FACTORS, AND THEIR RECEPTORS WITH DISEASE STATUS (TABLE II)

Compelling evidence suggests that autocrine and paracrine signaling between the EGF-family and the EGFR family are widespread and may play pivotal roles in both ER+ and ER− human breast carcinomas (Table I). Members of the EGF family-TGFα, EGF,

Table II. Summary of Growth Factor, Growth Factor Receptor and Sex Steroid Receptor Expression in Breast Cancer

Growth factor/ receptor	Positivity in breast cancer (% of cases)
EGF	60
TGFα	50–70
Amphiregulin	40–80
Cripto-1	80–90
Heparin binding-EGF	d[a]
Betacellulin	nd[b]
Heregulin α and β	30–70
Insulin-like growth factor-I	d[a]
Insulin-like growth factor-II	d[a]
TGFβ1	50–70
TGFβ2	d[a]
TGFβ3	d[a]
EGFR	50–60
ErbB2	30
ErbB3	75–90
ErbB4	75–90
IGF-IR	50–93
IGF-IIR	83
ER	50–60
PgR	25–30

[a] d = detected in human and mouse breast cell lines and human breast cancer tissue but no current percentages are available.
[b] nd = not determined.

ılin, cripto-1, the heregulins and HB-EGF
ɔen detected in human breast cancer biopsies.
VA has been detected in 60% of ER+/PgR+
he enhanced expression of TGFα mRNA and
.ve been detected in most breast hyperplasias,
% ER+/PgR+ tumors, in 70% of human
nors in general, as well as in the urine and
nd peritoneal effusions of breast cancer
72–76). Amphiregulin is expressed in both
ER− human breast cancer tissue (40–80%)
ın breast cancer cell lines, particulary those
ER+ (75–77). Cripto-1 mRNA and protein
elevated in 80–90% of infiltrating ductal
ar breast carcinomas and several ER+ and
nan breast cancer cell lines (8). Heregulin
ɔeen detected in 30% of primary breast carci-
ı sum, all of these growth factors appear to
ɔsed to a greater degree in malignant than
reast epithelium.

uisition of independence from estrogen, e.g.,
to hormonal autonomy, commonly occurs in
ɔssion of breast cancer. This step may involve
n hormone receptors and signal transduction,
n alteration of growth factor expression itself.
known that overexpression of two growth
ɔeptors, the EGFR and the erbB2 receptor,
iated with hormone independence and poor
in human breast cancer (23, 78). As noted
ıodulation of steroid receptor function may
ɔ to this independence. Conversely, IGF-I
ɔxpression is associated with hormone depen-
d good prognosis (23). Furthermore, muta-
uppressor genes (p53, Rb-1), overexpression
transduction genes (such as PKC), and ampli-
in cell cycle genes (myc, cyclin D1), may
ibute to the inevitable process of progression
ıone independence (23).

USIONS

ɔummary, we have discussed the rapidly
g literature concerning growth factors, sex
and their interactions in mammary develop-
neoplasia. It should be clear that both types of
ıodulators function in diverse ways to promote
evelopment and the abnormal conversion of
mary gland to uncontrolled proliferation. In
rowth factors expression is modulated and sex
ɔgulate growth factor synthesis and secretion,
ɔactor binding proteins, and growth factor receptors. Conversely, growth factors can also regulate sex steroid receptor phosphorylation and function. Both classes of effectors probably cooperate in hormone dependent breast cancer. However, in association with malignant progression of the disease, growth factors are expressed autonomously, with certain growth factor receptors actively overexpressed or amplified, and additional aberrations occurring in signal transduction-related and cell cycle genes. Several critical questions remain. First, how do steroid-growth factor interactions in the normal mammary gland compare to those in hormone dependent breast cancer? Second, what are the detailed pathways involved in growth factor regulation of steroid receptors? Third, how do steroid receptors regulate growth factor, binding protein, and receptor gene transcription? Finally, what is the range of steroid effects on growth, survival, and differentiation that require interaction with growth factor pathways.

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Review

Defining a role for c-Myc in breast tumorigenesis

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Key words: apoptosis, cell cycle, amplification, overexpression, breast neoplasia, c-Myc

Summary

The proto-oncogene c-myc is commonly amplified and overexpressed in human breast tumors, and the tumorigenic potential of c-myc overexpression in mammary tissue has been confirmed by both *in vitro* and *in vivo* models of breast cancer. However, the mechanisms by which Myc promotes tumorigenesis are not well understood. Recent evidence indicates that Myc can promote cell proliferation as well as cell death *via* apoptosis. These studies provide new insight and impetus in defining a role for c-Myc in breast tumorigenesis and may point toward novel targets for breast cancer therapy.

Introduction

Cancer is a genetic disease which progresses through the accumulation of multiple alterations in the DNA of cells, including both hereditary (familial) and sporadic mutations. Many of the mutations found in human cancers lead to aberrant expression or activation of proto-oncogenes which are involved in the signal transduction pathways of mitogens and thus promote deregulated cell proliferation. Determining the specific contributions of such activated oncogenes to tumor initiation, progression, and metastasis is therefore an important step for understanding the process of tumorigenesis. The focus of this review is the c-myc oncogene, which is commonly amplified and overexpressed in many types of human cancers, including breast cancer.

The proto-oncogene c-myc

Initially identified as the cellular homologue of v-myc, the transforming gene of MC29 and other highly oncogenic avian retroviruses, c-myc is now known to belong to a family of sequence-specific transcription factors (reviewed in [1-3]). Other family members include N-myc and L-myc, which were identified as amplified sequences in neuroblastomas and small cell lung carcinomas, respectively.

Myc is a nuclear phosphoprotein with several functional domains which are commonly found in transcription factors (reviewed in [1,4,5]). It can associate with DNA through a non-specific DNA binding domain as well as a basic region which allows for sequence specific DNA binding. The amino terminal region of the protein functions as

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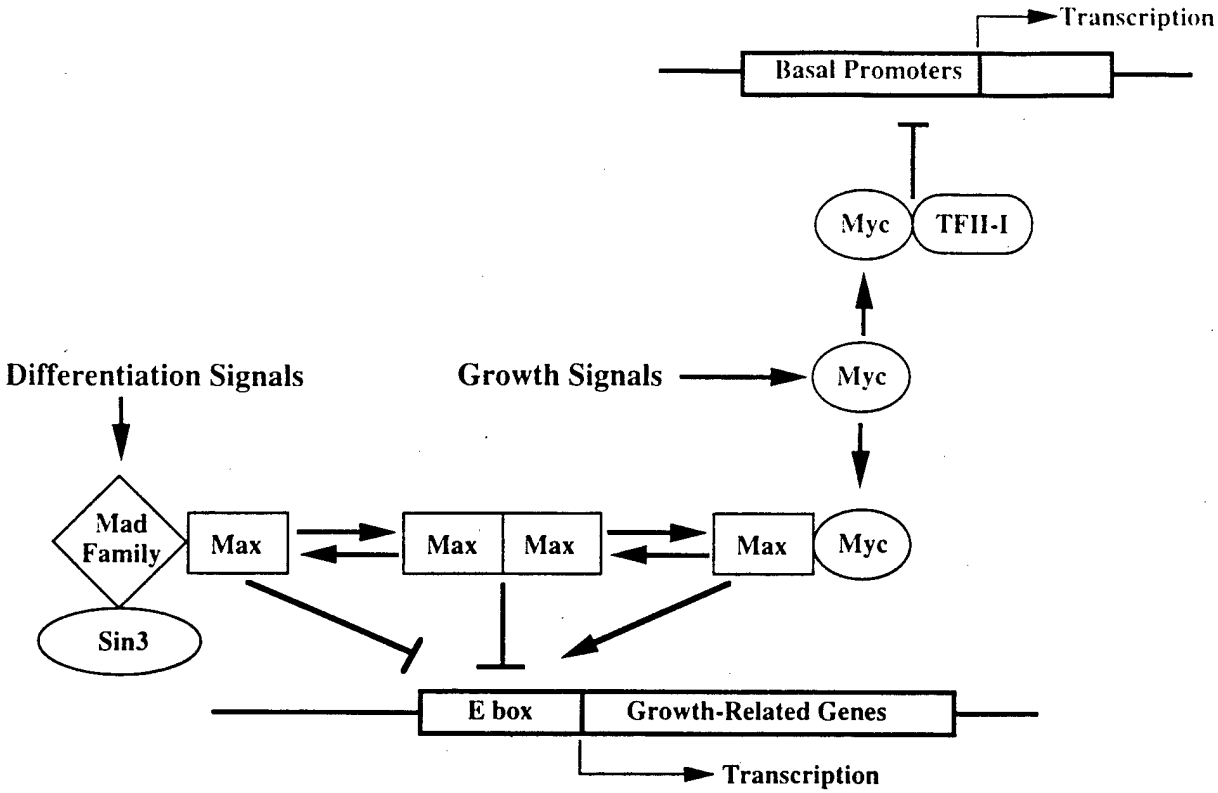

Figure 1. A model of transcription regulation by Myc, Max, and Mad. Max is constitutively expressed while Myc expression is stimulated by growth signals and expression of the Mad family of proteins is promoted by differentiation signals. Myc/Max heterodimers activate transcription from promoters which contain E box sequences. Max homodimers and Max/Mad heterodimers inhibit transcription from the same promoters. Inhibition by the Max/Mad complex is also dependent on its association with the corepressor Sin3. Myc can also interact with TFII-I and thereby inhibit transcription from basal promoters with initiator elements. Pointed arrows represent stimulation and blunted arrows indicate repression.

a transcriptional activation domain, while the carboxy terminal portion contains the leucine zipper and helix-loop-helix (HLH) structures which promote dimerization. When heterodimerized with another HLH protein named Max, Myc can specifically bind the E box DNA sequence [CAC(G/A)TG] and activate transcription (Figure 1). In contrast, Myc can also repress transcription from basal promoter elements (initiator element) through an interaction with the transcription initiator TFII-I [6]. Recently, this transcriptional repression by Myc was reported to play an important role in neoplastic transformation by the oncogene [7].

Homodimers of the constitutively expressed Max protein interact with the same DNA consensus sequence as Myc/Max dimers, but inhibit rather than promote transactivation (reviewed in [1,8,9]). Similarly, heterodimers of Max and members of another HLH family termed Mad inhibit transcription from the same sites [1,8,10]. In the latter case, repression is dependent on the interaction of Mad with the corepressor Sin3 [11-12]. The expression levels of Myc and the various members of the Mad family generally are inversely correlated, with Mad expression highest in cells which are committed to differentiation and with Myc expression highest in proliferating cells [10,13-17]. Mad proteins may therefore promote cell cycle arrest and differentiation. A

role for the Mad family of proteins in cell survival has also been hypothesized, but has not yet been demonstrated.

Although the core DNA recognition sequence for Myc/Max heterodimers has been identified, the specific transcriptional targets are not well defined. Transfection studies indicate that a number of promoters which contain the consensus recognition sequence can be activated by Myc, including the promoters for ornithine decarboxylase, α-prothymosin, lactate dehydrogenase, CAD, p53, ECA39, and cdc25A (reviewed in [18-20]. Some of those putative target genes are believed to play an important role in cellular proliferation and/or apoptosis, but the physiological relevance of each target gene remains to be elucidated. Furthermore, induction of expression of some genes by Myc appears to be cell type or context specific, and temporal expression of the different targets varies during the cell cycle, suggesting that other factors may in some cases cooperate with Myc to regulate transcription. For example, Myc has been reported to undergo cell cycle dependent phosphorylation, with altered transactivation activity as a consequence [21]. Recently, it was demonstrated that the tumor suppressor Rb and a related protein p107 can interact with Myc and differentially regulate Myc-mediated transcription [22-24]. Myc has also been shown to affect the expression of several genes which lack the consensus binding sequence, but the mechanism(s) of that action is unknown and may be indirect.

Thus, the exact function of Myc is not clear, but it is well established that Myc plays a central role in normal growth and development, as well as in cellular transformation and carcinogenesis. It apparently does so by promoting both cell proliferation and cell death, or apoptosis (Figure 2). That may seem to be a paradox, but it is becoming increasingly clear that the two opposing processes of proliferation and apoptosis may be intimately linked through their mutual dependence on the cell cycle (reviewed in [25]). Determining the contributions of the various Myc functions in tumorigenesis would therefore greatly facilitate our understanding of the molecular biology of cancer. In our studies, we have been particularly interested in deciphering the role of Myc in the initiation and progression of breast cancer.

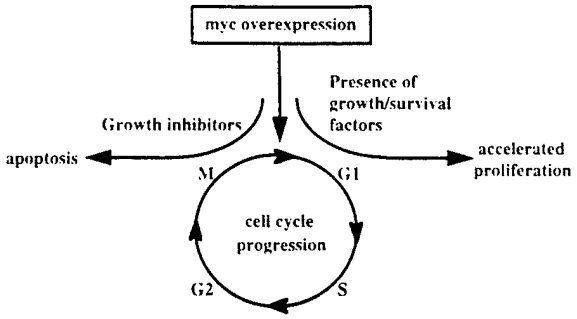

Figure 2. Hypothetical model for the dual action of c-myc overexpression on mammary epithelial cells. Deregulated c-myc expression promotes cell cycle progression through a poorly defined mechanism. The end result of such inappropriate cell cycle stimulation depends on a number of factors such as cell genotype and environment. For example, in the presence of growth or survival factors, Myc expression is proposed to accelerate cell proliferation. In the absence of such survival factors, or in the presence of growth inhibitors, constitutive c-myc expression is more likely to induce apoptosis.

Regulation of c-myc expression

In normal cells, c-myc expression is tightly regulated and is correlated with the proliferative state. Levels of the protein are very low in quiescent cells and are strongly induced following mitogen stimulation. Similarly, expression decreases as cells become growth arrested or undergo differentiation. The signal transduction pathways leading to mitogen-stimulated Myc expression are not well defined, but there is now evidence that Myc induction in fibroblasts by at least two different mitogens (PDGF and CSF-1) requires Src kinase activity [26,27, and references therein]. Whether the same pathway is utilized in mammary epithelial cells is not known, but there is some anecdotal evidence suggesting that may be the case. Neu (ErbB2) can interact with and activate c-Src [28], and mammary tumors which develop in MMTV-neu transgenic mice generally have elevated Src activity [29]. However, a

subset of tumors in this study did not show increased Src activity. Rather, those tumors expressed high levels of Myc protein, suggesting that Myc was downstream of Src and that the need was eliminated for Src activity in tumor growth.

Regulation of Myc levels is quite complex (reviewed in [2,3]). Both the mRNA and protein have very short half lives, and multiple control points of expression have been noted, including initiation and elongation of transcription and translation, and processing of the mRNA and protein. Control of transcription initiation is complicated because c-myc is transcribed from four independently regulated promoters, two major (P1 and P2) and two minor (P0 and P3). Many putative effector sites, both positive and negative, have been identified upstream of the promoters as well as in the first exon and intron. The specific protein complexes which interact with the effector sites are not well defined, but c-myc expression has been shown to be differentially regulated by such proteins as E2F and the ets family of proteins [30,31], the zinc finger protein YY1 [32], and the rel-oncogene-related family of NF-κB factors [33]. However, since promoter usage varies with cell type, many of the binding proteins may interact with the promoter region in a cell type specific manner.

In the normal mammary gland, increased c-myc expression is associated with the proliferative phases of development as well as with the normal apoptotic process of involution [34-35]. *In vitro*, c-myc expression can be induced in normal and tumorigenic breast cells by a variety of growth factors and hormones which stimulate their growth, including EGF, TGFα, IGF-I, insulin, and estrogen [36-40]. Linoleic acid, a fatty acid which can stimulate breast cancer cell growth through a non-hormonal mechanism, can also induce c-myc expression, presumably through a lipoxygenase pathway [40]. In contrast, TGFß, which inhibits mammary epithelial cell proliferation *in vitro*, can suppress EGF-induced c-myc expression [42]. The apparent association between c-myc expression and proliferation is not absolute, however, since several agents which inhibit breast cancer cell growth do not suppress c-myc or even up-regulate its expression. For example, the inhibitory action of retinoids [43] and vitamin D_3 analogs [44] is not accompanied by lowered c-myc levels, and TPA has been reported to induce c-myc expression while inhibiting MCF-7 cell growth [38]. Progestins stimulate a biphasic effect on both growth and c-myc expression, with an early response of up-regulated c-myc and progression through the cell cycle and a long term growth arrest with suppression of c-myc expression [45-47]. Antiprogestins, on the other hand, simply arrest cells in G_1 and down-regulate c-myc expression [45].

Many studies indicate that growth inhibition of estrogen receptor positive breast cancer cells by antiestrogens is also accompanied by a decrease in c-myc expression, both *in vivo* [48] and *in vitro* (T-47D cells [43-45,47]). The estrogen responsive cell line MCF-7 exhibits a transient increase in c-myc expression in response to some antiestrogens [49,50], perhaps as a result of their partial ER agonist activity in that cell line [51]. However, it is now recognized that the effects of antiestrogens may be quite complex and may include activity which is independent of the estrogen receptor. For example, high concentrations of tamoxifen can inhibit the growth of some estrogen receptor negative breast cancers and cell lines [52,53], and have been reported to inhibit growth factor-stimulated proliferation in estrogen receptor positive cell lines [54]. In the estrogen-independent cell line MDA-MB-231, the mechanism of tamoxifen action may be quite different from that of ER positive cells, since tamoxifen was found to elevate c-myc expression with concomitant induction of apoptosis [55]. Furthermore, inhibition of c-myc expression by antisense oligonucleotides prevented tamoxifen-induced apoptosis in that system. In the case of antiestrogen inhibition of growth factor-stimulated proliferation, it was found that pretreatment of MCF-7 cells with tamoxifen or droloxifene actually increased the level of c-myc induction by EGF or IGF-I [50]. Those results suggest that the point

of inhibition under those circumstances may be downstream of c-myc.

Of all the factors which have been shown to modulate c-myc expression in breast cells, the mechanism of estrogen action is the best characterized [56]. In estrogen responsive cells (MCF-7), estradiol stimulates a rapid and transient increase in c-myc transcription in the absence of protein synthesis [39,57]. In contrast, ER negative cells (MDA-MB-231) have constitutively high levels of c-myc expression, apparently due to an increase in the mRNA half life (approximately three-fold) rather than increased transcription [57]. TGFα, in contrast to estrogen, can increase c-myc RNA levels without altering the transcription rate, suggesting that the growth factor can exert its effects at the post transcriptional level [39]. Alternatively, the time course of transcription induction by TGFα may simply be different from that of estradiol.

The mechanism of c-myc transcriptional regulation by estrogen has been further characterized by Dubik and Shiu [51]. These authors found that the DNA binding region of the estrogen receptor was required for c-myc induction and that the P2 promoter region of the c-myc gene contained an ERE half-site and a CG-rich Sp1 binding site which could potentially cooperate to induce transcription. In both hormone-dependent and independent breast cancer cells, Sp1 or Sp1-like proteins appear to have an important role in regulating the activity of all three of the c-myc gene promoters [58]. Several additional breast cancer nuclear proteins have been reported to bind to the c-myc promoter regions of both MCF-7 and MDA-MB-231 cells, but a region in the P0 promoter showed different nuclear protein binding activity in the two cell lines, with higher levels in the MDA-MB-231 nuclear extracts. That same region was shown to contain a DNAase hypersensitive site which was more accessible in MDA-MB-231 cells, indicating that nuclear protein was specifically interacting with the P0 promoter in those cells [59]. However, it should be noted that in both ER positive and negative cells, the P2 promoter is the strongest, while the P0 promoter is the most infrequently used [59].

Whatever the mechanism of its regulation, c-myc expression appears to be essential for the growth of both estrogen-dependent and estrogen-independent breast cancer cells [60]. Oligonucleotides antisense to c-myc were able to inhibit estrogen-stimulated growth of MCF-7 cells and also had cytostatic effects on MDA-MB-231 cells. The results suggest that antisense therapy directed against c-myc may be useful in the treatment of breast cancer. New antisense strategies, such as polyamine-complexed triplex forming DNA, may dramatically improve the efficacy of antisense therapy and have recently been shown to be quite effective in inhibiting c-myc expression in MCF-7 cells [61]. Using a different approach, it has been demonstrated that c-myc expression is also necessary for tumor growth *in vivo*. Breast cancer cells which had been transfected with an expression vector for MBP-1, a c-myc P2 promoter-binding protein which negatively regulates transcription, lost their ability to grow in soft agar and nude mice [62].

Suppression of c-myc may also be an important component of traditional chemotherapy. A number of genotoxic drugs (such as the topoisomerase II inhibitors doxorubicin, amsacrine, and teniposide) currently being used to treat cancer have been shown to suppress c-myc in the MCF-7 breast cancer cell line [63-66]. Clinically relevant concentrations of these drugs reduce c-myc RNA levels with concomitant inhibition of cell proliferation. In the case of teniposide, a reduction in c-myc transcription has been observed [67]. However, the mechanism by which the drugs alter c-myc levels is not known, and it is unclear whether the decrease is the cause or only an effect of growth arrest. A potential mediator of such effects may be p53. Many DNA-damaging agents induce p53 expression, which has been shown to repress c-myc expression [68]. If normal regulation of both p53 and c-myc are required for the cytostatic or cytotoxic effects of the drugs, the prevalence of c-myc amplification and/or p53 mutation in human cancers may partially explain the refractoriness of

Table 1. Summary of clinical studies on c-myc amplification in human breast cancer

Reference	Study size	Frequency of myc amplification (%)	Correlations	Method
70	121	32	age	Southern
71	53	16	—	Southern
72	41	22	poor short term prognosis	Southern
73	48	41	—	Southern
74	116	6	poor prognosis	Southern
75	292	16	PR negative tumors, high tumor grade	Southern
76	125	18	inflammatory carcinoma	Southern
77	52	21	high tumor grade, survival, aneuploidy	Southern
78	176	4	shortened disease-free and overall survival	slot blots
79	157	6	—	Southern/slot blots
80	140	23	high cathepsin D levels	Southern
81	30	50	—	Southern
82	282	20	early recurrence/death, lymph node metastasis tumor size, negatively with erbB-2 amplification	Southern
83	1052	17	PR negative tumors	Southern
84	311	8	high S phase fraction, c-erbB-2 co-amplification early recurrence/death (especially postmenopausal)	slot blots
85	60	35	high proliferation rate	Southern
86	154	7	—	Southern,PCR
87	122	23	LOH of chromosome 1p32-pter	Southern
88	54	6	—	Southern
89	162	16	early recurrence/death, lymph node metastasis	PCR

many cancers to genotoxic chemotherapy. An alternative mechanism for drug-induced c-myc suppression may be gene specific DNA damage, which may simply reflect a higher susceptibility of genes which are being actively transcribed in proliferating cells [66].

Amplification and overexpression of c-myc is common in breast cancer

Myc expression is activated in many types of cancer through amplification, rearrangement, translocation, and proviral insertion [2,4]. Recently, multiple point mutations in the c-myc transcription activation domain have also been identified in a variety of lymphomas [69, and references therein], but it is not known whether such mutations are involved in epithelial cancers such as breast carcinoma.

Amplification of the c-myc locus in breast cancer tissue has been observed in many studies [70-89]. The reported frequency of amplification varies greatly (from 4% to 52%) among these studies, but the overall mean appears to be about 20%. Several studies have also reported genetic rearrangements of the c-myc gene in breast tumors [70,73,76,77,90], but the incidence appears to be less frequent than amplification (about 5%). One study also looked for alterations in other Myc family members [72]. A two-fold amplification of L-myc was identified in 1 out of 41 breast tumors, but no changes were observed for the N-myc locus. The frequency of c-myc amplification in human breast cancer cell lines has not been systematically investigated, but one study [91] found an amplified and overexpressed c-myc locus in one of five cell lines investigated (SKBR-3). However, it was not determined whether tumorigenicity of the cell line was dependent on c-myc expression.

There is also considerable variability in the predictive value of c-myc amplification and correlation with other prognostic markers of breast

cancer (summarized in Table 1). Some reports indicate that c-myc amplification is predictive for shortened relapse free and/or overall survival [72,74,77,78,82,84,89]. Depending on the study, c-myc amplification has also been correlated with highly proliferative tumors [84,85], high tumor grade [75,77], node status [82,89], steroid receptor status [75,83], tumor size [82], age [70], cathepsin D expression [80], aneuploidy [77], and a particularly aggressive form of breast cancer, inflammatory carcinoma [76]. A correlation has also been reported between c-myc amplification and loss of heterozygosity at a specific locus on chromosome 1, suggesting the possibility of a tumor suppressor whose inactivation may lead to c-myc amplification [87]. Furthermore, some studies have found that co-amplification of c-myc and erbB2 is relatively rare [76,82,85], while one study found a positive correlation for amplification of the two loci [84], and still others have found no correlation [74,81]. The variation in results is not surprising given the broad range of sample size, composition, and follow up, as well as inconsistencies in experimental and statistical methodology, as previously discussed by Callahan and Campbell [92]. For example, most studies have used Southern analysis of biopsy material, a method which does not take into consideration the variation in cellular composition and may therefore underestimate the frequency of amplification. PCR methods may be useful in reducing the error associated with amplification measurements, since they can facilitate analysis of very small and specific areas of the tumor ("tumor imprints" [86, 89]) or can even be performed *in situ* [93]. PCR analysis of imprints from primary tumors and lymph node metastases from the same patients suggested that c-myc amplification was maintained in the transition from carcinoma *in situ* to invasive cancer, but not necessarily in the transition to metastatic tumors [86]. However, the size of the study was quite small and the results must be repeated and confirmed before any definitive conclusions can be drawn. Newer methods such as fluorescence *in situ* hybridization (FISH), primed *in situ* labeling (PRINS), and comparative genomic hybridization (CGH) may also provide more accurate assessment of gene alterations [93].

Since amplification of a gene is not necessarily associated with or required for its overexpression, a number of studies have examined c-myc expression in breast cancer, at both the mRNA and protein levels. However, given the extremely short half life of c-myc protein and RNA, these results should also be interpreted with caution. Northern analysis indicated that c-myc mRNA expression was elevated compared to normal breast tissue in 70% ([70], n=14) or 45% ([74], n=116) of breast tumors. In the latter study, overexpression was correlated with lymph node involvement. *In situ* hybridization of the tissues used for the former study confirmed that c-myc mRNA levels were often higher in tumor cells compared to surrounding stromal cells [94], but that method also clearly demonstrated the potential for error associated with differences in tumor cellularity. All three studies indicated that c-myc could be overexpressed in the absence of detectable gene amplification.

Immunohistochemistry has been frequently used to examine the relative levels of Myc protein in specific cells of mammary tumor specimens [95-100]. The first published report on Myc immunohistochemistry in breast cancer showed moderate to strong staining in all tumors but only slightly positive signal in adjacent normal tissue [95]. Interestingly, these authors observed moderate to strong staining in a large proportion of cystic disease specimens as well (66%). The same group also used an ELISA assay to measure relative Myc protein levels in tumors and found that all breast tumors had elevated Myc expression compared to normal breast tissue [101]. In all three of the studies by Pavelic [96-98], Myc staining was found to be primarily nuclear and was observed in all ductal carcinomas examined as well as in 7 out of 11 normal breast tissues. In all cases, however, expression was higher in tumor cells than in normal cells. Staining was also heterogeneous within individual tumors, with more intense signal in invasive regions compared to non-invasive regions. In a larger study by

Pietilainen et al. [100], nuclear Myc staining was observed in only 12% of breast cancer samples, while cytoplasmic staining was predominant (95% of tumors). The reason for the difference in subcellular localization in these studies is not known, but it may be due to differences in tumor fixation and antibody source. They reported that nuclear Myc staining was correlated with lack of estrogen receptor, while strong cytoplasmic staining at the invasive margin was associated with low mitotic index and longer relapse-free survival. Those results suggest that regulation of Myc activity and its role in tumorigenesis may be highly complex. Another recent study of 85 breast tumors suggests that Myc overexpression is less common (17%) than the above studies indicate, perhaps because a different scoring system was used [99]. In that study, Myc overexpression was not, by itself, predictive for outcome, but co-overexpression of Myc with other oncogenes such as Ha-ras or c-fos was correlated with reduced disease-free and overall survival. As the number of overexpressed oncogenes increased, survival time decreased. There is also now evidence that the related family member N-myc is widely overexpressed in breast cancer in the absence of its amplification [102]. The level of N-myc expression was also correlated with clinical stage, histological grade, and clinical outcome.

Thus, amplification, rearrangement, and overexpression of c-myc are clearly present in a significant number of breast tumors, but the predictive value and pathophysiological consequences of amplification are not at all clear at this time. A recent study of benign breast disease, which has been associated with breast cancer risk, found none of the genetic alterations commonly identified in breast cancer, including c-myc amplification [103]. Those results suggest that such genetic changes may be late events in mammary tumorigenesis, but it has not been definitively demonstrated that benign lesions constitute precancerous tissue. Animal and *in vitro* models of breast cancer may therefore be very useful in deciphering the role of c-myc in breast tumorigenesis.

c-myc overexpression contributes to a transformed phenotype in mammary epithelial cells

The transforming activity of c-myc *in vitro* has been well documented in a variety of cell types (reviewed in [2,3,5]). However, the transformation of normal cells by c-myc requires co-activation or overexpression of additional oncogenic proteins such as Ras [104-105], Bcl-2 [106-109], or oncogenic viral proteins [2]. It has also been reported by a number of investigators that c-myc can cooperate with several peptide growth factors such as EGF to promote transformation of various cell types [110-114].

A similar phenomenon has been observed in normal human (A1N4 [115]) and mouse (MMEC [116]) mammary epithelial cells. A1N4 cells which had been infected with a c-myc construct under the transcriptional control of the Moloney Murine leukemia virus LTR could proliferate under anchorage independent conditions, but only in the presence of EGF, TGFα, or bFGF. Those same growth factors could stimulate the anchorage dependent growth of both the parental cells and the c-myc-infected cells, but only the A1N4-myc line could grow in soft agar. The differential response of the two cell lines was not due to changes in growth factor receptor expression (EGFR and FGFR). Interestingly, co-culture of A1N4-myc cells with primary mammary fibroblasts, or addition of fibroblast conditioned medium, could substitute for the exogenously added growth factors to induce colony formation. Those results suggest a possible mechanism by which stromal tissue might influence transformation and tumor progression in the epithelial tissue of the mammary gland. However, Myc overexpression was not sufficient to induce tumorigenic growth in nude mice.

In contrast, MMEC cells which constitutively overexpress c-myc could grow as tumors in nude mice [116]. The difference in results is most likely due to changes that the cell lines have undergone during the process of immortalization. The MMEC-myc cells could also grow in soft

agar in the absence of exogenous growth factors, but addition of EGF or TGFα stimulated colony formation about three-fold over basal levels. When grown under anchorage dependent conditions, the c-myc expressing cells were also less dependent on exogenous growth factors, grew to a higher saturation density, and exhibited a faster doubling time.

Another mouse mammary cell line (HC14) transfected with a v-myc construct could not grow as tumors in nude mice and showed only limited growth in soft agar, but produced abnormal hyperplastic glands when reintroduced into cleared mammary fat pads of syngeneic mice [117]. The effects of exogenous growth factors were not tested, but the authors found that v-myc expression synergized with lactogenic hormones to superinduce ß-casein expression, an indicator of differentiation. That was a surprising result, since c-myc expression is often down-regulated in conjunction with differentiation and c-myc expression can block differentiation in some cell types [4, 118]. However, the results are consistent with the Wap-myc transgenic mouse model [119]. Mammary tumors from those mice constitutively express ß-casein, independent of lactogenic hormone stimulation.

Transgenic mouse models of c-myc in mammary tumorigenesis

Since *in vitro* studies cannot simulate the complex environment that tumor cells normally experience *in vivo*, transgenic models have been developed to study the effects of deregulated oncogene expression in specific tissues. However, phenotypes observed in transgenic mouse lines should always be viewed in light of the genetic background, since different mouse strains can have inherently different susceptibility to cancers. Transgenic mouse lines which direct c-myc overexpression to the mammary gland frequently develop mammary tumors with a relatively long latency (7-14 months) after multiple pregnancies [119-121]. In the case of WAP-c-myc, the requirement for pregnancy is not surprising since the milk protein promoter does not become active until the late stages of pregnancy. However, driving expression of c-myc from the relatively constitutive MMTV promoter does not eliminate the latency or the role of pregnancy in tumor formation. v-myc expression has also been targeted to the mammary epithelium using a slightly different approach [122]. Primary mammary epithelial cells were infected *in vitro* with a recombinant retrovirus and then injected into the cleared mammary fat pads of syngeneic mice. The resultant cells produced hyperplastic glands with abnormal morphology, but frank tumors were not observed. Taken together, the results suggest that c-myc overexpression can contribute to mammary gland tumorigenesis, but it is not sufficient by itself for tumor development. The long latency suggests that other genetic changes must occur prior to tumor outgrowth, and the effects of pregnancy indicate that mitogens may promote the tumorigenesis of c-myc expressing cells.

That conclusion is not surprising, given the "multihit" hypothesis that neoplasia develops through a series of genetic alterations. Transgenic mouse lines have therefore been crossed to produce double or even triple transgenic strains in order to examine the cooperation among potential oncogenes. To date, c-myc has been co-overexpressed in the mammary gland with v-Ha-ras, c-neu (ErbB2), and TGFα [123-127]. For all three combinations, the double transgenic mice exhibited an accelerated tumor onset compared to the parental strains. Nevertheless, with the exception of the MT-TGFα/MMTV-myc cross [123], tumor formation was still stochastic, suggesting that additional genetic events are required. However, a triple transgenic strain (MMTV-myc/MMTV-ras/MMTV-neu) did not appear to have a significantly shortened latency compared to the parental myc-ras strain [125]. Consistent with the in vitro transformation studies, the most dramatic interaction was observed for the myc-ras and myc-TGFα crosses (mean tumor onset of 46 and 60 days, respectively). In both cases, tumors were observed in both

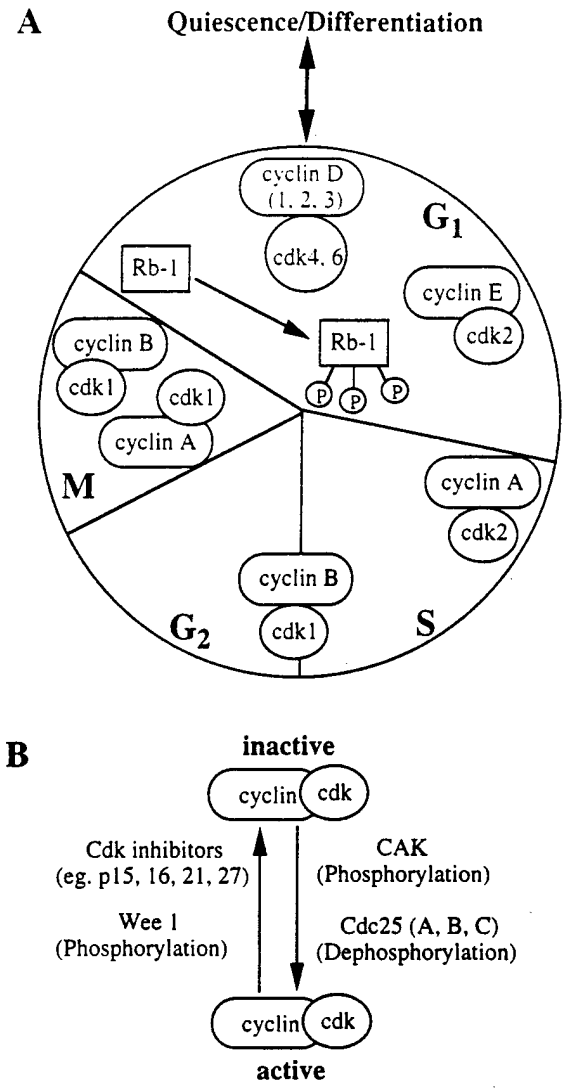

Figure 3. A model of cell cycle regulation. Panel A: Progression through the cell cycle is orchestrated by periodic expression of the various cyclins which activate their associated cyclin dependent kinases. Complete withdrawal from the cell cycle leads to quiescence and in some cases, differentiation. Panel B: Cdk activity is further modulated by several low molecular inhibitors, as well as kinases (such as CAK and Wee1) and phosphatases (Cdc25 family). Myc has been reported to modulate expression of several key cell cycle regulators, as noted in the text.

males and females, suggesting that ovarian hormones were no longer necessary for tumorigenesis. It is also interesting to note that signaling pathways for EGF receptor include activation of Ras.

Gene knockout mouse models can also be used to study the effects of loss of function mutations in tumor suppressor genes in conjunction with gain of function alterations in oncogenes. Mutations in the p53 gene are among the most common genetic alterations in many types of cancer, including breast cancer (reviewed in [128]). It has been hypothesized that p53 mutations may cooperate with c-myc overexpression to induce mammary tumors since Myc-induced apoptosis is dependent on p53 in some systems [129-132] and since activated Myc expression and p53 deletion can synergize to induce thymic lymphomas [133]. However, a cross between the MMTV-myc mice and p53 null mice did not result in accelerated mammary tumorigenesis [134], suggesting that loss of p53 may not be required for onset of Myc-driven breast neoplasia. A caveat in this study was that lymphomas arose so rapidly in the absence of p53 that consequences of the bitransgenic Myc cross could only be evaluated over a short interval of time. Interestingly, p53 knockout mice have also been used to demonstrate that the normal apoptotic process of mammary gland involution, which is associated with high levels of c-myc expression [35], is not dependent on the p53 protein [135].

Myc and cell cycle regulation

It has been proposed that Myc controls the expression of genes which are important for cell cycle progression (see Figure 3), since Myc induction is sufficient to drive quiescent cells into the cell cycle [136] and constitutive expression of c-myc prevents growth arrest of serum-deprived fibroblasts [137]. A reduction in c-myc levels due to disruption of one copy of the gene results in a lengthened G1 phase [138], while ectopic expression of c-myc leads to a shortened G1

phase in fibroblasts [139]. Furthermore, inhibition of c-myc expression blocks cell cycle progression and leads to G1 arrest [140-141].

Myc has been implicated in the expression of several genes involved in cell cycle control, but it is believed to do so indirectly, since most of the genes in question lack Myc-Max consensus binding sites in the promoter region. For example, constitutive overexpression of c-myc in fibroblasts resulted in overexpression of cyclin A and, in one case, cyclin E [142-144]. Similarly, fibroblasts in which Myc expression was reduced by disrupting one copy of the c-myc gene had a lengthened G1 phase, with concomitant delayed expression of cyclin E and A and phosphorylation of Rb [138]. There are conflicting reports about the effects of Myc on cyclin D1 expression, with some suggesting repression [142-143] and others indicating upregulation of the gene by Myc [145]. However, it has been recently shown that Myc cooperates with cyclin D1 in transformation, suggesting that the two proteins function in complementary rather than linear pathways [146].

Myc may also be involved in the regulation of cyclin dependent kinase (cdk) expression and activity. Expression of cdk1 (previously termed cdc2) and cdk2 is up-regulated when Myc is expressed [147,148]. In accordance with the cell transformation studies, it has been shown that activated Ras is required for Myc-induced expression of cdk1, indicating that the Map kinase pathway may cooperate with Myc to regulate cdk expression, perhaps by direct phosphorylation of the Myc transactivation domain [21,148]. Cdk activity may be modulated by Myc through increased cyclin expression, as noted above, through decreased expression of cdk inhibitors such as p27 [149], or by altered expression or activity of the kinases and phosphatases which regulate cdk activity, such as cdc25 [20].

Recent results from our laboratory indicate that de-regulated c-myc expression results in altered cell cycle regulation in MECs as well. The growth rate of both mouse and human MECs was accelerated by constitutive c-myc overexpression [150]. The mechanism behind that phenomenon has been further investigated in an immortalized human MEC model system (184A1N4 and 184A1N4-myc cell lines, [115,151]). The difference in doubling time appears to be due to a shortened G1 phase, as A1N4-myc cells reach S phase more quickly than parental cells when released from a G1 growth arrest. In unsynchronized cells, the effect of c-myc overexpression on cyclin A or D1 mRNA expression was minimal. Cyclin expression was nearly undetectable in arrested cells and induction was correlated with cell cycle progression in both cell lines. However, Rb was hyperphosphorylated in A1N4-myc cells at all time points tested, in contrast to the parental cells which exhibited a typical phosphorylation shift as they traversed G1 [152]. Our results further indicated that the abnormal phosphorylation status of Rb in c-myc-overexpressing cells was associated with reduced p27, increased cyclin E, and premature activation of cdk2 kinase activity.

In Figure 4, a model is proposed to explain the effects of constitutive c-myc expression on passage through the G1 phase of the cell cycle. We suggest that Myc promotes the expression of cyclin E while it suppresses the level of the p27 cdk inhibitor, leading to increased cdk2 kinase activity and thus increased Rb hyperphosphorylation. However, the mechanism by which Myc targets those two proteins is not clear and may be indirect. The primary functions of Myc are thought to be dependent on its ability to act as either a transcriptional activator or repressor, but a consensus binding sequence for Myc/Max has not been identified in the cyclin E promoter, and p27 levels are regulated primarily at the transcriptional and post-transcriptional stage [153,154]. That suggests that Myc is somehow activating other pathways which lead to altered expression of the two proteins in question.

Our results may provide at least a partial explanation as to why Myc and EGF can cooperate to transform cells and similarly, why there is such a strong synergism between Myc and TGFα in mammary tumorigenesis, as demonstrated by transgenic mouse models. Myc overexpression, in

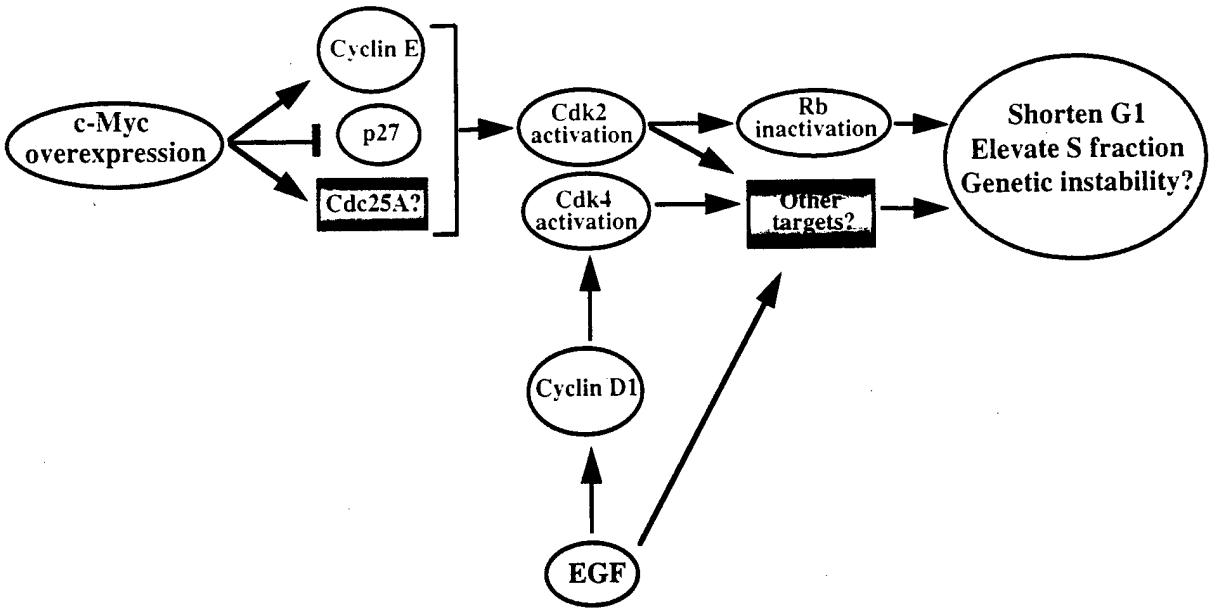

Figure 4. Proposed model for the effects of c-myc overexpression on cell cycle regulation in MECs. Myc induces cdk2 activation, via increased cyclin E expression and decreased p27 expression, which in turn can alter the phosphorylation state of Rb. Myc also elevates Cdc25A expression, which can further stimulate cdk2 activity. EGF is required for cyclin D1 expression. Additional targets of the EGF receptor signaling pathways may also be necessary for progression into S phase. The combination of c-myc overexpression and EGF signaling leads to accelerated proliferation and may therefore promote genetic instability.

conjunction with EGF receptor stimulation, forces the cells through G1 at a faster rate, resulting in accelerated growth and thus an elevated S-phase fraction. If this phenotype allows the cells to proliferate under conditions which would normally induce a G1 arrest and block DNA replication, increased genetic instability may also be a logical endpoint, analogous to the phenomenon which was demonstrated for p53 mutations [155,156]. In support of that hypothesis, a recent study demonstrated that ectopic expression of p27 suppressed the accumulation of aneuploid cells and tumor growth in a brain tumor model [157]. Furthermore, it was recently demonstrated that prolonged overexpression of Myc in rat fibroblasts promoted a variety of irreversible chromosomal alterations, including numerical changes, chromosomal breakage, and the formation of circular chromosomal structures, chromosome fusions, and extrachromosomal elements [157a].

Deregulated expression of c-myc can promote the onset of apoptosis

Myc has also been implicated in the control of apoptosis (reviewed in [18,158]). Suppression of Myc synthesis by antisense oligonucleotides can block apoptosis in Burkitt lymphoma cells [159] as well as T cell receptor-induced apoptosis in normal lymphocytes [160]. In contrast, Myc overexpression can induce or accelerate apoptosis in fibroblasts or lymphocytes when combined with a negative growth signal [137,161]. Since Myc-induced apoptosis can be rescued by a variety of growth factors [162], it has been proposed that cell death is the result of conflicting cell cycle regulatory signals. Cells normally arrest in the absence of a growth signal, but often c-myc overexpressing cells are unable to withdraw from the cell cycle and instead undergo apoptosis. Thus, it would appear that the impact of

Myc on both proliferation and apoptosis may be through control of cell cycle regulatory proteins. Myc has been shown to affect the expression of cyclin A [137,144], cdk 1 [147,148], and cdc25A [20], three regulators of the cell cycle which have also been implicated in apoptosis [20,144,163, 164]. However, it should be noted that a recent study demonstrated that inhibitors of the G1 cdks could block Myc-driven cell cycle progression, but not apoptosis [165].

Ornithine decarboxylase (ODC), a direct target of Myc transactivation, has also been implicated in both proliferation and apoptosis (reviewed in [18]). Like cdc25A, ODC expression is tightly regulated and mimics the expression of Myc. Both proteins can also substitute for Myc in the induction of apoptosis, as well as in the transformation of cells with Ras. ODC activity regulates polyamine biosynthesis and is essential for S-phase progression, as demonstrated by antisense oligos and specific enzyme inhibitors. However, those same inhibitors only partially inhibited Myc-dependent apoptosis, despite the fact that polyamine synthesis is associated with the production of reactive oxygen species which can promote apoptosis. Furthermore, overexpression of ODC was not sufficient to drive cells into S-phase in the absence of a growth signal [166].

Myc can transactivate the p53 promoter [167], and the promoter of the death-inducing bax gene also contains a putative Myc response element [168]. Myc expression has also been associated with stabilization of the short-lived p53 protein [130]. Thus, Myc may up-regulate bax expression and activate the p53 pathway as a safeguard to prevent the growth and survival of cells with oncogenic activation. In accordance with that theory, Bcl-2 can cooperate with Myc to transform cells and promote tumorigenesis by blocking the apoptotic response to c-myc overexpression without affecting the proliferative response [106-109,169].

The mechanism by which Myc triggers cell death is not universal, however, since the induction of apoptosis by c-myc overexpression is dependent on wild type p53 in some, but not all, systems. In normal fibroblasts, lymphoma cells, and hepatocarcinoma cells, Myc-induced apoptosis was dependent on wild type p53 function [129-132]. However, in primary rat kidney epithelial cells, uncontrolled c-myc expression was found to induce apoptosis via both p53-dependent and p53-independent mechanisms [170].

In cases where Myc-induced apoptosis is dependent on p53, one would predict that p53 mutation would be required for the formation of aggressive tumors. Such a cooperation between c-myc overexpression and p53 mutation has been demonstrated in lymphomagenesis [133,134,171]. However, in the study by Hsu et al., the synergistic effects of the two genetic changes were reported to be due to enhanced proliferation, rather than the abrogation of apoptosis. Furthermore, some other cancer models of c-myc overexpression do not show such cooperativity with loss of p53. For example, p53 mutation is rare in mouse plasmacytomas, which invariantly overexpress c-myc [172]. Similarly, macrophage-monocyte tumors generated by a c-myc containing retrovirus showed a paucity of p53 mutations. In a prostate tumor model, activated Myc and Ras could induce tumorigenesis in the absence of mutated p53, while hyperplasia induced by Ras alone required genetic alteration of the p53 gene [173]. Unfortunately mutation of the p53 gene was not examined in cells with c-myc overexpression alone.

The explanation for this variation in Myc/p53 cooperativity is not clear, but most likely depends on cell specificity, especially with respect to additional genetic changes which have taken place in the transformed cells. In addition, since the regulation of p53 activity is highly complex, alterations in the wild type protein via post-translational modifications or other mechanisms must also be considered. For example, it has been shown that Myc and Bcl-2 can regulate p53 function by altering its subcellular trafficking during the cell cycle [174]. That is particularly interesting in light of the observation that p53 can be found sequestered in the cytoplasm rather than

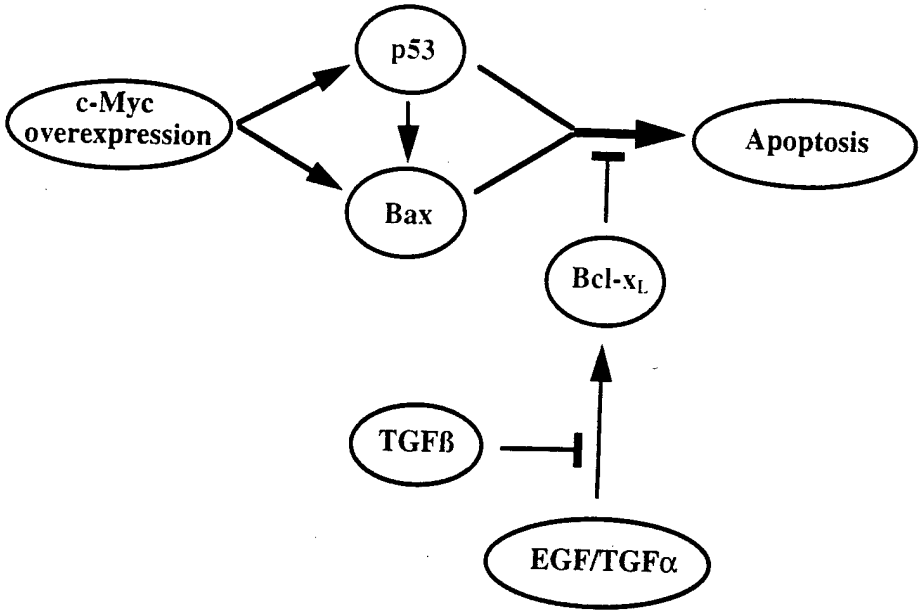

Figure 5. Proposed model for apoptosis induction by c-myc overexpression in MECs. Myc is postulated to stimulate the expression of the pro-apoptotic regulators p53 and Bax. p53 can itself also induce Bax expression. Apoptosis ensues unless a growth/survival factor such as EGF or TGFα is present to induce expression of the anti-apoptotic regulator Bcl-xL. The growth inhibitor TGFß can block the effects of EGF, thereby promoting apoptosis.

in the nucleus in primary breast cancer specimens [175]. As previously noted, the complete absence of p53 in c-myc-overexpressing mammary tissue did not accelerate tumor formation [134], but studies are currently under way in our lab to determine whether altered p53 function plays a role in Myc-induced mouse mammary tumors which express high levels of the wild type protein.

Recent results from our laboratory suggest that a second component of the Myc-EGF synergy in mouse mammary tumorigenesis may be the inhibition of Myc-induced apoptosis by TGFα. We found that mouse mammary tumors induced by c-myc overexpression contained many apoptotic cells [176]. In contrast, the more rapidly arising and aggressive tumors in Myc-TGFα double transgenic mice had essentially no apoptotic cells, suggesting that TGFα can act as a survival factor for mammary cells which constitutively express Myc. Similarly, MEC lines derived from tumors of single transgenic Myc mice show a propensity to undergo apoptosis, and TGFα and EGF function as survival factors for those cells in vitro, while the growth inhibitor TGFß promotes Myc-induced apoptosis even in the presence of EGF. The cell lines therefore provide a useful model for investigating the mechanism by which Myc induces apoptosis in MECs and by which TGFα acts as a survival factor for such cells.

Further characterization of such a cell line suggested a role for Bcl-xL in the regulation of Myc-induced apoptosis by EGF and TGFß in MECs [177]. In Figure 5, a model based on our recent findings is postulated to explain the ability of those growth factors to regulate the apoptotic pathway when c-myc is constitutively overexpressed in MECs. We propose that Myc stimulates the expression of the pro-apoptotic proteins Bax and p53. This is most likely a direct stimulation, since the promoters of both genes contain Myc E-box sequences [165,166]. The bax promoter region also contains a p53 recognition site, suggesting that Bax expression may be additionally stimulated by p53 itself [166]. The con-

stitutive expression of Myc, and thus Bax and p53, should make the cells highly susceptible to apoptosis unless a survival factor such as EGF is present to elevate levels of the survival-promoting Bcl-xL protein. The inhibitory TGFß signal is apparently dominant to that of EGF since it blocks the ability of the growth factor to elevate Bcl-xL levels and therefore promotes Myc-induced apoptosis. However, the mechanism by which EGF modulates Bcl-xL protein levels and the point at which TGFß interferes with that modulation are not known, and are thus the focus of ongoing investigations.

Conclusions

In recent years, significant progress has been made in elucidating the molecular mechanism of c-myc function. However, much work remains to be done before we can fully understand the role that Myc plays in normal regulation of the divergent cellular fates of proliferation, differentiation, and apoptosis. Deciphering the contributions of aberrant Myc expression or function to tumorigenesis will undoubtedly prove to be even more complicated.

There is substantial evidence that the c-myc gene is frequently amplified and overexpressed in breast cancer, and that such Myc overexpression can contribute to breast neoplasia. However, the evidence from both in vitro and in vivo studies indicates that Myc alone is not sufficient for tumorigenesis. Recent reports suggest that c-myc overexpression will not be advantageous for a breast tumor cell unless it is counter balanced by additional signals which promote cell survival, such as growth factor stimulation, or other genetic changes, such as loss of Bax expression or elevated Bcl-xL expression. Clinical studies which examine Myc expression in conjunction with a survey of apoptosis regulators and growth factors or markers of signal transduction pathway activation may therefore provide new prognostic information for human breast cancer and may identify potential new targets for therapy.

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MOLECULAR CARCINOGENESIS 17:163–173 (1996)

Potentiation of Diethylstilbestrol-Induced Alterations in the Female Mouse Reproductive Tract by Transforming Growth Factor-α Transgene Expression[1]

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Neonatal estrogen exposure causes numerous abnormalities in the female reproductive tract, including carcinogenesis. One mechanism by which neonatal estrogen elicits teratogenic and carcinogenic effects is epigenetic and involves the modulation of a number of estrogen-regulated genes including epidermal growth factor (EGF). Because of the evidence that there is an integral relationship between the EGF family, estrogen action, and the regulation of the growth and differentiation of the reproductive tract, we used transforming growth factor-α (*TGFα*) transgenic mice to investigate the interaction of constitutive *TGFα* expression with the potent estrogen diethylstilbestrol (DES) in the induction of reproductive-tract alterations. Our study was designed to determine whether *TGFα* expression could modulate DES-induced carcinogenesis of the female mouse reproductive tract. The animals were homozygous *TGFα* transgenic female mice from the MT42 line and the parental CD-1 outbred mice. The presence of the *TGFα* transgene significantly increased the incidence of DES-induced vaginal adenosis, uterine endometrial hyperplasia, uterine polyps, hypospadia, benign ovarian cysts, and pituitary adenomas. However, constitutive *TGFα* expression did not promote reproductive-tract neoplasia. This study demonstrates that TGFα participates in the regulation of developmental and morphogenic events in the Müllerian duct and urogenital sinus, suggesting a role for TGFα in the pathogenesis of reproductive-tract diseases. Furthermore, we showed that although constitutive expression of the *TGFα* transgene did have an effect on the reproductive tract, *TGFα* overexpression alone could not substitute for DES as a reproductive-tract carcinogen or as a promoter of uterine neoplasia, indicating that DES-induced carcinogenesis requires events in addition to the overexpression of this single peptide growth factor.

Key words: TGFα, DES, reproductive tract carcinogenesis

INTRODUCTION

Exposure to estrogens during critical periods of development induces teratogenic and carcinogenic lesions in the reproductive tracts of humans and experimental animals [1–4]. Genital-tract abnormalities, including vaginal adenosis, cervical ectropion, ridges, pseudopolyps, uterine growth alterations, and occasional vaginal and cervical clear cell adenocarcinoma occur in women whose mothers received the synthetic estrogen diethylstilbestrol (DES) during the first trimester of pregnancy for prevention of threatened miscarriages. Prenatal or neonatal exposure of mice to DES causes similar lesions, making the rodent a good model system for exploring the mechanism of the developmental toxicity of estrogens. The actual mechanism for the toxicity of DES remains unknown, but both genotoxic and nongenotoxic pathways have been proposed [3–5]. The possible epigenetic mechanisms are hypothesized to involve the premature and permanent induction of genes normally under steroid-hormone control in older animals such that the persistent induction or "imprinting" of estrogen-regulating genes contributes to the establishment of the ovarian-independent, "estrogenized" phenotype in the reproductive tract [3]. Support for the idea that DES acts as an initiator of transformation in the genital tract comes from

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Abbreviations: DES, diethylstilbestrol; EGF, epidermal growth factor; TGFα, transforming growth factor-α; SIN, salpingitis isthmica nodosa.

studies that showed that DES is metabolized to reactive intermediates in animals and can induce chromosomal abnormalities and neoplastic transformation in estrogen receptor–negative Syrian hamster embryo fibroblasts [4,5].

Substantial evidence points to a critical role for epidermal growth factor (EGF) and transforming growth factor-α (TGFα) and the EGF receptor pathway in the developing and adult reproductive tracts [3–14]. EGF and TGFα are members of a growing family of homologous growth factors that mediate their effects by binding to and activating the tyrosine kinase intrinsic to the EGF receptor. Data that support a role for these peptides in estrogen-induced uterine growth demonstrate that the expression of EGF and TGFα and their receptor are enhanced by estrogen, that EGF is a potent mitogen for uterine epithelial cells both in culture and in vivo, and that estrogen-induced mitogenesis can be inhibited in vivo by TGFα- and EGF-specific antibodies. Furthermore, many of the cell types in the uterus contain high-affinity binding sites specific for EGF [8,10]. The fact that EGF receptor protein is detectable in the genital tract as early as day 13 in fetal life suggests that most uterine cell types are potential targets of EGF/TGFα action from very early development throughout the life of the animal [10]. Like the adult uterus, the neonatal uterus is also quite responsive to EGF in that a significant growth response can be elicited by treatment with exogenous EGF [3,11,12]. In vitro studies have clearly shown that EGF and TGFα can elicit transcriptional activation of an estrogen-responsive element in the absence of estrogen [13]. Although not as thoroughly studied, estrogen regulation of the growth of the vaginal epithelium appears to involve many of the autocrine/paracrine growth factors found in the uterus, including both EGF and TGFα [3,6,7]. Collectively, these data suggest the existence of an integral relationship between the EGF family and estrogen action.

These observations may be crucial to understanding the developmental toxicity of estrogens in that the abnormal, inappropriate induction of estrogen-regulated genes such as *EGF* and *TGFα* may lead to permanent disruptions in growth and differentiation. Dysregulation of autocrine/paracrine growth pathways due to either increased expression of growth factors or increased synthesis or mutation of their receptors is a consistent alteration observed in many neoplastic cells, including those in reproductive tissues [3].

Recent studies of transgenic mice expressing growth-factor genes have shown direct links between the overexpression or inappropriate expression of a single growth factor and the development of lesions marked by abnormal proliferation and differentiation and neoplasia [15–26]. In various *TGFα* transgenic mouse strains, preneoplastic and neoplastic changes have been demonstrated in mammary gland, salivary gland, stomach mucosa, coagulating gland, liver, and pancreas. Studies of hepatocarcinogenesis have shown that TGFα can facilitate both initiation and promotion in male mice as well as enhance malignant conversion associated with tumor progression [19,21]. Most transgenic mouse studies have revealed that the expression of a growth factor transgene alone does not result in neoplastic lesions unless there are very long latency periods. For example, TGFα overexpression must be combined with wounding, phorbol-ester tumor promotion, or treatment with an initiator to elicit papilloma formation in mouse skin [15,20]. Similarly, the development of mammary-gland neoplasia in *TGFα* transgenic female mice requires sexual maturity and pregnancy [16,21–25], indicating that a growth-promoting, physiological hormonal environment is needed to synergize with TGFα before transformation can occur and that additional events are necessary before cancer can arise. A distinguishing characteristic of mammary tumorigenesis in *TGFα* transgenic mice is the development of hyperplastic alveolar nodules, which is proposed to be due to a TGFα-mediated inhibition of complete terminal differentiation of the secretory epithelium, an increase in the DNA-labeling index, and escape from normal postlactational involution or apoptosis [22,23]. These nodules generate an expanding secretory epithelium population that is proposed to be more susceptible to acquisition of additional transforming mutations.

In addition to having neoplastic lesions, MT42 *TGFα* transgenic animals exhibit changes that reflect disruption of the mechanisms that regulate tissue homeostasis, including effects on cell proliferation, cell differentiation, and senescence [15–26]. A role for TGFα in cell differentiation is supported by the finding that pancreatic acinar cells undergo redifferentiation to simple ductal cells, mucin-producing cells, and occasionally to insulin-producing cells in transgenic animals [17,18]. Disorganization of the gastric mucosa with pathological accumulation of surface mucous cells and reduction in the number of differentiated parietal and chief cells is another characteristic of *TGFα* mice. These results have led to the proposal that chronic overexpression of TGFα disrupts the dynamic balance between growth and differentiation in some organs.

A recent study of MT42 *TGFα* transgenic mice also provided evidence that TGFα acts on multiple components with the hypothalamic-pituitary-ovarian axis to affect reproductive function [26]. Although TGFα was found to facilitate hypothalamic release of luteinizing hormone–releasing hormone, TGFα reduced the ovarian responsiveness to gonadotropins, which influenced the progression of sexual development in the transgenic animals.

Because of the evidence that TGFα may influence reproductive function and that sex steroid hormones regulate the expression of TGFα expression, we used

MT42 *TGFα* transgenic mice to investigate the influence of constitutive *TGFα* expression on DES-induced alterations in the mouse uterus, vagina, and oviduct. These transgenic mice have been shown to express the human *TGFα* transgene in a wide variety of tissues, including the liver, lung, kidney, stomach, brain, mammary gland, salivary gland, and, most importantly for our study, the reproductive tract [21]. In comparison with the nontransgenic animals, the presence of the *TGFα* transgene was found to promote certain reproductive organ–specific pathological abnormalities. Significant increases in the incidence of DES-induced vaginal adenosis, uterine polyps, hyperplasia, and benign ovarian cysts were observed, but no increase in the incidence of neoplasia occurred. The increase in proliferative and differentiation lesions in the transgenic animals provides further support for an important role for TGFα in reproductive physiology.

MATERIALS AND METHODS

Animal Treatment and Tissue Preparation

This study was designed to determine whether *TGFα* expression could modulate DES-induced carcinogenesis of the reproductive tract. The animals used were homozygous *TGFα* transgenic female mice from the MT42 line generated as described previously [21] and the parental nontransgenic CD-1 outbred mouse strain. The transgene consists of a 917-bp human *TGFα* cDNA cloned into an expression vector (pEV142) with a zinc-inducible mouse metallothionein-1 promoter and a DNA fragment containing the human growth hormone polyadenylation signal. The MT42 transgenic mouse line contains the metallothionein–human *TGFα* transgene stably integrated at a single site with two copies per haploid genome. Only homozygous animals were used in this study, and zinc was not needed for induction, because the *TGFα* transgene is constitutively expressed in reproductive tissues of these mice.

The study duration was 52 wk, and some mice were killed at 39 wk. However, other mice were killed when they became moribund. There was no difference between the mice killed at 39 and 52 wk, and so the data were pooled for presentation. The mice were given NIH31 rodent chow (Ziegler Brothers, Gardeners, PA) and water ad libitum. The experimental animal protocol that was followed was in accordance with the National Institutes of Health approved procedures.

At death, standard dissection methods were used to remove the reproductive tract from each animal in one unit that included the ovary, oviduct, uterus, cervix, and vagina. Wolffian duct remnants were often observed in association with the ovary or embedded in the stroma adjacent to the cervix and vagina. The reproductive tracts were fixed in Bouins' fixative for 24–48 h, washed in water for at least 24 h, and stored in 70% ethanol until embedded in paraffin. Serial sections (5–7 μm) of the embedded tissues were stained with hematoxylin and eosin and evaluated for histopathological alterations.

Unless designated, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO). The data were analyzed by chi square and considered significantly different at $P < 0.05$ with one degree of freedom.

There were four experimental groups of the parental CD-1 and TGFα transgenic mice treated neonatally (days 1–5 of age) with either DES (2 μg/pup) dissolved in sesame oil or with the vehicle alone by using established protocols shown to result in numerous reproductive-tract abnormalities [2,3,12]. The experimental groups and treatment regimens are outlined below:

Group #1: CD-1 mice, vehicle control, received daily subcutaneous sesame seed–oil injections on days 1–5 of age.

Group #2: TGFα transgenic mice, vehicle control, treated as described for Group #1.

Group #3: CD-1 mice, neonatal DES treatment, received daily DES treatment by subcutaneous injections from days 1–5 of age.

Group #4: TGFα transgenic mice, neonatal DES treatment, treated as described for Group #3.

RESULTS

Effects of Neonatal DES Exposure on the Uteri of *TGFα* Transgenic and CD-1 Mice

The most distinctive histological changes observed in the uteri of CD-1 and *TGFα* transgenic mice in the various treatment groups are summarized in Table 1. It is noteworthy that clear differences in uterine histology were seen between the vehicle-control CD-1 mice (Group #1) and the vehicle-control *TGFα* transgenic animals (Group #2). The uteri of the transgenics exhibited a number of abnormalities, the most significant being endometrial hyperplasia (in five of 25 mice) ranging from microcytic to atypical, uterine polyps (in two mice of 25 mice), and an unusual increase in the number of glands (in four of 12 mice; data not shown). Although not quantified, the morphology of the uterine epithelium of the *TGFα* transgenics consistently resembled that of estrogen-treated epithelium in its epithelial hypertrophy, increased luminal epithelial cell height, and increased number of epithelial cells per unit of basement membrane. Examples of atypical endometrial hyperplasia exhibited by *TGFα* transgenic mice (middle and lower panels) and compared with the uterine epithelium (upper panel) of a nontransgenic CD-1 animal are shown in Figure 1. The hyperplastic uterine lesions of the *TGFα* mice were characterized by papillary projections and piling-up of cells with hyperchromatic nuclei into the lumen and by numerous mitotic figures.

An unusual rare lesion found in the TGFα transgenic

Table 1. The Most Significant Uterine Histological Changes*

Group #	Treatment	No. of mice with lesions/total no. (%)		
		Polyps	Hyperplasia	Adenocarcinoma
#1 CD-1	Vehicle	0/26 (0%)	2/26 (8%)	0/26 (0%)
#2 *TGFα*	Vehicle	2/25* (8%)	5/25* (20%)	0/25 (0%)
#3 CD-1	DES	0/16 (0%)	6/16 (38%)	7/16 (44%)
#4 *TGFα*	DES	0/15 (0%)	10/15* (67%)	7/15 (47%)

*Endometrial hyperplasia ranging from microcytic to cystic with and without atypia was observed in these animals. For tabulation purposes, the values obtained for the various histological types of endometrial hyperplasia have been combined and classified as "Hyperplasia." The asterisk indicates that the *TGFα* animals exhibit significantly more lesions than the parental CD-1 mice did, as determined by chi square analysis.

animals was uterine polyps (in two of 25 mice) (Table 1), although the incidence was low and of borderline significance. It is noteworthy that treatment with DES blocked the development of polyps. Photomicrographs of uterine polyps induced in *TGFα* transgenics are shown in Figure 2A and B. The polyp shown in Figure 2A contained dilated vessels filled with blood known as angioecstasis. This condition was found often in the uteri of transgenic animals. The uterus containing this polyp also had cystic endometrial hyperplasia, as seen by the enlarged gland lined by flattened epithelium situated beside the base of the polyp. Another polyp containing cystic glands (Figure 2B) was arrowhead shaped and protruded from the uterus through the cervical canal into the vaginal lumen. Vaginal, cervical, and uterine epithelium are indicated in the micrographs by abbreviations and arrows.

After exposure to DES for the first 5 d of life, the uteri of both the CD-1 mice (Group #3) and the *TGFα* transgenic mice (Group #4) displayed classical, DES-induced lesions, including an increased amount of connective tissue designated as sclerotic stroma, disorganized muscle layers (data not shown), endometrial hyperplasia ranging from microcytic to cystic with and without atypia, adenocarcinoma, squamous metaplasia of the epithelium, and fewer glands (data not shown). Neonatal DES treatment of *TGFα* transgenic mice elicited a significantly greater combined incidence of cystic endometrial hyperplasia (in 10 of 15 mice; 67%) than did the same treatment of the CD-1 parental animals (in six of 16 mice; 38%), further indicating that the *TGFα* transgenic mice had a propensity for developing endometrial hyperplasia (Table 1). In contrast to the differences in the occurrence of endometrial hyperplasia, the incidence of the uterine adenocarcinomas was found to be similar in the DES-treated CD-1 mice (in seven of 16 mice; 44%) and the *TGFα* transgenic mice (in seven of 15 mice; 47%). Representative micrographs of histological sections showing two uterine adenocarcinomas induced in *TGFα* transgenic mice by neonatal DES exposure are shown in Figure 3C–F.

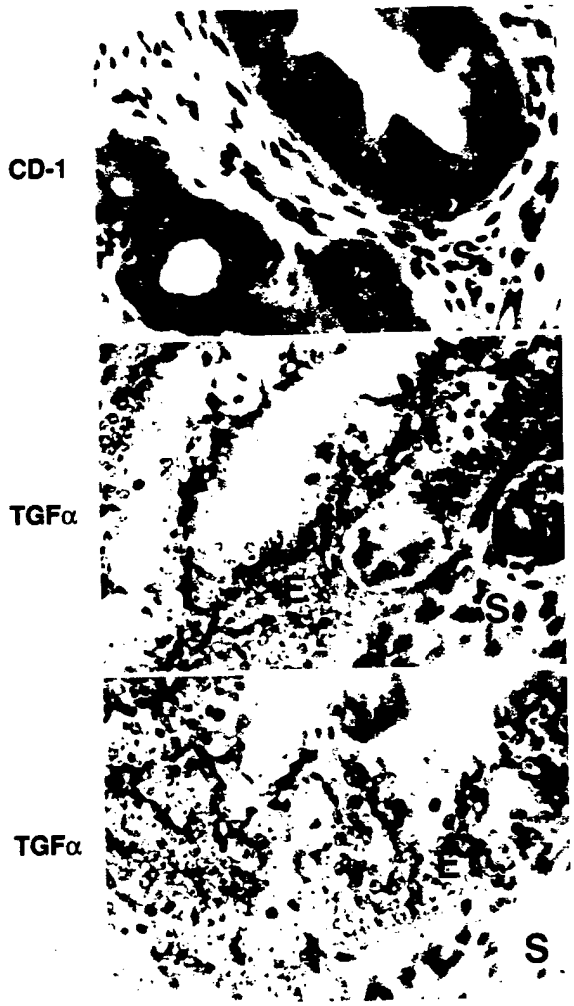
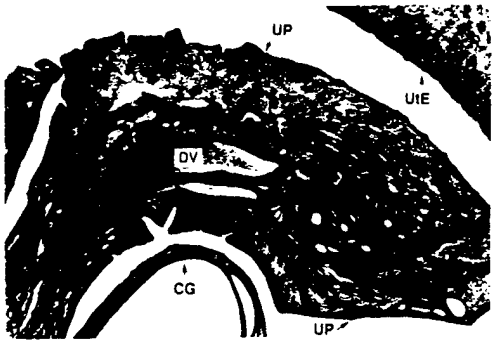
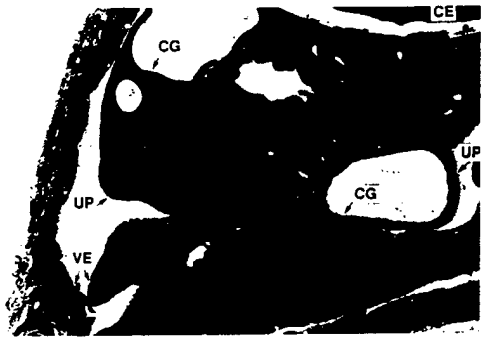


Figure 1. Examples of atypical endometrial hyperplasia exhibited by vehicle-treated *TGFα* transgenic mice (middle and lower panels) compared with the uterine epithelium (upper panel) of a nontransgenic CD-1 animal. The atypical hyperplastic epithelium of the uteri of *TGFα* mice is characterized by papillary projections and piling-up of cells with hyperchromatic nuclei in the lumen and by the presence of numerous mitotic figures. Magnification, 100×. S, stroma; E, epithelial cells.

A. UTERINE POLYP



B. UTERINE POLYP



C. ABNORMAL DIFFERENTIATION OF THE VAGINAL EPITHELIUM

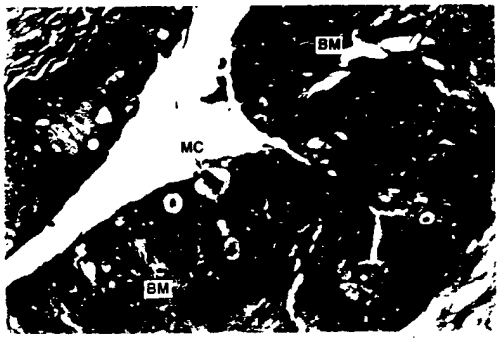


Figure 2. Photomicrographs of uterine polyps (A and B) and of abnormal vaginal epithelium (C) observed in *TGFα* transgenic mice. (A) A large uterine polyp (UP) extends into the lumen of the uterus from one side coming close to the uterine epithelium (UtE) of the opposite side. The polyp contains dilated vessels (DV) filled with blood and known as angioecstasis that were often in the uteri of transgenic animals. This uterus also has cystic endometrial hyperplasia, as indicated by the enlarged gland (CG) lined by flattened epithelium below the base of the polyp. Magnification, 25×. (B) An arrowhead-shaped uterine polyp (UP) protruding from the uterus through the cervical canal into the vaginal lumen. The polyp contains cystic endometrial glands (CG). Vaginal epithelium (VE) and cervical epithelium (CE) are also denoted. Magnification, 25×. (C) An example of abnormal differentiation of the vaginal epithelium often observed in the *TGFα* transgenics. This epithelium shows an unusual, rather disorganized mixture of mucous-cell differentiation (clear foamy cells) and squamous differentiation with distinct microcyst formation (MC). There is crowding of the basal cells, some nuclear pleomorphism, and irregularities in the basement membrane (BM). Magnification, 50×.

Alterations in Vaginal Histology After Treatment of *TGFα* Transgenic and CD-1 Mice With DES

A summary of the vaginal abnormalities observed in the CD-1 parental and transgenic animals after neonatal DES treatment is given in Table 2. A notable difference between these mice is that the vaginal epithelium of *TGFα* transgenics often exhibited abnormalities in differentiation and resembled a confused mixture of squamous and mucous cells arranged in a disorganized manner that was quite uncharacteristic of normal cycling vaginal epithelium. An example of the abnormal differentiation of the vaginal epithelium often observed in the vehicle-treated *TGFα* transgenic mice is shown in Figure 2C. This epithelium has an unusual, rather disorganized assortment of cells, demonstrating mucous differentiation (clear foamy cells) and squamous differentiation with distinct microcyst formation. There is also crowding of the basal cells, some nuclear pleomorphism, and irregularities in the basement membrane.

Another striking abnormality exhibited by some *TGFα* transgenic animals was the retention of the Wolffian duct, also known as Gardner's duct. In our study, Wolffian-duct remnants, which normally regress during development in females, were found only in the *TGFα* transgenic mice with or without exposure to DES (in three of 25 mice in Group #2 and four of 15 in Group #4) and not in the CD-1 parental animals (26 in Group #1 and 16 in Group #3 examined) (Table 2). Interestingly, retention of the Wolffian duct is documented to be one of the pathological alterations induced by neonatal DES exposure of nontransgenic CD-1, albeit at a very low incidence. These data implicate TGFα in the abnormal occurrence of Wolffian ducts in the female reproductive tract.

Exposure of transgenic animals to DES induced many of the features commonly attributed to neonatal estrogen exposure, such as increased vaginal keratinization and squamous metaplasia of the endocervical canal; increased prevalence of Wolffian-duct remnants; marked hypertrophy of the cervix; cervical ectropion; abnormal urethral openings (hypospadia), often associated with the formation of vaginal concretions; and vaginal adenosis (Table 2).

Adenosis is the abnormal appearance of columnar and glandular epithelium within a region of the vagina normally lined with squamous epithelium. Vaginal adenosis was consistently observed after neonatal DES exposure in both the CD-1 mice and *TGFα* transgenics. In comparison with the parent CD-1 strain (Group #3), in Group #4 the presence of the *TGFα* transgene significantly potentiated the appearance of vaginal adenosis upon exposure to DES (in three of 16 Group #3 mice vs seven of 15 Group #4

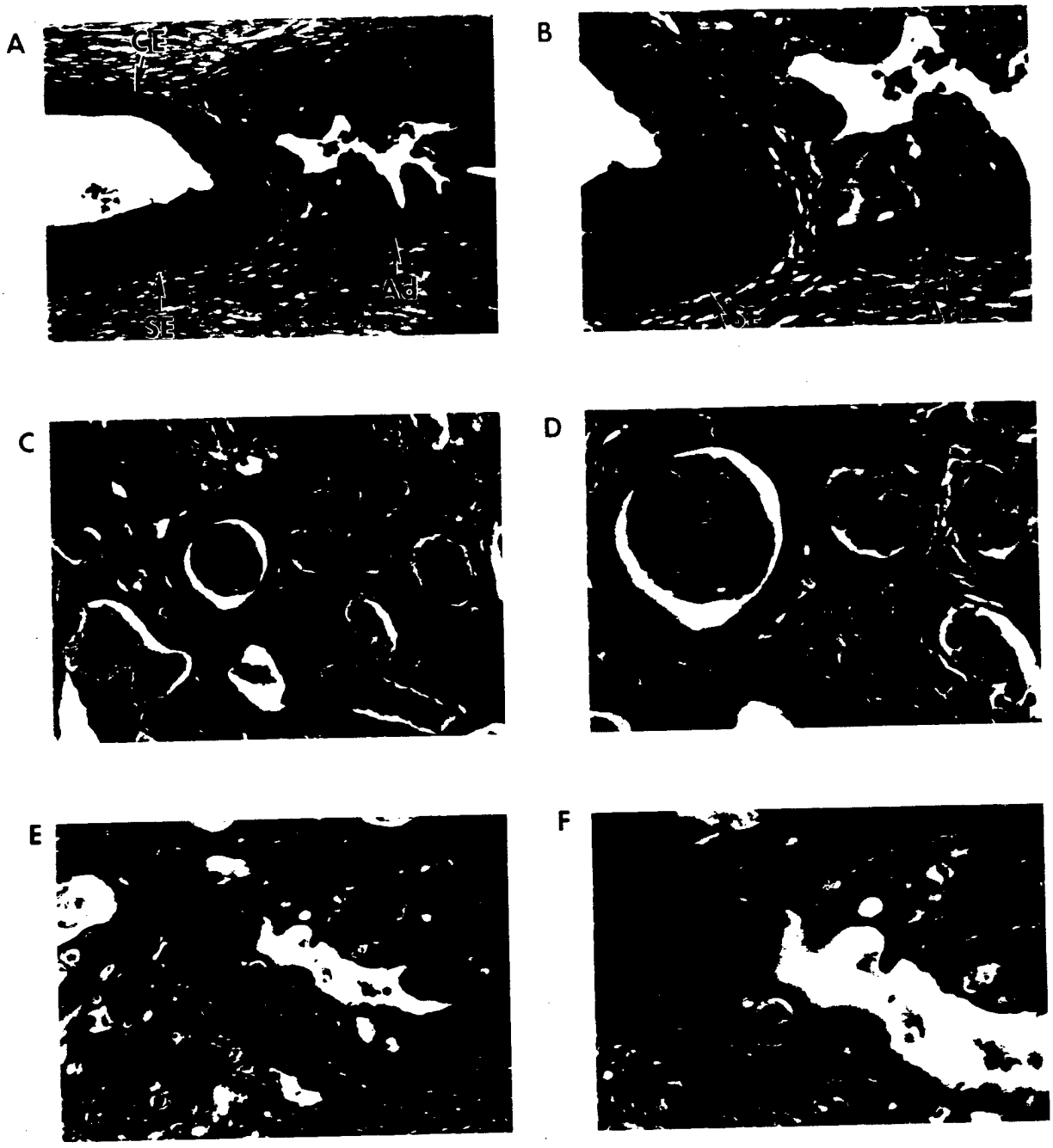


Figure 3. Representative low- and high-power micrographs of histological sections showing vaginal adenosis (A and B) and two uterine adenocarcinomas (C–F) induced in *TGFα* transgenic mice by neonatal DES exposure. (A, C, and E) Magnification, 50×; (B, D, and F) Magnification, 100×. (A and B) In this cross section of a vaginal fornix the arrows denote the region of adenosis (Ad) showing uterine-like gland formation with squamous metaplasia, the presence of cervical epithelium (CE) due to cervical ectropion and extension of the transition zone into the vagina, and the presence of typical vaginal squamous epithelium at the metestrus stage (SE) of the estrous cycle. (C and D) A typical uterine adenocarcinoma with squamous differentiation containing a mixed population of squamous and cuboidal cells arranged in a gland-like pattern that was often observed in mice treated neonatally with DES. (E and F) A uterine adenocarcinoma without squamous differentiation and with regions containing relatively well-defined glandular structures with secretory material interspersed with areas of invasive tumor cell nests with no lumens.

mice). Figure 3A and B presents a cross section of a vaginal fornix containing a region of adenosis in a *TGFα* mouse, showing uterine-like gland formation with squamous metaplasia, the presence of cervical epithelium known as cervical ectropion due to the abnormal extension of the transition zone into the vagina, and the presence of typical vaginal squamous epithelium at the metestrus stage of the estrous cycle.

Table 2. The Most Significant Vaginal Histological Changes*

Group #	Treatment	No. of mice with lesions/total no. (%)		
		Adenosis	Wolffian or Gardner's duct	Concretions (hypospadia)
#1 CD-1	Vehicle	0/26 (0%)	0/26 (0%)	0/26 (0%)
#2 *TGFα*	Vehicle	0/25 (0%)	3/25* (12%)	0/25 (0%)
#3 CD-1	DES	3/16 (19%)	0/16 (0%)	0/16 (0%)
#4 *TGFα*	DES	7/15* (47%)	4/15* (27%)	4/15* (27%)

*The asterisk indicates that the *TGFα* animals exhibit significantly more lesions than the parental CD-1 mice did, as determined by chi square analysis.

Besides adenosis, *TGFα* mice also exhibited enhanced sensitivity to DES exposure for the development of other lesions. DES treatment resulted in a significantly greater number of *TGFα* animals (four of 15 in Group #4) having vaginal concretions, which are thought to be caused by abnormal urethral development. No concretions were found in the DES-treated CD-1, but this lesion has been reported to occur in CD-1 mice at a very low incidence as a result of DES exposure. This is the first evidence that inappropriate expression of TGFα interferes with normal urethral development.

Abnormalities of the Oviduct and Ovary

Exposure to neonatal estrogen induces histological alterations in the oviduct associated with hyperplasia, and aberrant differentiation and gland formation (diverticuli) of the oviductal mucosa resembling the human clinical lesion termed salpingitis isthmica nodosa (SIN). Examples of SIN-like oviductal lesions found in two neonatally estrogenized *TGFα* transgenic mice are shown in Figure 4. Both oviducts were characterized by epithelial hyperplasia with mucosal folds and gland-like structures extending into the muscularis, which disrupted the normal architecture of the oviduct. Furthermore, one of the oviducts (Figure 4D–F) exhibited abnormal mucinous differentiation resembling cervix, which demonstrated the ability of the oviduct to differentiate along an alternative pathway characteristic of Müllerian-duct epithelium. Unlike in the uterus and vagina, the *TGFα* transgene did not significantly increase the incidence of SIN-like lesions induced by DES (in five of 10 mice) over that observed in the parental CD-1 mice (in four of 11 mice) (Table 3).

Neonatal DES-treatment of *TGFα* transgenic mice

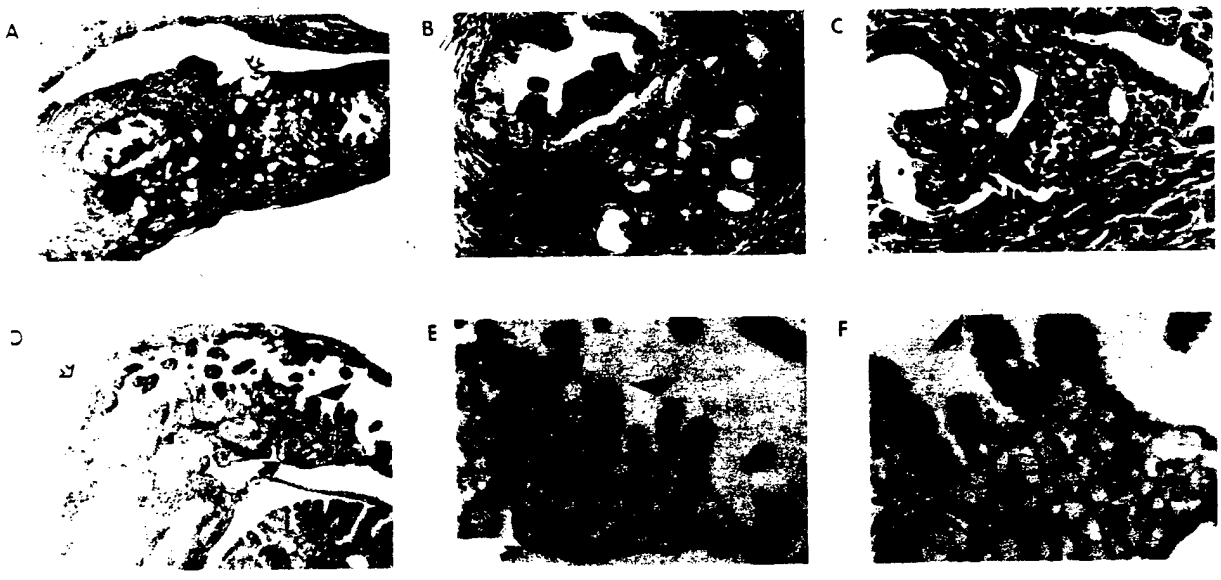


Figure 4. Representative photomicrographs showing examples of oviductal abnormalities observed in two *TGFα* transgenic animals after exposure to DES. Panels A–C and D–F show increasing magnifications of two different oviducts, Panels A and D, 25×; panels B and E, 50×; and panels C and F, 100×. Both oviducts are characterized by epithelial hyperplasia with mucosal folds and gland-like structures extending into the muscularis (A and D, open arrows), which resembles the clinically described lesion SIN in women. Furthermore, one of the oviducts (D–F) exhibits abnormal mucinous differentiation resembling cervix (denoted by the clear cytoplasm), which demonstrates the ability of the oviduct to differentiate along an alternative pathway characteristic of the Müllerian duct epithelium. The dark and open arrows in panel A denote regions that are magnified further in panels B (50×) and C (100×), respectively. The regions indicated by the solid arrow and arrowhead in panel D are magnified further in panels E (50×) and F (100×).

Table 3. Significant Lesions of the Oviduct and the Ovary

Group #	No. of mice with lesions/total no. (%) Oviduct SIN-like lesions	Benign ovarian cysts
#1 CD-1	0/7 (0%)	4/26 (15%)
#2 *TGFα*	0/16 (0%)	12/25* (48%)
#3 CD-1	4/11 (36%)	5/16 (31%)
#4 *TGFα*	5/10 (50%)	12/15* (80%)

*The asterisk indicates that the *TGFα* animals exhibit significantly more lesions than the parental CD-1 mice did, as determined by chi square analysis.

also induced many of the ovarian lesions previously reported in wild-type mouse strains, including inflammation, more interstitial tissue; fewer follicles, oocytes, and corpora lutea; and more ovarian cysts derived from the mesonephros rather than ovarian surface epithelium. The most notable effect of the *TGFα* transgene on the ovary was that the transgenic mice (Groups #2 and #4) had significantly more benign ovarian cysts (in 12 of 25 and 12 of 15 mice, respectively) than were found in the parental CD-1 mice (in four of 26 Group #1 mice and five of 16 Group #3 mice) with or without DES treatment (Table 3).

DISCUSSION

Neonatal estrogen exposure causes numerous reproductive-tract abnormalities such as squamous metaplasia and hyperplasia of the uterine epithelium, persistent cornification and hyperplasia of the stratified epithelium of the cervix and vagina, formation of vaginal adenosis, malformation of the urethral opening (hypospadia), altered steroid receptor levels, and induction of uterine and vaginal adenocarcinoma, but the mechanism responsible for these diverse effects is not yet known [1–5,12]. A considerable amount of data suggests that steroid hormones regulate the local production of a variety of multifunctional growth regulatory peptide factors, and of these, EGF and TGFα appear to be intimately associated with estrogen action [3,6–11,13,14,27]. In fact, neonatal DES treatment has been shown to deregulate synthesis of EGF in the reproductive tract [3]. Based on the documented role of the EGF family in reproductive tract physiology, we used the MT42 *TGFα* transgenic mouse as a model for further evaluating the effects of one member of this family, TGFα, as a mediator of the pathological changes induced by neonatal estrogen exposure. Specifically, we wanted to investigate the effects of constitutive expression of this growth factor in the reproductive tract, and to determine whether TGFα would potentiate DES-induction of abnormalities of growth and differentiation including various preneoplastic and neoplastic lesions. We show here that deregulation of TGFα expression did enhance the incidence of both basal and DES-mediated hyperplastic and differentiation changes in the reproductive tract including uterine hyperplasia, vaginal adenosis, uterine polyps, ovarian cysts, hypospadia, and retention of the Wolffian ducts. However, the incidences of neoplasia and oviduct SIN-like lesions were not increased by the *TGFα* transgene.

Our data clearly document that there is a relationship between the development of endometrial hyperplasia in the mouse uterus and aberrant expression of TGFα. This is of interest because of the association between unopposed estrogen and the development of uterine hyperplasia and carcinoma in women [28–30]. In fact, aberrant TGFα has been proposed to be involved in the development of uterine adenocarcinomas in women [29]. Although our results support a role for TGFα as a mediator of events that accompany the development of hyperplastic endometrial lesions, that neoplasia was not promoted in the mouse leaves open the question of whether deregulation of TGFα is sufficient for the development of endometrial cancer. It is possible that hyperplastic endometrial lesions may not be precursors of uterine adenocarcinomas induced by DES. The role of estrogen in mediating the transition between excessive growth and neoplastic transformation of the endometrium is still unclear. In women, endometrial cancer is proposed to include not only estrogen-dependent endometrial tumors that progress through distinct precursor stages but also cancers that arise independently of estrogen without evidence of intermediate hyperplastic preneoplastic stages. Consequently, the etiology of these tumors may be quite different, and they may not involve persistent induction of peptide growth factors that stimulate hyperplasia. In our study, the lack of an association between endometrial hyperplasia and neoplasia suggests that DES-induced carcinogenesis involves more than just hyperplasia and overexpression of growth factors.

It is also noteworthy that the *TGFα* transgene in CD-1 mice appeared to be instrumental in the induction of a rare lesion in mice, uterine polyps, albeit at a low incidence. That one of the common side-effects of tamoxifen treatment of women with breast cancer is the development of uterine polyps leads us to propose that upregulation of TGFα may be involved in the etiology of these lesions [30]. Surprisingly, DES treatment suppressed the development of uterine polyps in the *TGFα* transgenic animals by an unknown mechanism.

Interestingly, DES- and vehicle-treated *TGFα* transgenic animals exhibited a significantly greater incidence of benign ovarian cysts than the corresponding nontransgenic mice did. This finding, along with the published observations that *TGFα* plays a role in ovarian function, that unopposed estrogen in humans is associated with follicular polycystic degeneration of the ovary, and that TGFα is regulated by estrogen in many hormonally sensitive

tissues, suggests that dysregulation of TGFα may be one of the etiologic factors involved in polycystic ovarian disease [26,31].

Alterations in the vaginal squamous epithelium was another characteristic feature of the *TGFα* transgenic animals. The most important of these abnormalities was the significant potentiation of DES-induced vaginal adenosis, which has been proposed to be a precursor lesion for the generation of vaginal adenocarcinoma in women [1]. In light of the well-documented role of TGFα as a major regulator of epidermal keratinocyte growth and differentiation, it is not surprising that we found that the mesodermal-derived squamous epithelium of the vagina underwent alterations after constitutive TGFα expression in the transgenic animals [15,20]. Furthermore, that the *TGFα* transgenics had more vaginal concretions due to malformation of the urethra and retention of Wolffian ducts also indicates that TGFα plays an important role in the regulation of developmental and morphogenic events not only of the Müllerian duct but also of the urogenital sinus in the female animal.

We can only speculate about the mechanism by which TGFα potentiates basal and DES-induced nonneoplastic lesions of the reproductive tract. Our findings in the reproductive tract support the results of other studies that showed that TGFα overexpression apparently can alter growth and differentiation of glandular and squamous epithelia in several different organs, often redirecting epithelia along alternative differentiation pathways. For example, TGFα disrupts the normal program of cellular differentiation in the gastric mucosa by interfering with the terminal differentiation of parietal and chief cells while promoting the growth of surface mucous cell progenitors [17,18]. Similar effects of TGFα in the uterus, vagina, and urogenital sinus may have contributed to the development of the abnormalities found in our study.

Overexpression of TGFα has been linked to neoplastic transformation of multiple tissue types, including the gastrointestinal tract, pancreas, skin, mammary gland, reproductive tissues, kidney, lung, and brain. Transgenic studies have clearly demonstrated that the carcinogenic, co-carcinogenic, and promoting effects of TGFα vary depending on the organ, carcinogen, promoter, and hormonal environment [15–26]. For example, the most impressive characteristic of mammary carcinogenesis in *TGFα* transgenic mice is that tumor development is clearly dependent on exposure of the mammary gland to multiple pregnancies involving repeated cycles of lobular-alveolar growth, differentiation, lactation, and involution [16,22–25]. Inappropriate TGFα expression appears to potentiate the development of preneoplastic hyperplastic alveolar nodules not only by increasing the proliferative index but also by enhancing survival during postlactationally induced apoptosis [22,23]. The ability of TGFα to inhibit complete terminal differentiation of the secretory epithelium of the mammary gland is thought to be one mechanism by which TGFα blocks normal involution and promotes the persistence of glandular structures. This TGFα-mediated expansion of hyperplastic nodules of secretory mammary epithelial cells is proposed to generate a population of cells more susceptible to acquiring additional transforming mutations that lead to malignant transformation.

Carcinogenesis of the reproductive tract has several features in common with mammary-gland tumorigenesis in that genital-tract neoplasia is clearly a multistage process involving pathologically distinct intermediate preneoplastic stages consisting of hyperproliferative premalignant lesions. Also, long-term exposure to endogenous hormones is instrumental in DES oncogenesis in the reproductive tract. Thus, we speculate that inappropriate expression of TGFα may contribute to the development of pathological lesions in the reproductive tract as it does in the mammary gland, by conferring a selective growth advantage and enabling cells to escape apoptosis. However, it is clear that the role TGFα plays in hormonal carcinogenesis of the mammary gland is different from that played in the uterus and vagina. Unlike in the mammary gland, TGFα did not significantly increase the development of uterine carcinomas in the transgenic animals compared with the parental mice during the investigated time-frame of 12 mo, even though the incidence of various proliferative and differentiation lesions increased. We therefore ask why the increase in endometrial hyperplasia seen in the transgenics does not translate into increased tumorigenesis. It is likely that DES induces specific genetic changes that contribute to neoplastic conversion of uterine cells that cannot be mimicked by TGFα overexpression and that prolonged exposure to TGFα does not amplify this population of cells at risk for neoplastic transformation.

That a general effect of the TGFα transgene on the hypothalamic-pituitary-ovarian axis contributes to the development of uterine and vaginal lesions by altering the endocrine environment of the animal cannot be ruled out [26,28–33]. In fact, a recent study by Ma et al. [26] clearly demonstrated that overexpression of the *TGFα* transgene disrupts hypothalamic and ovarian function and that chronic TGFα expression is deleterious to female reproduction. Although perturbations in the secretion of endocrine hormones may contribute to changes in reproductive capacity, Ma and colleagues also found inherent defects in ovarian function independent of altered hypothalamic-pituitary function by transplantation of transgenic ovaries into nontransgenic hosts. Furthermore, we have also found a high incidence of pituitary adenomas in female *TGFα* transgenics, which not only implicates TGFα in growth regulation of the pituitary but also suggests

that pituitary hormone production may become progressively disrupted as the adenomas develop [33]. That *TGFα* transgenics develop pituitary tumors is not surprising, because estrogen regulates TGFα expression in the normal pituitary and also will, if unopposed, produce pituitary adenomas [31]. More in-depth studies are clearly required to determine the exact roles of TGFα-mediated alterations in the hypothalamic-pituitary-ovarian axis versus direct organ-specific effects in the induction of reproductive-tract lesions in *TGFα* transgenic mice.

In this report, we further elucidated the role of TGFα in the evolution of preneoplastic and neoplastic lesions in the reproductive tract. Previous studies suggested that one mechanism by which neonatal estrogen treatment elicits teratogenic and carcinogenic effects is epigenetic and involves modulation of the expression of growth and differentiation regulatory genes such as EGF, whose inappropriate, persistent expression may lead to the development of epithelial abnormalities of the genital tract. That the presence of the *TGFα* transgene potentiated the basal as well as the DES-induced nonneoplastic lesions further supports the hypothesis that deregulation of peptide growth factors is involved in the pathogenesis of reproductive-tract diseases. Although it is well documented that estrogens upregulate the expression of various EGF family ligands, the constitutive overexpression of TGFα in the reproductive tracts of transgenic animals may enhance the growth factor environment and thus increase proliferation over that induced by DES alone in nontransgenic mice. Alternatively, the inappropriate expression of TGFα may create specific cellular autocrine growth-factor loops within the uterus and vagina that did not exist in these tissues before, resulting in dysregulation of growth. However, constitutive expression of TGFα did not translate into more uterine or vaginal tumorigenesis over the time course studied. It is clear that overexpression of the *TGFα* transgene cannot substitute fully for DES as a reproductive-tract carcinogen or act as a promoter of DES-induced neoplasia. These data suggest that DES carcinogenesis involves more than aberrant expression of this single peptide growth-factor. Furthermore, the results of our investigation emphasize the value and importance of transgenic animals in studies of hormonal teratogenic and carcinogenic effects, and the interactions between estrogens and peptide growth factors.

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Cripto-1 Activity in the Intact and Ovariectomized Virgin Mouse Mammary Gland

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In the virgin mouse mammary gland, proliferation and differentiation of secretory epithelium are necessary for the establishment of the architectural design of the mammary tree. The initiation of this growth phase occurs between weeks 3 and 13 postnatally, and is coincident with the expression of the epidermal growth factor (EGF)-like peptide cripto-1 (CR-1). CR-1 has been shown to be localized in the putative multipotent stem cell/cap cell region of the growing Terminal End Bud (TEB). To ascertain the biological effects of the CR-1 protein on mammary gland development, a CR-1 peptide that corresponds to the EGF-like domain of this protein was incorporated into Elvax slow-release implants and locally inserted into the #4 inguinal mammary glands of 5 week-old ovariectomized C57 BL6 mice. The results demonstrate that implantation of the CR-1 pellet promoted a new network of branching ducts, all of which were actively migrating towards the pellet. In support of these findings, we also investigated the morphogenic effects of the CR-1 gene during the development of the mammary tree by transducing primary mouse mammary epithelial cells *in vitro* with an CR-1 expression vector and reintroducing these cells into cleared mammary fat pads of virgin mice. Here we demonstrate that the overexpression of the CR-1 gene resulted in the appearance of atypical mammary outgrowths. Mammary aberrations included increased lateral branching, loss in ductal spacing and hyperplastic-like terminal ducts. These data, in combination with previous results on endogenous CR-1 localization, suggest that the CR-1 gene may be involved in initiating the early stages of hyperplasia by effectively modulating the behavior of ductal progenitor cells.

Keywords: Cripto, ductal morphogenesis, mammary gland hyperplasia

INTRODUCTION

The mammary gland is a unique organ that postnatally undergoes periodic cycles of growth, morphogenesis and differentiation, followed by involution (Williams and Daniel, 1983; Robinson *et al.*, 1996; Smith *et al.*, 1995, Daniel and Silberstein, 1987). In the developing or pregnant mouse mammary gland, rapid and extensive proliferation of the mammary ductal epithelium is systematically under the influence of hormones (Topper and Freeman, 1980; Nandi, 1958). The initial hormonal response of pubescent mammary epithelium to these stimuli leads to the growth of a network of multi-directional branching ducts. At the tip of each of these ducts is a large bulbous-like structure, the Terminal End Bud (TEB), which contains two potential mammary epithelial progenitor cell

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types, each derived from the commitment of the multipotent stem cell/cap cell:secretory progenitors and ductal progenitors (Williams and Daniel, 1983; Smith, 1996). These end buds terminate either when they encounter other lateral or terminal ducts or upon reaching the limits of the mammary fat pad.

During the influence of pregnancy, lactogenic hormones such as prolactin, insulin, glucocorticoids, and progesterone induce secretory progenitors to undergo a process of further maturation into milk-synthesizing alveoli. These alveoli then elaborate milk proteins such as β-casein and whey acidic protein (WAP) (Kordon *et al.*, 1995; Robinson *et al.*, 1996; Yang *et al.*, 1995). In contrast, ductal progenitors are involved in the architectural design of the mammary tree and participate in the process of sequestering and delivering milk to the nipple. In addition to systemic pituitary and ovarian hormones, there are a host of additional signals such as growth factors, that control the function of the mammary gland. The production and/or function of some of these factors are facilitated by estrogen regulation (Kenney and Dickson, 1996).

Growth factors that may contribute to the maturation and functional differentiation of the mammary gland include members of the epidermal growth factor (EGF) family [Transforming Growth Factor α (TGFα), Heregulin β1 (HRG), and Amphiregulin (AR)], Hepatocyte Growth Factor (HGF), Insulin-like Growth Factors (IGFs), Transforming Growth Factor betas (TGFβ1–3), and Mammary-Derived Growth Inhibitor (MDGI) (Daniel and Silberstein, 1987; Robinson *et al.*, 1991; Coleman *et al.*, 1990; Vonderhaar, 1987; Snedeker *et al.*, 1991; Yang *et al.*, 1994; Kennet *et al.*, 1995; 1996c; Spitzer *et al.*, 1995). EGF, HRGβ1, TGFα, HGF, and AR are important growth factors and cytokines that regulate ductal growth and branching in the virgin mouse and rat mammary gland, while MDGI is involved in modulating alveolar development during pregnancy.

Recently, Cripto-1 (CR-1) a new member of the EGF-like family of growth factors has been shown to be expressed in the mouse mammary gland (Kenney *et al.*, 1995). However, the role of CR-1 in mammary gland development, function and etiology of mammary tumorigenesis has not been fully elucidated. The mouse CR-1 gene encodes a 171 amino acid protein that exhibits 93% homology to human CR-1 protein (Ciccodicola *et al.*, 1989; Dono *et al.*, 1991). Both mouse and human CR-1 proteins possess a conserved EGF-like repeat that lacks an A-loop and contains a truncated B-loop (Dono *et al.*, 1991). The mouse CR-1 protein like human CR-1, is predicted to be a secreted growth factor based on its primary sequence. The CR-1 protein has been shown to function as a potent mitogen for nontransformed and transformed human and mouse mammary epithelial cells and does not bind to any members of the EGF receptor tyrosine kinase family, which includes EGFR, *erb* B-2, *erb* B-3, and *erb* B-4, nor to heterodimeric combinations of these receptors (Brandt *et al.*, 1994). We have previously demonstrated that the CR-1 gene gland is expressed in the virgin, pregnant and lactating C57/BL6 mouse mammary gland (Kenney *et al.*, 1995). In 4-week-old virgin mouse mammary ducts, CR-1 is immunohistochemically localized in the compartment of the end bud tip, specifically in the cap cells, which are the putative multipotent stem cells (Williams and Daniel, 1983; Medina and Smith, 1990; Kenney *et al.*, 1995). In addition, CR-1 is present in the luminal epithelial cells, myoepithelial cells, and lumen of the 12-week-old mammary duct, and its expression is enhanced in secretory epithelium following pregnancy and lactation (Kenney *et al.*, 1995). We have also recently reported that the CR-1 gene may be involved in mammary epithelial cell transformation and neoplasia. Overexpression of the CR-1 gene in the nontransformed mouse NOG-8 mammary epithelial cell line can lead to transformation *in vitro* (Ciardiello *et al.*, 1991). Additionally, elevated expression of CR-1 has been detected in mouse mammary tumors that arise in TGFα, *neu*, *int*-3, SV/40 large T and polyoma middle T transgenic mice (Kenney *et al.*, 1996b). Finally, a number of human breast cancel cell lines and nearly 80% of

primary human breast carcinomas over-express CR-1 mRNA and/or protein (reviewed in Kenney and Dickson, 1996). In light of these findings, there is no evidence to date of an effect of the CR-1 protein in vertebrate/organ morphogenesis. Thus far, preliminary reports suggest CR-1 transcripts are actively expressed in mouse embryo epiblast cells of the primitive streak during midgestation and FRL1, a murine related CR-1 growth factor, is expressed in Xenopus *laevis* throughout gastrulation and early neuralization (Kinoshita *et al.*, 1996). The FRL1 protein, which has 24% sequence homology to CR-1 and more importantly, 100% identity in the EGF-like domain, can also induce the formation of neural and mesoderm tissue via interaction with the FGF receptor-1 (Kinoshita *et al.*, 1996).

The goal of this study was to investigate the effects of the Cr-1 growth factor and its contribution to the growth and development of the mammary gland. To ascertain this, we used two approaches: 1) slow-release Elvax pellets that contained a biologically active CR-1 peptide which was implanted into the mammary gland of ovariectomized (OVX) virgin mice to determine mammogenic activity, and 2) the isolation of primary mouse mammary epithelial cells and infection of these cells with a replication-defective amphotropic retrovirus that contained the mouse CR-1 cDNA to observe the morphogenic effects of this gene following their reintroduction into a cleared mammary fat pad in virgin mice. The results obtained from both experimental approaches indicate that CR-1 can induce multi-directional ductal branching and/or facilitate the appearance of hyperplastic-like lesions in the mammary gland.

MATERIALS AND METHODS

Animals, Pellet Implant Preparation, Tissue Transplantation and Surgical Procedures

A total of 75 pathogen-free, 5-week-old OVX C57/BL6 mice (Charles River Labs) were utilized in the Elvax slow-release pellet experiment. The animals were housed an additional 2.5 months to clear the mice of systemic ovarian hormones. Before each experiment, a small L-shaped incision was made on the abdominal wall of the mouse exposing the 4th inguinal mammary gland (DeOme *et al.*, 1959; Daniel and Silberstein, 1987; Kenney *et al.*, 1996a,c). A small pocket within the mammary tissue was then made with small forceps and used for manipulations.

Ethylene acetate copolymer Elvax pellet was a gift of Dupont Chemical Co., Wilmington, DE. Biologically-active CR-1 peptide was synthesized as previously described (Brandt *et al.*, 1994). Elvax pellets were prepared by dispersing 100 µg of either EGF (Collaborative Research, Waltham, MA) or CR-1 peptide with 100 µg of BSA in 125 µl of Elvax that had been emulsified previously in 20% (w/v) dichloromethane. The mixture was quick frozen, dried and cut into 0.5 mg pieces (equal to 5 µg of growth factor per pellet) and surgically implanted into the #4 mammary gland (Kenney *et al.*, 1996c). Contralateral #4 mammary glands received control BSA pellets. For each experiment (repeated 3 times) a total of 10 ovariectomized mice contained CR-1 Elvax pellets, 10 OVX mice contained EGF pellets, and 5 ovariectomized mice contained BSA Elvax pellets.

In transplantation experiments that used retroviral CR-1 transduced mammary primary cultures, pNO4 (empty vector) transduced mammary primary cultures, and control nontransduced mammary primary cultures, a total of seventy-five 3-week-old virgin female C57/BL6 mice were utilized. In each experiment (repeated 3 times), a total of 10 mice received CR-1 transduced primary cells (1×10^6 cells per site), 10 mice received pNO4 (empty vector) transduced primary cells (1×10^6 cells per site), and 5 mice received control nontransduced primary cells (1×10^6 cells per site). All animals were previously cleared of the host's #4 mammary epithelium before transplantation.

In the second set of experiments that used CR-1 transgenic serially transplanted outgrowths into syngeneic hosts, a total of 10 mice were utilized.

Five hormonally intact 3-week-old female mice and five 3-week-old ovariectomized mice received a pooled epithelial portion (2×10^5 cells) of CR-1 hyperplastic outgrowths from 3 donors. In addition, three 3-week-old female mice received a pNO4 pooled epithelial portion (2×10^5 cells), and three 3-week-old ovariectomized mice received a pNO4 pooled epithelial portion (2×10^5 cells), respectively. All animals were previously cleared of the host's #4 mammary epithelium before transplantation. Outgrowths were allowed to grow within the host mammary gland for 4–12 weeks or 12 weeks in mice that were eventually mated and sacrificed after 5 or 28 days parturition. In all experiments, animals were supplied with food and water *ad libitum* and were housed under 12 hr light, 12 hr dark cycle. Upon surgery or completion of the experiment, animals were either anesthetized with metofane or euthanized with CO_2 and metofane.

Primary Mammary Epithelial Cell Cultures

Primary mouse mammary epithelial cell cultures were obtained and transplanted as previously described (Smith *et al.*, 1992; Kenney *et al.*, 1996c). Cells were grown in Improved Modified Eagle's Medium (IMEM) supplemented with 10% FBS, bovine insulin (10 μg/ml), transferrin (10 μg/ml), hydrocortisone (5 μg/ml), and EGF (10 ng/ml) at 37°C in 5% CO_2.

Retroviral Vectors

A 900-bp mouse CR-1 fragment that includes the EGF-like domain and the remaining coding sequence for CR-1 protein was derived from a full-length 4.8-Kb mouse CR-1 cDNA as previously described (Dono *et al.*, 1991). The fragment was introduced in the 5′ to 3′ orientation into the Moloney sarcoma virus (MSV-LTR)-derived pNO4 recombinant plasmid at the 3′ end of the CMV promoter (McGeady *et al.*, 1989; Smith *et al.*, 1992; Kenney *et al.*, 1996c). The pNO4 expression vector contains MSV-LTRs and an internal 650-bp kpn 1-Bgl II fragment of the CMV promoter that is 3′ to an SV40 promoter-driven neomycin (neo) gene conferring resistance to the antibiotic G418. Transfection of the 900-bp CR-1 sense plasmid was carried out into the PA-317 amphotropic packaging cell line, which produce amphotropic, replication-defective retrovirus stocks. Viral supernatants were obtained from these mass-transfected G418-resistant PA-317 cells and screened for helper virus and found to be negative (Markuntz *et al.*, 1988). Mouse primary mammary epithelial cells were infected with supernatants (1.5×10^5 neomycin-resistant colonies/ml) as previously described (Kenney *et al.*, 1993). Primary cultures were maintained in G418 (400 μg/ml) for 4 days and then transplanted into cleared mammary fat pads as previously described (Smith *et al.*, 1992; Kenney *et al.*, 1996c).

Histology, BrdU Labeling and Immunohistochemistry

Mammary glands were excised and fixed overnight in 4% phosphate-buffered formalin. Whole mount preparation of mammary glands were prepared by defatting the glands in acetone and hydrating through a graded series of ethanol as previously described (Smith *et al.*, 1992; Kenney *et al.*, 1996a). Whole mount preparations were stained with hematoxylin, dehydrated and mounted for photography or the glands were embedded in paraffin, sectioned horizontally for immunohistochemistry or stained with hematoxylin and eosin (H&E).

For BrdU labeling, mice were injected i.p. with BrdU (100 mg/kg) (Sigma Chemical Co., St. Louis, MO) 2 hr prior to sacrifice. Mammary tissue was then extracted, fixed in 4% paraformaldehyde, sectioned horizontally at 5 μm and reacted against an anti-BrdU antibody (1:1000) (Sigma Chemicals). A total of 20 random fields from each treatment group that surrounded the periphery of the Elvax pellets were used for BrdU scoring. All fields that were scored contained

more than 100 cells. The percentage of labeled cells (positive cells stained brown over purple negatively stained cells) was calculated by dividing the number of labeled nuclei by the total number of nuclei counted as previously described (Yang *et al.*, 1994; Kenney *et al.*, 1996c).

Western Blot Analysis

Transplanted mammary glands that contained transduced CR-1, pNO4, or nontransduced (control) outgrowths were homogenized in 1 ml hypotonic buffer containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM PMSF, 1% NP-40, and 20 μg/ml aprotinin. Samples (50 μg of protein) were boiled, reduced, and resolved on a 4–15% SDS/PAGE gel. Immunoreactive isoforms of CR-1 were detected as previously described with an affinity-purified rabbit anti-CR-1 IgG (67CR) that was generated against a peptide corresponding to amino acids 93–118 in the CR-1 human sequence (Brandt *et al.*, 1994; Kenney *et al.*, 1995) using 0.5 μg/ml of the antibody or blocked by pre-absorbing CR-1 67CR with a 20-fold excess of the synthetic 93–118 peptide (Brandt *et al.*, 1994; Kenney *et al.*, 1995).

Southern Blot Analysis

Genomic DNA was extracted from transplanted outgrowths using the proteinase K-phenol extraction method (Maniatis *et al.*, 1989). The DNA was then digested with Hind III/Xho I, separated by electrophoresis in 1% agarose and transferred to nylon filters. Hybridization was then carried out at 42°C in hybridization buffer (Clontech, Palo Alto, CA) containing 100 μg/ml of salmon sperm DNA and 0.5 μg/ml DNA probe labeled by 32P dCTP random priming (Boehringer Mannheim, Indianapolis, IN) and hybridized overnight. The blots were washed in 0.2 X SSC-1% SDS for 20 min at 65°C then 0.1 X SSC-0.1% SDS for 5 min at 65°C. Autoradiography was carried out by exposing the blots to Kodak films at –70°C with an intensifying screen. A pNO4 complimentary DNA probe (2.2-kb fragment) which contained the neo-CMV promoter region was generated by Hind II/Xho I digestion (Kenney *et al.*, 1996c).

RESULTS

Effect of the Cripto-1 Protein on Regressed Ductal Epithelium

Previous studies have demonstrated that EGF-like peptides (EGF, TGFα, and AR) that have been incorporated into slow release Elvax pellets, can either induce ductal morphogenesis or initiate lobuloalveolar formation in the mammary glands of intact or OVX virgin mice (Coleman *et al.*, 1990; Snedeker *et al.*, 1991; Vonderhaar, 1987). To investigate whether the administration of exogenous CR-1 can provide a similar epithelial response, biologically active CR-1 peptide was incorporated into Elvax pellets. The pellets were then surgically implanted into the fourth inguinal mammary gland of 5-week-old C57/BL6 OVX mice and the animals were sacrificed 5 days later. By utilizing the whole mount technique, our observations indicate that the CR-1 peptide effectively initiated ductal morphogenesis. Details in Figure 1c–d demonstrate that in close proximity (2mm) of the CR-1 pellet, pre-existing mammary ducts developed newly formed lateral branching andbi furcated end buds that migrated towards the pellet. When we searched fields 5mm distal from the CR-1 pellet (midgland region) we observed that the secondary mammary ducts also displayed similar migratory patterns towards the pellet although newly formed lateral buds and ducts on existing terminal ducts were not as intense (not shown). This was an intriguing observation since the concentration of exogenous CR-1 growth factor released from the Elvax pellet is decreased several orders of magnitude in the midgland region (Coleman *et al.*, 1990). In the contralateral #4 inguinal mammary glands that received the

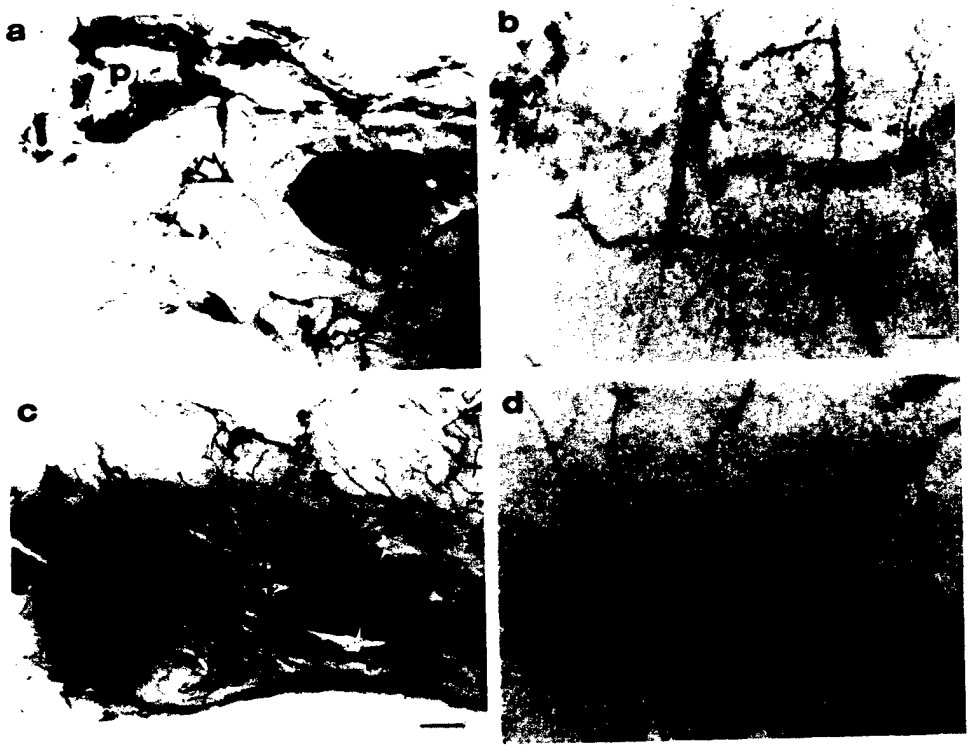

FIGURE 1 Whole mount analysis of OVX mammary glands that received either BSA or CR-1 Elvax pellets. Elvax pellets containing BSA (5 μg/pellet) (**a–b**) (negative control) were implanted into the inguinal #4 mammary gland contralateral to Elvax pellets containing recombinant CR-1 (5 μg/pellet) (**c–d**). Photos in **c–d** show the CR-1 protein stimulated ductal morphogenesis. Open arrows in **a** and **c** indicate areas enlarged in **b** and **d**, respectively. **P** represents pellet, **Nd** represents normal mammary duct. Arrows in **d** indicate new lateral branches that have developed in response to CR-1. **a** and **c** scale bar = 200 μm. **b** and **d** scale bar = 25 μm.

control BSA pellets, these responses did not occur (Figure 1a–b). Aspects of several growth-induced characteristics that appeared at various distances from CR-1 Elvax pellet are presented in Table I.

In the next set of experiments, mice that had been implanted with either BSA pellets, EGF pellets (not shown), or CR-1 pellets, were injected with BrdU 2 hr prior to sacrifice and their mammary glands removed, sectioned, and immunohistochemically assayed in order to assess DNA synthesis. The results suggest that in comparison to control BSA mammary ducts, both EGF and CR-1 responding mammary ducts showed an approximate 20-fold increase in the number of epithelial cells undergoing DNA synthesis (Figure 2). Stromal cells adjacent to CR-1 newly formed ducts were also undergoing DNA synthesis, but the activity was strictly confined to the tip of the end bud or stroma that was adjacent to the subtending ducts (data not shown).

TABLE I Growth-induced response of ductal epithelium by CR-1 Elvax pellets

	Control (BSA)	CR-1
Average # of secondary ducts migrating towards the pellet	2 ± 1	5 ± 2
Average # of newly formed lateral ducts on existing tertiary ducts *	19 ± 6	65 ± 15
Ratio of terminal ducts near the pellet versus the midgland region	9:2	17:3

* Represents the number of lateral ducts found on preexisting tertiary ducts 700 μm or less from the pellet.
The table represents the average of three separate experiments with OVX mammary glands that received either BSA or CR-1 Elvax pellets as described in *Materials and Methods*. Measurements represent a total of 20 mammary glands and 8 BSA mammary glands that were scored by whole mount analysis.

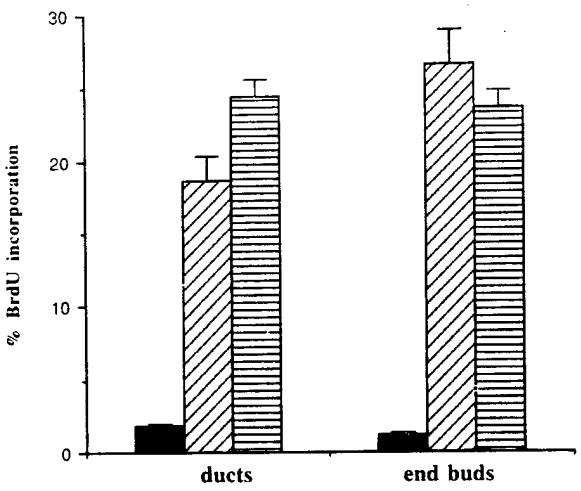

FIGURE 2 Percentages of BrdU incorporation by immunohistochemical detection in mammary glands that received BSA, EGF or CR-1 pellets. The percentages of labeled cells for BrdU incorporation were derived according to *Materials and Methods*. The histogram represents the percentages of immunostained anti BrdU cells located in the mammary ductal and end bud compartments, (■) indicates BSA treatment, (□) indicates EGF treatment, and (□) indicates CR-1 treatment.

Detection of Cripto-1 Expression in Transgenic Outgrowths

Transgenic mice that overexpress TGFα, *int*-3, c-*neu*, SV40 large T antigen, or polyoma middle T antigen spontaneously develop mammary carcinomas with short latency periods (Jhappan *et al.*, 1990; reviewed by Cardiff and Munn, 1995). Mammary tumors in these disparate transgenic animals exhibit an enhanced expression of CR-1 protein and process the CR-1 protein in a transgene-model-specific manner, suggesting that CR-1 might be one common distal effector or marker in the conversion of malignant mammary epithelium (Kenney *et al.*, 1996b). To study the consequence of CR-1 overexpression in the transgenic mammary gland *in vivo*, primary mouse mammary epithelial cells were infected with an amphotrophic, replication-defective retrovirus expression vector containing a 900 bp mouse CR-1 cDNA. Our controls during these experiments were transgenic outgrowths that were transduced with the empty vector alone (pNO4) or primaries that were not transduced with the virus. To initially confirm that pNO4 or CR-1 transgenic mammary outgrowths incorporated the viral expression vector, Southern blot hybridization of genomic DNA was performed. As illustrated in Figure 3a (arrow), details from DNA extracts that were hybridized with a ^{32}P dCTP 2.7 kb *Hind* III/*Xho* I random prime-labeled probe containing the neo-CMV sequence detected the 2.7 kb fragment in pNO4 and CR-1 transduced outgrowths, suggesting the presence of the expression cassette. As a second means to confirm expression cassette insertion, we immunostained serial tissue sections from pNO4 and CR-1 transgenic outgrowths with an antibody that immunoreacts with the neo protein. Tissue sections from both pNO4 and CR-1 transgenic outgrowths contained a small percentage of transduced cells that expressed the protein (10%) (data not shown). The ratio of stable-expressing transduced to nontransduced cells *in vivo* were similar to the findings previously reported by Smith *et al.* (1992) and Edwards *et al.* (1996).

We next determined whether the CR-1 outgrowths expressed higher endogenous levels of the CR-1 protein or underwent additional pro-

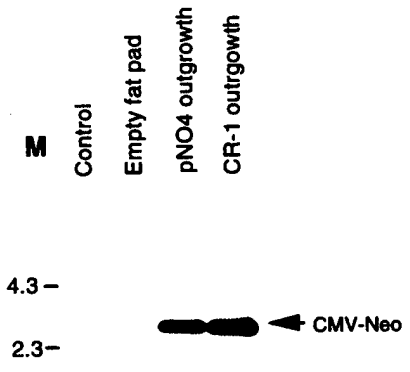

FIGURE 3 Southern Blot detection of genomic DNA extracts derived from pNO4 or CR-1 transgenic outgrowths hybridized to a 2.7 kb *Hind* III/*Xho* I random ^{32}P dCTP labeled neo-CMV probe. Arrow indicates a 2.7 kb fragment isolated in both CR-1 outgrowths and pNO4 outgrowths. **M** represents molecular weight in kilobases.

cessing. To examine this, Western blot analysis was performed on pNO4 outgrowths, CR-1 outgrowths, and extracts from transgenic TGFα mammary tumors (Figure 4) (Jhappan *et al.*, 1990; Kenney *et al.*, 1996b). As previously mentioned, TGFα mammary tumors enhance expression of and process the CR-1 protein in a transgene-model-specific fashion and this was used as a positive control. As shown in Figure 4, four prominent (30-, 24-, 21-, and 14kDa) isoforms of the immunoreactive CR-1 protein were detected. The 21kDa isoform apparently appeared as a result of the transgene. More importantly, several of these isoforms migrated in a similar fashion as seen with extracts derived from TGFα mammary tumors. In the control pNO4 protein extracts, only the prominent 30-kDa and weaker 24- and 14kDa bands were detected.

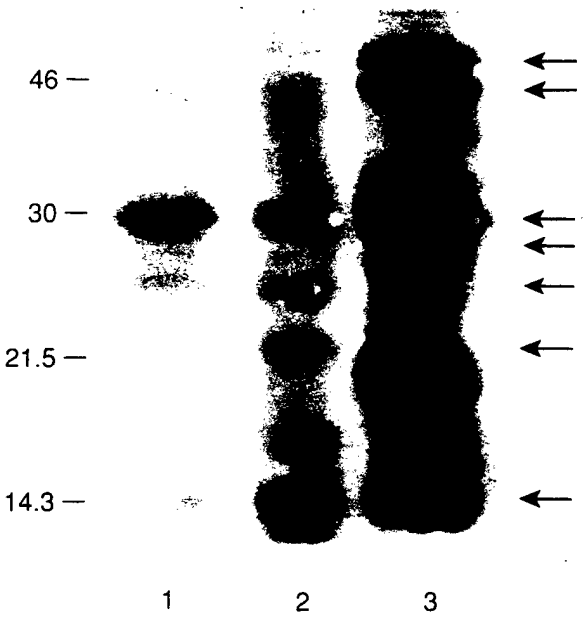

FIGURE 4 Western blot detection of the CR-1 protein in control outgrowths, pNO4 (empty vector) outgrowths, CR-1 transgenic outgrowths, and MT 100 TGFα transgenic mammary tumors. Total protein extracts were derived according to *Materials and Methods*. All lanes were loaded with 50 μg total protein. *Lane 1* control pNO4 extracts, *lane 2* CR-1 extracts, and *lane* 3 TGFα tumor extracts. The extracts from MT-100 TGFα tumors were utilized to detect various transgene-induced CR-1 isoforms (Kenney et al., 1996b). An apparent 21 kd isoform occurred in CR-1 transgenic outgrowths as a result of the CR-1 transgene (*lane 2*). Arrow indicates 47-, 45-, 30-, 28-, 24-, 21-, and 14- kDa species. **M** represents molecular weight markers in kilodaltons.

Cripto-1 Overexpression and the Induction of Hyperplastic Ducts

The CR-1 protein is heterogenously expressed in all epithelial regions (end bud tip region, transitional and finished duct) of the TEB during ductal morphogenesis (Kenney *et al.*, 1995). The main aim of this study was to determine the various morphogenic effects of the CR-1 transgene in primary mammary epithelial cells. To address this posit, we initially transplanted CR-1-transduced mammary epithelial cells into epithelial-free mammary fat pads of hormonally intact C57/BL6 3-week-old mice (DeOme *et al.*, 1959; Smith *et al.*, 1992; Daniel *et al.*, 1968; Kenney *et al.*, 1996a; Edwards *et al.*, 1996). The results of our study suggest that after 9 weeks post-transplantation, CR-1 transgenic outgrowths displayed an atypical ductal growth pattern. In CR-1 outgrowths we observed focal areas of ductal hyperproliferations that affected the development of secondary, tertiary, and quaternary ducts in 6 out of 9 (66%) mammary glands. Details from one of the six CR-1 transgenic outgrowths is presented in Figures 5c–e. Further, morphometric analysis suggested that CR-1 transgenic outgrowths showed patterns of slightly narrower ducts in conjunction with newly formed multi-directional terminal ducts, lateral buds, or lateral branches. We also detected an obvious loss in the spatial distance between the CR-1 ducts relative to the spatial distance between ducts that appeared in the control pNO4 outgrowths. Apparently, the majority of CR-1 transgenic lateral ducts had migrated towards, around, and above each other (Figure 5e, open arrowhead). In addition, upon examining tissue sections from CR-1 outgrowths, we also detected several substantial areas of atypical ductal hyperplasias (enlarged ductules that contain benign hyperplasia) (Figure 6c–d) or hyperproliferative ducts that contained surrounding sites of hyperproliferative stroma (Figure 6a–b). Details of the lesions

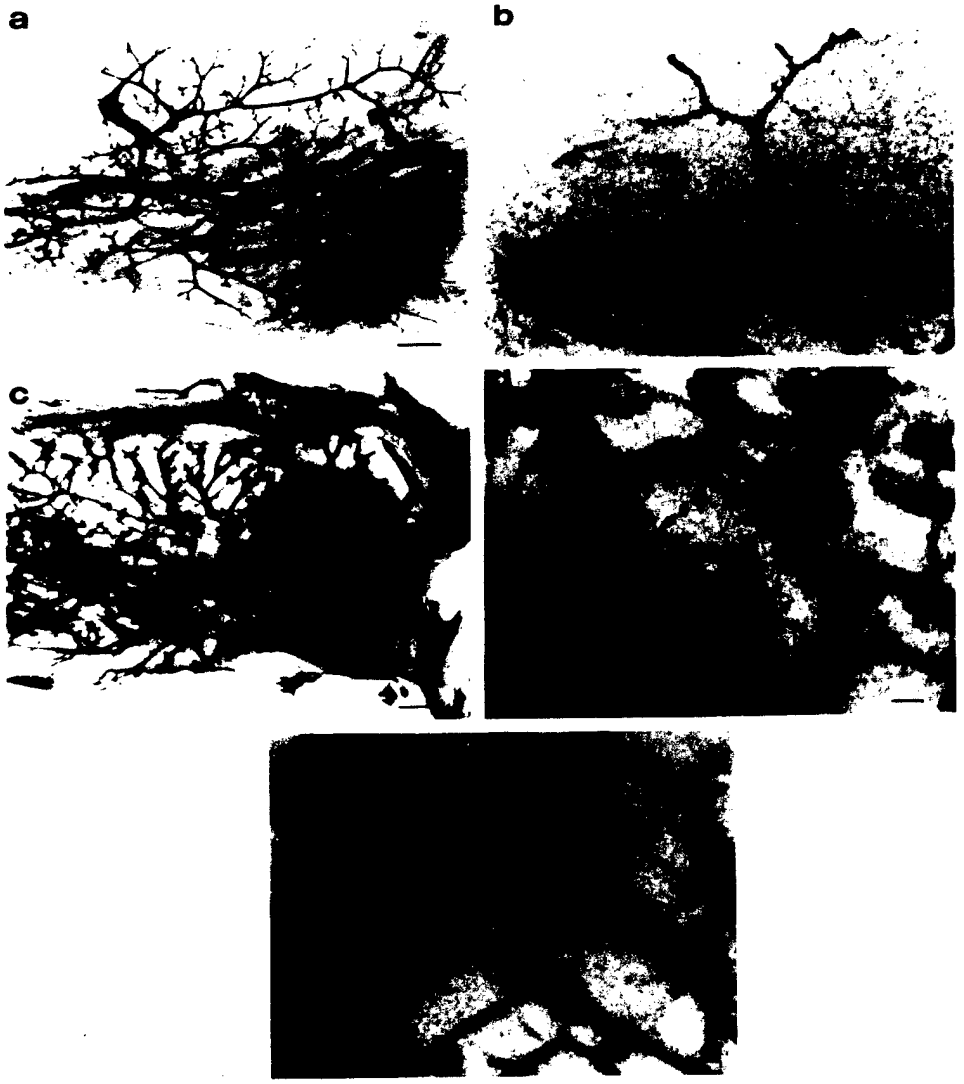

FIGURE 5 Whole mount analysis of intact control pNO4 and CR-1 transgenic mammary outgrowths 12 weeks after transplantation into the # 4 mammary gland of 3-week-old intact mice. Mammary glands were excised, fixed and stained according to *Materials and Methods*. **a** and **b** represent pNO4 outgrowths that show normal ducts (**Nd**) with no apparent mammary aberrations. **c–e** represent CR-1 transduced outgrowths that show limited hyperplasia (**H**) and loss in ductal spacing (open arrow head). Scale bars in **a** and **c** = 200 μm, scale bar in **b** 32.5 μm, scale bars in **d** and **e** = 25 μm.

observed in CR-1 transgenic outgrowths are described in Table II and CR-1 induced morphometric characteristics are presented in Table III.

Serial Transplantation of Cripto Outgrowths

The next set of experiments investigated whether serially transplanted CR-1 transgenic mammary epithelium had the regenerative capacity to maintain or modify the growth and subsequent appearance of the hyperplastic outgrowth (DeOme *et al.*, 1959; Daniel *et al.*, 1968). To address this question, we serially transplanted small collagenase digested tissue fragments (2×10^5 cells) from 20-week-old CR-1 hyperplastic transgenic outgrowths into epithelial-cleared

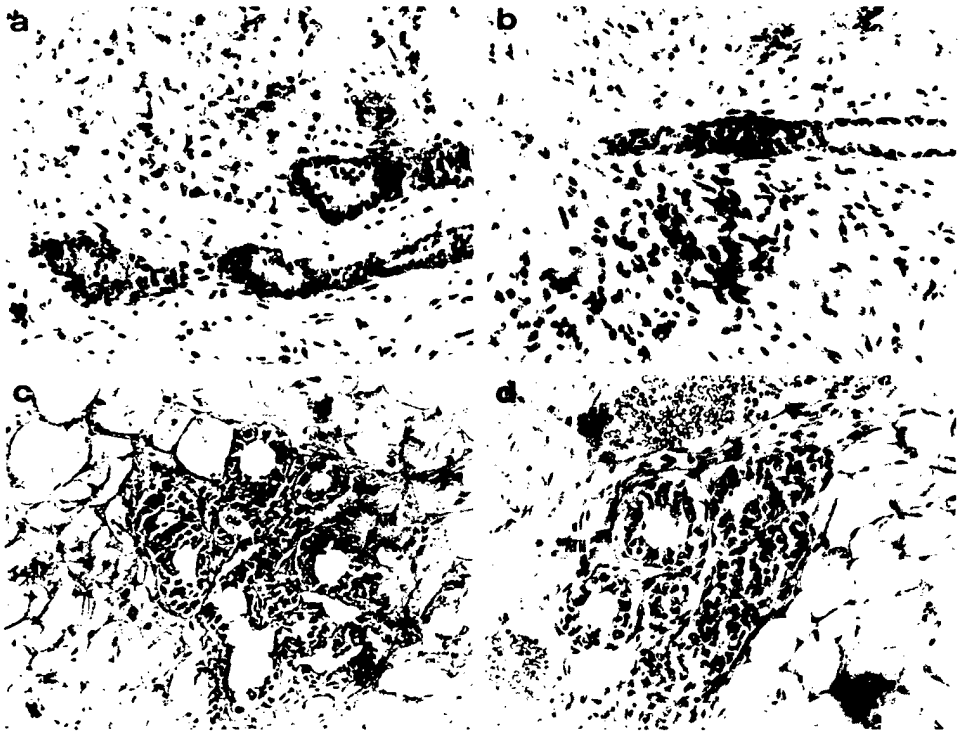

FIGURE 6 Cross section analysis of intact pNO4 or CR-1 transgenic mammary outgrowths. Paraffin embedded mammary glands were sectioned at 5 μm and stained according to *Materials and Methods*. **a** and **b** represent control pNO4 normal end bud (**a**) and normal mammary duct (**b**). **c** and **d** represent CR-1 atypical hyperplastic lesions (**ATH**) and several sites of hyperproliferative stroma surrounding the mature disorganized duct. Magnifications in **a–d** ×400.

3-week-old hormonally intact and epithelial-cleared 3-week-old OVX syngeneic mice. After 4–12 weeks post-transplantation, each gland receiving a transgenic tissue fragment was then microscopically examined for abnormalities. Our data suggests that serially transplanted CR-1 transgenic outgrowths could form hyperplastic mammary lesions although focally, when introduced into cleared fat pads (Fig. 7c–d). In light of this, we also observed that the total ductal epithelium in CR-1 outgrowths repopulated the fat pad (70%) which was similar to the control pNO4 serial outgrowths (Figure 7a–b). However, when we examined the repopulation of CR-1 transgenic outgrowths transplanted into OVX fat pads, mammary aberrations were not observed (data not shown). Essentially, these results indicate that the growth of CR-1 transgenic tissue may depend upon the availability of sex steroids. In Table II, the cumulative data obtained from serially transplanted CR-1 outgrowths or pNO4 outgrowths are summarized.

DISCUSSION

Clearly, the Elvax slow-release pellet model has proven that growth factors can participate in the mitogenic, inhibitory, or differentiation pathways in the mammary gland (Daniel and Silberstein, 1987; Snedeker *et al.*, 1991). In conjunction with these findings, several reports have contributed to the detailed patterns of localization, receptor activities, and mesenchymal interactions which growth factors participate in during mammary growth and neoplasia (Pringent and Gullick, 1994; Kenney and Dickson 1996). Emerging detailed molecular evidence has

TABLE II Hyperplastic lesions in CR-1 outgrowths

Outgrowth	outgrowths/take	Time period	Lesions
Primary transplants	6/9	8–36 weeks	hyperproliferative lateral buds benign hyperplasia
Serial transplants intact	5/5	8–12 week	hyperproliferative lateral buds benign hyperplasia
OVX	2/5	4–12 weeks	none
Lactating mice*	2/2	5 and 28 days	none

* Represents mice that were sacrificed during or after lactation.
The table represents the average of three separate experiments with primary cultures that were transduced and then transplanted as described in *Materials and Methods*. Numbers indicate transplants that were positive for epithelial outgrowths over total number of transplants (takes) in all experiments. Outgrowths were examined by whole mount analysis and sectioned and stained with H&E.

also demonstrated that germline alterations in growth factor expression contributes to the development of mammary hyperplasias and adenocarcinomas (Jhappan *et al.*, 1990; Sandgren *et al.*, 1995; Smith *et al.*, 1992; Cardiff and Munn, 1995). Similarly, several groups (Smith *et al.*, 1992; Edwards *et al.*, 1996; Strange *et al.*, 1989) have also successfully shown that the tissue reconstitution technique can be exploited to examine the effects of transgenic mammary epithelium or follow the cellular fate of mammary epithelial cells *in vivo*. In the present report, we have utilized both the slow-release pellet model and the tissue reconstitution technique to examine the biological and morphogenic effects of the EGF-related CR-1 growth factor in the mouse mammary gland.

TABLE III Morphometric characteristics of CR-1 outgrowths

	Control	CR-1
Number of lateral buds per branch	2	>15
Number of lateral branches from secondary ducts	2	4
Width of secondary ducts (μm)	30 ± 5	20 ± 5

The table represents the average of three separate experiments with primary cultures that were transduced and then transplanted as described in *Materials and Methods*. Measurements represent 10 random fields of 20 mammary secondary ducts from 5 mammary glands that were examined by whole mount analysis and sectioned and stained with H&E.

CR-1 is a novel, mammary tumor-associated protein that is structurally different from other members of the EGF family of growth factors and fails to directly bind to any of the known type 1 receptor tyrosine kinases (Brandt *et al.*, 1994). In addition, the CR-1 peptide has been previously shown to stimulate moderately the *in vitro* proliferation of nontransformed human 184A1N4 mammary epithelial cells (Brandt *et al.*, 1994). The CR-1 gene has also been shown to be overexpressed in the aged mammary gland from 24-month-old nulliparous or multiparous Balb/c mice and in nearly 50% of noninvasive ductal carcinomas *in situ* (Kenney *et al.*, 1996a; Herrington *et al.*, 1996; D. Salomon unpublished observations). Together with these and other findings, an indirect correlation between CR-1 expression and the genesis of spontaneous mammary tumors has been suggested (Salomon *et al.*, 1995). Is CR-1 overexpression universally restricted to neoplasia? Two significant results suggest CR-1 overexpression may only partially account for the onset of epithelial hyperplasia or neoplasia in the context of the mammary gland. We have observed that during various time periods, CR-1 transgenic outgrowths failed to give rise to either micro nodules or palpable tumors. Equally important, CR-1 outgrowths also failed to give rise to transplantable hyperplasias in OVX mice. These results

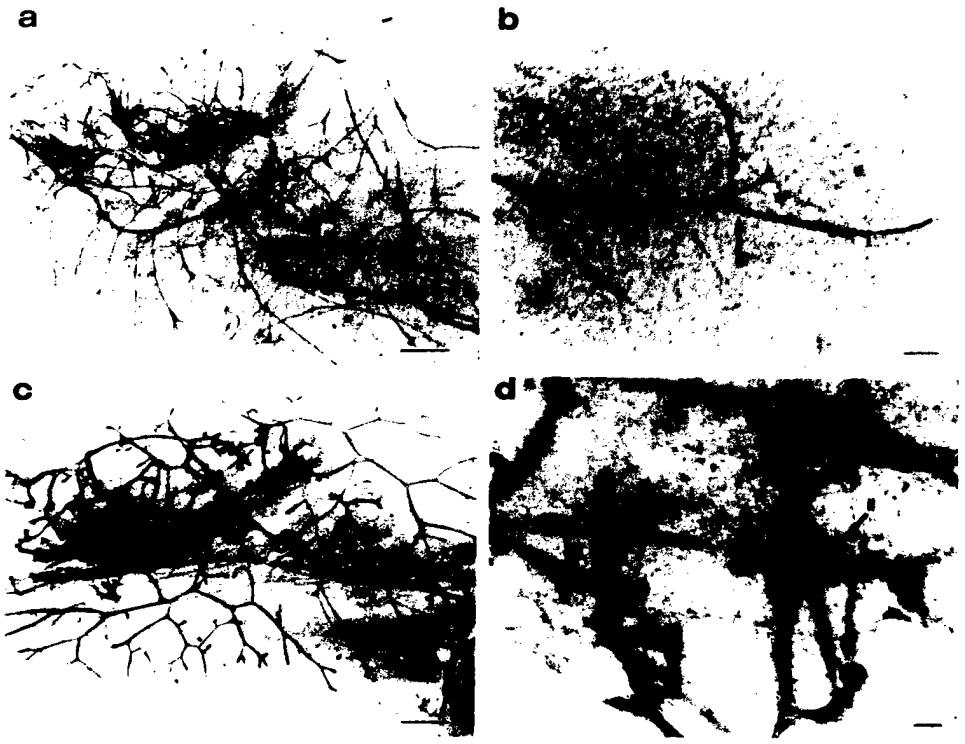

FIGURE 7 Whole mount analysis of 12 week-old control pNO4 and CR-1 transgenic outgrowths serially transplanted into hormonally intact 3 week-old epithelial-cleared mammary fat pads. Pooled portions from intact pNO4 and CR-1 transgenic donors were transplanted according to *Materials and Methods*. **a** and **b** represent normal transplanted outgrowth that show normal terminal ducts (**Nd**) with no apparent mammary aberrations. **c** and **d** represent CR-1 transplanted outgrowths that show focal zones of hyperplasia (**H**). Scale bars in **a** and **c** = 200 μm, and scale bars in **b** and **d** = 25 μm.

suggest that the development of CR-1 induced mammary lesions may also depend upon appropriate ovarian steroid hormones. Consistent with our data, Ciardiello *et al.* (1990) also showed that the overexpression of the CR-1 gene in mouse NOG-8 mammary epithelial cells lead to their transformation *in vitro* but failed to form tumors in nude mice (Ciardiello *et al.*, 1991). Collectively, the results demonstrate that additional genetic alterations and hormonal stimuli in conjunction with CR-1 overexpression, are required for completing the tumorigenic process.

Ductal Aberrations Induced by Cripto-Expressing Progenitors

What are the modulatory elements during the development of the mammary gland? Essentially, several regulatory factors and processes have been elucidated, which include epithelial-mesenchymal interactions, hormones, and local regulation by growth factors (Bernfield *et al.*, 1973; Topper and Freeman, 1980; Daniel and Silberstein, 1987; Kenney and Dickson, 1996). During the establishment of the mammary network, partitioning between ducts must be maintained so that lobuloalveolar structures can develop before and at the onset of pregnancy. The maintenance of this open pattern of ductal growth requires cell-cell signaling that actively suppresses lateral bud migration (Faulkin and DeOme, 1960). One must also consider that the resourcefulness of the mammary network to undergo repeated rounds of alveolar development and subsequent involution implies that the ductal network must maintain an abundant supply of self-renewing multipotent mam-

mary stem cells. These stem cells must move to a new position and either become a secretory progenitor or ductal progenitor cell (Smith, 1996). Considering that the aberrations observed in the mammary ducts were attributed to the transduced CR-1 multipotent mammary stem cells, it is our assumption that their progeny (the ductal progenitor) also participate in this process (pictured in Figure 5e open arrow). Combined with the growth-inducing effects of the CR-1 Elvax pellet, the results of our study suggest that the CR-1 growth factor influences mammary gland ductal architecture and plays a prominent role in the initiation of ductal hyperplasias.

Transgenic Outgrowths and the Effects of Growth Factors

In the rodent mammary gland, the effects of growth factor expression on secretory epithelium have not been fully elucidated. Current evidence suggests that the TGFβ/EGF family (TGFα, TGFβ, and *int-3*) plays a prominent role in arresting the maturation of secretory progenitors (Sandgren *et al.*, 1995; Smith *et al.*, 1995; Robinson *et al.*, 1996). For example, when the mammary glands from the WAP/int-3 and WAP/TGFβ1 transgenic mice were histomorphometrically examined for secretory structures, these mice lacked considerable lobuloalveolar development and were unable to suckle their pups (Robinson *et al.*, 1996). In contrast, WAP/TGFα transgenic mammary glands have only marginal lobuloalveolar morphologies, suggesting a failure of secretory progenitors to undergo complete differentiation (Robinson *et al.*, 1996) (G. Robinson, G. Smith and L. Henninghausen unpublished observations). In comparison, in our model, CR-1 overexpression did not impair lobuloalveolar formation or modulate the ability of secretory epithelium to undergo terminal differentiation (Table II). These results imply that CR-1 expression may not have a dominant inhibitory function on normal secretory progenitor maturation.

It is possible that other growth factors can promote dysfunctional ductal progenitor positioning? The current use of the outgrowth system as a bioreactor for mammary epithelial cells that are overexpressing additional EGF, IGF, FGF, or other growth factor superfamilies should provide an additional assay to systematically screen for other genes that can compliment or synergize with the CR-1 transgene. In this light, we suggest that in the future, particular attention should be taken in understanding the progenitors that are involved in the structure/function of the mammary gland.

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Regulation of Cell-Cycle Progression and Cell Death in Breast Cancer

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■ **Abstract:** Tumorigenesis is often characterized by a combination of aberrant proliferation and the inappropriate suppression of apoptosis. In breast cancer, a variety of growth factors and hormones, as well as attachment to extracellular matrix, can regulate both cell growth and cell death via apoptosis, suggesting that the two processes may be closely linked. A better understanding of the mechanism by which those factors regulate the cell cycle and the apoptotic pathways could therefore be very useful in designing novel therapies for breast cancer. ■

Key Words: breast cancer, cell cycle, apoptosis, *c-myc*, growth factors, ECM, estrogen, progestins

Tissue homeostasis is maintained through a balance of cell proliferation, differentiation, and death. Although cancer has traditionally been viewed as a disease of abnormal growth, it is now understood that abrogation of the cell-death pathways can also contribute to tumorigenesis. It is also becoming increasingly clear that the two opposing processes of cell proliferation and cell death may be intimately linked through their dependence on the cell cycle (reviewed in 1). In normal tissues, apoptosis occurs almost exclusively in association with proliferating cells, and deregulation of the cell cycle often results in induction of apoptosis. In the mouse mammary gland, expression of many immediate early response genes, including *c-myc* and the D-type cyclins, has been observed in association with the proliferative phase of puberty as well as with the apoptotic process of involution following cessation of lactation (2).

Since the cell cycle is involved in both cell growth and death, it is not surprising that many hormones and growth factors that have been implicated in the regulation of mammary cell growth can also regulate the induction of apoptosis. For example, growth promoters such as estrogen and epidermal growth factor (EGF) can act as survival factors for mammary epithelial cells (MECs), while the growth inhibitor transforming growth factor β (TGFβ) can induce apoptosis in some cases. The focus of this article is to examine the mechanism(s) by which those factors modulate cell growth and cell death in MECs and breast cancer.

CELL-CYCLE REGULATION IN BREAST CANCER

Normal Cell-Cycle Regulation

Passage through the cell cycle is orchestrated by the cyclins and their associated cyclin-dependent kinases (cdks). Expression of the various cyclins is tightly regulated and is indicative of specific stages of the cell cycle (Fig. 1). The cyclins and their periodicity were first described in yeast, and the cell cycle control mechanisms in mammalian cells have proven to be quite analogous to those of the budding yeast *Saccharomyces cerevisiae* (reviewed in 3). In both models, cells must pass through a G_1 checkpoint, after which they are committed to DNA synthesis. In yeast, this START point is dependent on

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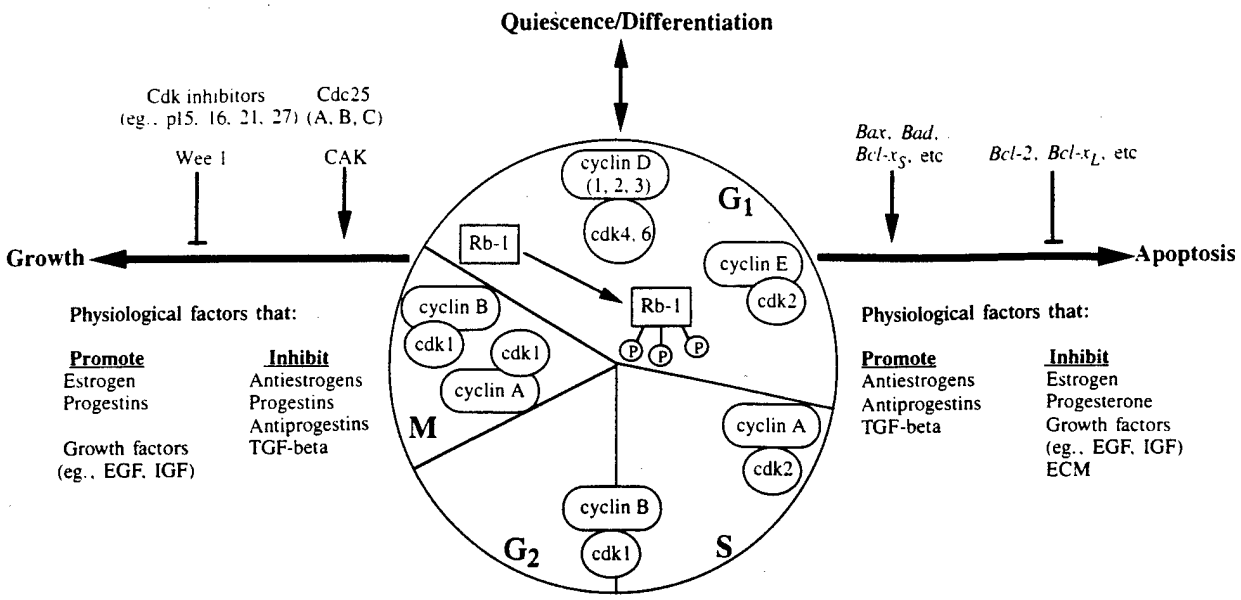

Figure 1. A model of cell-cycle regulation. Progression through the cell cycle is orchestrated by periodic expression of the various cyclins that activate their associated cyclin-dependent kinases. Expression and activation of the cell-cycle regulatory proteins can lead to two distinct outcomes: cell proliferation or cell death via apoptosis. Proteins that further modulate those outcomes are listed with pointed arrows (stimulation) or blunted arrows (inhibition). Hormones and growth factors that either promote or repress cell growth and cell death in MECs are listed on each side. Note that most factors that promote growth also inhibit apoptosis, and vice versa. Complete withdrawal from the cell cycle leads to quiescence and in some cases, differentiation. Although not shown, overexpression of *c-myc* activates several regulatory components of the cell cycle, and therefore also leads to either cell growth or cell death, depending on the presence of survival factors.

nutrient availability. The analogous mammalian restriction point is also dependent on the presence of mitogens (hormones and growth factors).

The D-type cyclins (1–3) function in G_1 and can associate with *cdk* 4 and 6 (3). Expression of cyclin D is dependent on the presence of mitogens and has therefore been associated with the restriction point of cell growth in culture. Cyclin D synthesis begins during the G_0 to G_1 transition and persists with only minor oscillations as long as growth factors are present in the media. Cyclin E is maximally expressed late in G_1 and activates cdk2 in late G_1 phase. The tumor suppressor Rb-1, which arrests cells in G_1 in its hypophosphorylated state (reviewed in 4), has been proposed as one of the targets of cyclin D-activated *cdk* 4 and 6 as well as cyclin E/cdk2 (5–8). Overexpression of either of these two G_1 cyclins can result in shortened G_1 and reduced dependency on mitogens (9–11).

As cells enter S phase, cdk2 forms a complex with cyclin A. Cyclin A-cdk2 is required for progression through S phase and cyclin A-cdk1 activity also participates in the G_2/M transition (12). The primary regulator of mitosis is cyclin B, which also activates cdk1 (reviewed in 13). Its synthesis begins late in S phase and must accumulate above a threshold level for cells to enter mitosis. At metaphase, cyclin B initiates its own destruction by activating a ubiquitin-dependent proteolytic cascade, thereby triggering anaphase.

In addition to the positively acting cyclins, a number of negatively acting cdk inhibitors have recently been identified (reviewed in 3,14). They are small proteins (e.g., p15, 16, 18, 19, 21, and 27) that interact with cyclin-cdk complexes. p21 and p27 are structurally related and their ability to inhibit cdk function is dependent on the stoichiometry within the multimeric complexes. However, differences in regulation suggest that they perform different functions in the cell. For example, p21 is up-regulated during the G_0 to G_1 transition (15) and its expression is also directly regulated by the tumor suppressor p53 (16), indicating that it plays a role in the p53-controlled G_1 checkpoint. In contrast, p27 levels are high in quiescent cells and decline during the G_0–G_1 transition. p27 is also released from a sequestered state by TGF-beta treatment or by contact inhibition (17,18).

Cdk activity is also regulated by phosphorylation at multiple sites (reviewed in 19). Phosphorylation by cdk-activating kinase (CAK, or cyclin H/cdk7) positively modulates cdk activity, while phosphorylation by Wee1 inhibits activity. Removal of the latter phosphate groups is catalyzed by the cdc25 family of phosphatases.

Altered Cell-Cycle Regulation in Breast Cancer

Since cancer is generally characterized by abnormal proliferation, it is not surprising that the cell cycle is deregulated in many cancers. Loss of cell-cycle control could be due to changes in expression or activity of any key regulatory protein, but the most common changes in cancer appear to modulate the G_1 components that contribute to insensitivity to external growth signals (reviewed in 20).

It is well known that a high rate of proliferation in breast tumors, as determined by the S-phase fraction, is correlated with poor prognosis, but unfortunately, little is known about the role of cell-cycle deregulation in breast cancer. The cyclin D1 locus is commonly amplified and overexpressed in breast cancer (21–22; reviewed in 23), but the functional consequences of such alterations are not known. Some studies have observed a weak correlation with poor prognosis, but others have found an association with ER- and PR-positive tumors (21,24,25). Cyclin E has also been shown to be amplified, overexpressed, or found in variant forms in primary human breast tumors and cell lines, and overexpression has been correlated with poor prognosis (26; reviewed in 23). Overexpression or deletion of several other cell-cycle regulatory proteins (including cyclins A and B, cdk1, cdk4 and p16) have been observed in human breast tumors, or cell lines, or both (27; reviewed in 23), but their contribution to breast neoplasia is unknown. One of the difficulties with these studies is that we cannot determine whether cyclin overexpression is driving breast-tumor proliferation or is simply a consequence of cell-cycle re-entry. That problem could potentially be addressed with transgenic mouse models. Overexpression of cyclin D1 in the mammary glands of transgenic mice resulted in hyperplasia and tumorigenesis only after a long latency, indicating that additional genetic events are required for full transformation of MECs by cyclin D1 (28). Transgenic mice with mammary-directed cyclin A overexpression displayed many cells with nuclear abnormalities, but the resultant phenotype was one of elevated apoptosis frequency rather than hyperplasia (29).

The recently identified breast and ovarian cancer susceptibility gene *BRCA-1* may also play a role in the regulation of cell-cycle progression, since its expression varies in synchrony with the cell cycle (30,31). Both normal and tumor-derived MECs contain low levels of *BRCA-1* when growth arrested, and expression dramatically increases as cells re-enter the cell-cycle, with a peak just prior to the S-phase transition. However, a clearly defined function for the protein must be identified before any serious conclusions can be drawn about its role in aberrant cell-cycle progression.

Since passage through the cell cycle is dependent on hormones and growth factors, it is possible that changes in cell-cycle regulation in cancer cells may also be related to altered expression or activity of the receptors or signal transduction pathways through which those agents promote growth, as discussed below.

Regulation of the Cell Cycle by Estrogen and Progestins

Variations in DNA synthesis in breast epithelium during the menstrual cycle indicate that ovarian hormones promote MEC growth. Although estrogen and progesterone are both required for proliferation, maximal growth is observed during the luteal phase when progesterone levels are high and estrogen levels are relatively low. However, supraphysiological doses of progesterone are growth-inhibitory for breast cancer (reviewed in 32). Both antiestrogens and antiprogestins can arrest cells in G_1 in vitro and can reduce tumor growth in vivo, in conjunction with a reduced S-phase fraction (33–36). In the case of antiprogestins, it has been proposed that the G_0/G_1 cell-cycle arrest is associated with a differentiation pathway, since the tumor cells form dysplastic secretory glandular structures following treatment.

In vitro, estrogen stimulates cell-cycle progression in ER-positive breast cancer cells, but this action is temporally limited to early G_1 phase, suggesting that it modulates expression or activity of G_1 regulatory proteins (reviewed in 23). Similarly, antiestrogens block proliferation during the same window of the cell cycle, with a concomitant decrease in cyclin D1 and E expression and reduced Rb-1 phosphorylation. Such observations suggest that overexpression of those cyclins could interfere with the efficacy of antiestrogen therapy. Estrogen can reverse the negative effects of antiestrogens on cyclin D1 expression, but since the promoter of that gene does not contain any known ER response elements, that action is probably not through direct transcriptional control by ER.

Progestins produce a biphasic growth response in T-47D breast cancer cells, with an initial increase in cell-cycle progression followed by growth arrest (23). As in the case of estrogen, cells are only sensitive to the action of progestins in the G_1 phase of the cell cycle and respond with changes in cyclin D1 expression. The initial growth spurt is accompanied by a transient increase in cyclin D1 expression, while the long-term growth arrest is associated with decreased cyclin D1 expression, as well as inhibition of cdk2 and -4 activity and a reduc-

tion in Rb-1 phosphorylation. The antiestrogen RU486 also leads to Rb-1 hypophosphorylation and growth inhibition, but does not target cyclin D1. Rather, cell cycle arrest by that agent is accompanied by a decrease in cyclin D3 expression.

Growth Factors in Cell-Cycle Regulation

Many growth factor receptors, such as ErbB-2, EGF receptor (EGFR), and IGF-I receptor, are commonly overexpressed in breast cancer (reviewed in 37,38). In the case of the two former receptors, that overexpression is often associated with a high S-phase fraction and poor prognosis, but elevated IGF-I receptor expression has actually been correlated with low S-phase fraction and good prognosis. Stimulation of those receptors by their specific ligands induces MEC proliferation in vitro, but their ability to regulate the cell cycle has not been well characterized. However, insulin, IGF-1, and EGF stimulate cell-cycle progression of serum-deprived, G_1-arrested MECs, with concomitant induction of cyclin expression and cdk activity with a temporal pattern similar to that induced by serum (18,39,40). From a therapeutic standpoint, it would be useful to determine the specific components of the receptor-signaling pathways that target the various cell-cycle regulators in breast cancer cells.

In contrast to the growth-promoting factors described above, TGF-beta inhibits the growth of most MECs in vitro and in vivo (41,42; reviewed in 43). In nontransformed mammary epithelial cells, TGF-beta has been reported to block cyclin A expression, but to only mildly inhibit expression of cyclins D1, D2, E (18, 44). Activity of existing G_1 cyclin-cdk complexes was repressed by TGF-beta-induced changes in *p27*, thereby blocking Rb-1 phosphorylation and thus the S-phase transition. However, it should be noted that Rb-1 function does not appear to be an absolute requirement for growth arrest by TGF-beta in all MEC lines (45). Furthermore, it has been demonstrated that TGF-beta expression is actually higher in tumor cells than in normal mammary tissue, and protein levels are positively correlated with disease progression (reviewed in 43), suggesting that breast cancer cells develop the ability to resist the negative signal of TGF-beta.

Cell Attachment and Cell-Cycle Regulation

Most normal cells cannot grow unless they attach to a substrate, implying that integrin-mediated signaling may be required for proper cell-cycle regulation (46; reviewed in 47,48). In contrast, transformed or malignant cells often demonstrate a reduced anchorage dependence. Anchorage dependent fibroblasts arrest in G_1 when grown in suspension because they are unable to phosphorylate Rb-1 due to inhibition of cyclin D1 and E-associated kinase activity by increased affiliation with *p21* and *p27* or by reduced cyclin expression (49).

The role of cell attachment to extracellular matrix (ECM) in the proliferation of MECs has not been characterized, but may be different from that observed in fibroblasts since epithelial cells attach to a basement membrane composed primarily of type IV collagen and laminin, while fibroblasts are surrounded by ECM in which fibronectin and collagen I predominate. In one study using an epithelial gastric adenocarcinoma cell line (50), removal of ECM proteins arrested cells in G_0/G_1. Addition of collagen I restored ability to enter S-phase. However, addition of neutralizing antibodies to either integrin α_2 or β_1 in the presence of collagen I arrested the cells in G_0/G_1, suggesting a possible role for integrin $\alpha_2\beta_1$ in the progression through the cell cycle for at last certain adenocarcinomas.

The Myc Oncogene in Cell-Cycle Regulation

Overexpression of proto-oncogene *c-myc* is thought to play a role in the development of breast cancer since it is commonly amplified, overexpressed, or both in breast tumor tissues (reviewed in 51). Its amplification is associated with high proliferation rates in mammary tumors and is correlated with poor prognosis. It has been suggested that enhanced c-myc expression may also allow cells to progress from a hormone dependent state to a more aggressive hormone independent phenotype (52). In addition, several groups have reported a tumorigenic action of *Myc* when overexpressed in the mammary gland of transgenic mice (reviewed in 53).

Myc is a nuclear phosphoprotein that acts as a transcriptional activator when complexed with its heterodimeric partner Max. Although the targets of Myc/Max are not well defined, they are believed to have an important function in the regulation of cell proliferation, differentiation, and death (reviewed in 54–56). Classified as an immediate early gene whose expression is induced following growth-factor stimulation, c-myc expression is tightly regulated and correlated with the proliferative state of the cell. A reduction in c-myc levels due to disruption of one copy of the gene results in a lengthened G_1, while inhibition of *c-myc* expression blocks cell-cycle progression and leads to G_1 arrest. Conversely, cells that constitutively overexpress *c-myc* cannot arrest

in G_1 and therefore continue to proliferate, or in the absence of survival factors they undergo apoptosis.

Myc has been implicated in the expression of several genes involved in cell-cycle control, although it is believed to do so indirectly, since most of the response genes in question lack Myc-Max consensus binding sites [CAG(G/A)TG] in the promoter region. For example, constitutive overexpression of *c-myc* in fibroblasts led to overexpression of cyclins A and E (57–59), while reduced Myc expression resulted in delayed expression of those cyclins. There are conflicting reports about the effects of Myc on cyclin D1 expression, but it has been shown recently that Myc cooperates with cyclin D1 in transformation, suggesting that the two proteins function in complementary rather than liner pathways (60).

Myc may also be involved in the regulation of cyclin-dependent kinase expression and activity. Expression of *cdk1* and *cdk2* is up-regulated when Myc is expressed (61–62). In addition, Cdk activity may be modulated by Myc through increased cyclin expression, as noted above, decreased expression of *cdk* inhibitors such as *p27* (63), or by altered expression or activity of the kinases and phosphatases that regulate *cdk* activity, such as *cdc25* (64).

Recent results from our laboratory indicate that the growth rate of MECs is accelerated by c-myc overexpression due to a shortening of the G_1 phase of the cell cycle. The rapid proliferation of Myc-expressing cells was associated with constitutive Rb-1 hyperphosphorylation, which was most likely due to the premature activation of *cdk2* kinase activity as a result of reduced *p27* expression and enhanced cyclin E expression (40).

APOPTOSIS IN BREAST CANCER

Regulation of Apoptosis

The induction of apoptosis during postlactational involution may reduce the risk of breast cancer (65), but recently, it has been recognized that breast tumors often contain many apoptotic cells (66). It is therefore important to characterize the nature of those apoptotic events and to determine how the occurrence of apoptosis relates to prognosis.

A cell undergoing apoptosis typically exhibits morphological and biochemical changes distinct from those observed in necrotic cells. In vivo, apoptosis usually takes place asynchronously in single cells or small groups of cells. The earliest changes involve loss of cell-cell contacts and specialized plasma membrane structures. The cytoplasm shrinks due to loss of water and ions and the nucleus condenses. The chromatin coalesces into several dense structures at the nuclear membrane and then the cell splits into multiple membrane-bound apoptotic bodies that contain intact cytoplasmic organelles and nuclear DNA fragments. The apoptotic bodies are phagocytosed almost immediately by neighboring cells or by macrophages without eliciting an immune response.

The induction of apoptosis is regulated by a family of proteins that either promote or inhibit cell death (67). The first and best characterized family member is the death suppressor *Bcl-2*. Initially recognized for its role in follicular B cell lymphomas, *Bcl-2* has since been found to be highly expressed in a variety of tumors, including breast cancer (68–70). When overexpressed, *Bcl-2* can protect cells from a variety of apoptotic signals, including unregulated *c-myc* expression (reviewed in 56, 71). *Bcl-x* is a unique family member in that the mRNA can be alternately spliced to produce two different proteins: a death suppressor ($Bcl\text{-}x_L$) and a death inducer ($Bcl\text{-}x_S$). The suppressive activity of *Bcl-2* and $Bcl\text{-}x_L$ can be modulated by death inducers such as Bax or Bad, family members that form heterodimers with the two former proteins (72,73). It is thought that the ratio of Bcl inducers to suppressers determines the fate of the cell (67,73). There is now evidence that Bax expression may be suppressed as breast cancer develops and progresses, tipping the cells toward increased survival (reviewed in 74).

The tumor suppressor *p53* has also been implicated in the regulation of apoptosis (75). *p53* differentially regulates Bax and *Bcl-2*, inducing *Bax* expression, while inhibiting *Bcl-2* synthesis (76,77). Not surprisingly, wild-type *p53* activity is required for apoptosis in many but not all circumstances (reviewed in 75). Whether p53 induces apoptosis or G_1 arrest seems to depend on the cell type or cellular context in which it is activated. For example, the level of *Bcl-2* expression and the presence or absence of growth factors can strongly influence the occurrence of *p53*-mediated apoptosis. Conversely, genetic changes can direct the cell toward the apoptotic pathway if the signals induced by those changes (e.g., oncogene overexpression) are in conflict with *p53*-induced growth arrest. Mutations in the *p53* gene are common in breast cancer and have been associated with increased resistance to cytotoxic drugs that induce apoptosis (reviewed in 78).

Once the cell has been committed to undergo apoptosis, it begins to cleave proteins that are important for cell growth and survival through a family of cysteine proteases, of which the prototype is interleukin-1β-converting enzyme (ICE) (reviewed in 79). The proteases share

a propensity for cleaving their substrates after an aspartyl residue and must be cleaved themselves at a critical aspartate to become activated, suggesting an autocleavage cascade. Trans-cleavage targets include the nuclear enzymes poly(ADP-ribose) polymerase and DNA-dependent protein kinase, U1 ribonucleoprotein, and nuclear lamins, as well as several cytoplasmic proteins, including protein kinase C-δ, and components of the cytoskeleton, such as actin. However, a direct relationship between cleavage of the various substrates and the morphological and biochemical changes that characterize apoptosis has not been defined.

Regulation of Cell Death by Estrogen and Progestins

Estrogen is thought to act as a survival factor for ER-positive breast tumor cells since regression of MCF-7 xenograft tumors following estrogen ablation was associated with the induction of apoptosis (80). *Bcl-2* is commonly expressed in human breast cancer, and that expression is associated with estrogen receptor-positive tumors (reviewed in 74), suggesting that estrogen may promote survival by regulating *Bcl-2* expression. In ER-positive breast cancer cell lines grown in vitro, estrogen promotes chemotherapeutic drug resistance by increasing *Bcl-2* levels without affecting *Bax* expression (81, 82). Furthermore, estrogen-induced increases in *Bcl-2* are significantly inhibited by the antiestrogens ICI164,384 and tamoxifen. At high concentrations, tamoxifen can also induce apoptosis in ER-negative cells (83), but the mechanism by which it does so is not clear.

It is not known whether progestins can also act as survival factors for breast cancer cells, but in the normal, involuting mammary gland, progesterone can inhibit apoptosis (84). In contrast, progesterone antagonists promote apoptosis and hence tumor regression in xenograft breast cancer models (33–35).

Regulation of Apoptosis by Growth Factors

Transgenic mouse models have clearly demonstrated that growth factors such as TGF-alpha and IGF-I can block postlactational involution, suggesting that the growth factors may be acting as survival factors for the mammary epithelium (85–87). In contrast, transgenic mice that overexpress the growth inhibitor TGF-beta show increased occurrence of apoptosis in the mammary epithelium, with a subsequent lack of secretory lobule development (88). Experiments with cell lines cultured in vitro have confirmed that those growth factors can indeed regulate the induction of apoptosis in MECs (89–91). In addition, those studies also demonstrated the ability of EGF, insulin, and basic fibroblast growth factor (bFGF) to act as survival factors for MECs. Several studies have reported increased TGF-beta expression in MECs that have been stimulated to undergo apoptosis by a variety of factors, including cytotoxic drugs and hormone ablation (33,80,92,93). However, it was not determined whether TGF-beta secretion was required for apoptosis induction in those systems.

Signaling through the EGFR-related receptor ErbB-2 (also known as Neu or Her2) may also regulate the induction of apoptosis, but the results are conflicting. Immortalized human MECs that expressed a mutated and thus constitutively activated form of ErbB2 were especially prone to apoptosis when serum-deprived (94). In contrast, overexpression of ErbB2 in MCF-7 breast cancer cells resulted in increased expression of *Bcl-2* and *Bcl-x*$_L$, and thus a greater resistance to tamoxifen-induced apoptosis (95). In the former study, apoptosis may simply be due to overstimulation of the cells, analogous to EGF-induced apoptosis in MDA-468 cells, which overexpress EGF receptor (93).

Attachment to ECM Promotes MEC Survival

Integrin-mediated attachment to ECM can promote cell survival in anchorage-dependent cells (reviewed in 47,48). Recent work with CHO cells indicates that integrins may regulate cell survival by directly influencing the expression of *Bcl-2* (96). Overexpression of integrin $\alpha_5\beta_1$ inhibited apoptosis in serum starved CHO cells plated on fibronectin. This was accompanied by increased *bcl-2* mRNA and protein expression, but *Bax* protein expression was not effected.

Involution of the mammary gland is characterized by matrix metalloproteinase activation and subsequent degradation of the basement membrane, suggesting that ECM attachment may also play a role in MEC survival. In accordance with that hypothesis, the mammary epithelium in vivo (97), as well as primary MECs from midpregnant mouse mammary gland grown in vitro (98) require attachment to basement membrane for survival. ECM also promotes survival of established MECs that have been serum-starved. Similarly, cells which are deprived of ECM attachment by addition of ECM-degrading enzymes or neutralizing antibodies against β_1 integrins become apoptotic in conjunction with increased expression of ICE. Furthermore, coaddition of ICE inhibitors prevents apoptosis in that system, indicating that ICE activity is required for apoptosis induction by

disruption of ECM attachment. However, the physiological induction of apoptosis during mammary gland involution can apparently be divided into two distinct phases: an initial phase characterized by the induction of apoptosis-associated genes such as ICE, and a secondary phase during which the ECM is remodeled and mesenchymal-epithelial interactions are thus disrupted (99).

There is also evidence that tissue architecture in combination with ECM attachment is important for the regulation of MEC growth, survival, and differentiation. When MECs are grown in basement membrane matrix, they form three-dimensional alveolar structures that become growth arrested and fully differentiated. In the absence of this morphology, cells continue to express cell-cycle related proteins and are susceptible to apoptosis (100). Overexpression of ErbB-2 in MECs can block the formation of these alveolar structures by reducing the expression of α_2 integrin RNA and protein (101). That suggests a mechanism whereby a growth factor can directly modulate growth regulation by integrins.

Two recent studies implicate a role for *p125* focal adhesion kinase (FAK) in the regulation of apoptosis by ECM attachment. FAK, a protein tyrosine kinase that participates in integrin-mediated signal transduction, is often overexpressed in breast cancer cells. Reduction of FAK expression in the human ductal breast cancer cell line BT474 by antisense phosphorothioate oligonucleotides caused the cells to detach from the tissue culture plastic and undergo apoptosis (102). Those observations support a proposed physiological mechanism based on recent data that demonstrates that FAK is proteolytically cleaved during c-myc induced apoptosis (103).

Myc and Apoptosis

Myc has also been implicated in the control of apoptosis (reviewed in 56, 75). Suppression of Myc synthesis by antisense oligonucleotides can block apoptosis in hematopoietic cells. In contrast, Myc overexpression can induce or accelerate apoptosis in fibroblasts or lymphocytes when combined with a negative growth signal. Since Myc-induced apoptosis can be rescued by a variety of growth factors, it has been proposed that cell death is the result of conflicting cell cycle regulatory signals. Cells normally arrest in G_1 in the absence of a growth signal, but often *c-myc* overexpressing cells are unable to withdraw from the cell cycle and instead undergo apoptosis. Since Myc has been shown to affect the expression of cyclin A and cdk1, two regulators of the cell cycle that have also been implicated in apoptosis (1), it seems likely that the impact of Myc on both proliferation and apoptosis may be through control of cell-cycle regulatory proteins.

However, Myc has also been implicated in the expression of several apoptotic pathway genes. Myc can transactivate the *p53* promoter (104) and the promoter of the *bax* gene also contains a putative Myc response element (77). Myc expression has also been associated with stabilization of the short-lived p53 protein (105). Thus, Myc may up-regulate bax expression and activate the *p53* pathway as a safeguard to prevent the growth and survival of cells with oncogenic activation. In accordance with that theory, *Bcl-2* can cooperate with Myc to transform cells and promote tumorigenesis by blocking the apoptotic response to *c-myc* overexpression without affecting the proliferative response (reviewed in 56). The mechanism by which Myc triggers cell death is not universal however, since the induction of apoptosis by *c-myc* overexpression is dependent on wild-type *p53* in some, but not all systems (56,106).

Recent results from our laboratory indicate that mouse mammary tumors induced by c-myc overexpression contain many apoptotic cells (90). In contrast, tumors arising in Myc-TGF-alpha double transgenic mice have essentially no apoptotic cells, suggesting that TGF-alpha can act as a survival factor for mammary cells that constitutively express *Myc*. Similarly, MEC lines derived from tumors of single transgenic Myc mice show a propensity to undergo apoptosis, and TGF-alpha functions as a survival factor for those cells in vitro. These cell lines therefore provide a useful model for investigating the mechanism by which Myc induces apoptosis in MEC and by which TGF-alpha acts as a survival factor for such cells. Recent results indicate that the survival-promoting activity of EGF and TGF-alpha is associated with changes in *Bcl-x_L* RNA and protein levels, but not in *Bax* or *p53* expression levels. We have also demonstrated that the growth inhibitor TGF-beta blocks the effects of EGF on *Bcl-x_L* expression (91).

SUMMARY

Certainly there are other modulators of proliferation and apoptosis in the mammary gland that have not been discussed here. For example, glucocorticoids have recently been shown to be survival factors for MECs in vitro and in vivo (84,94). However, the goal of this review was to focus on the physiological factors that have most commonly been implicated in breast cancer initiation and progression.

When considered together, the studies discussed here support the hypothesis that proliferation and apoptosis

are linked, presumably at least in part through the cell cycle. As summarized in Figure 1, many hormones and growth factors that stimulate growth also act as survival factors for MECs. Likewise, agents that inhibit growth often promote apoptosis as well. The former observation suggests that cells are less prone to undergo apoptosis when stimulated with appropriate growth signals, while the latter observation clearly indicates that the cellular context is critical in determining whether a given treatment will result in growth arrest or apoptosis. That suggests that the therapeutic induction of apoptosis could be quite problematic. The ideal therapeutic agent would both block cell proliferation and promote apoptosis. Much work needs to be done to clarify the intimate relationship between cell-cycle-mediated regulation of proliferation and cell death before we can obtain that goal.

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Characterization of a Novel Amphiregulin-Related Molecule in 12-*O*-tetradecanoylphorbol-13-acetate-Treated Breast Cancer Cells

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Amphiregulin (AR) can be induced at the mRNA level by 17-β-estradiol (E_2) or the phorbol ester tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). This study compares the effects of TPA and E_2 on the regulation of processing of AR isoforms and on subcellular localization in human MCF-7 breast cancer cells. AR was localized in the nucleus of MCF-7 cells after E_2 treatment, whereas it was predominantly secreted after TPA treatment. AR isoforms of 28, 18, and 10 kDa and an additional species of approximately 55–60 kDa were detected in the cellular conditioned media after TPA stimulation. Expression of this unusual AR isoform was inhibited by protein kinase C (PKC) inhibitors such as bryostatin or H-7. The biochemical properties of this isoform are consistent with it being an *N*-linked glycosylated form of the AR precursor that contains unprocessed mannose residues. The size of this large isoform is reduced to approximately 40 kDa after treating the TPA-induced MCF-7 cells with tunicamycin or treating the conditioned media of such cells with *N*-glycosidase F or with endoglycosidase H. Moreover, this isoform is able to bind several lectins with specificity for mannose residues. The 55–60 kDa glycosylated AR isoform, like lower Mr AR isoforms, is able to bind to heparin and to stimulate the growth of MCF-10A cells by interacting with the EGF receptor. These data suggest that TPA activation of PKC may be involved in post-translational modifications of AR, such as glycosylation, and in alteration of its subcellular routing to predominantly a secretory pathway.

Steroid hormones can regulate the expression of or response to locally-derived growth factors in breast cancer cells (Dickson and Lippman, 1995). In addition, phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) that activate protein kinase C (PKC) are important regulators of the expression and action of growth factors. In this context, epidermal growth factor (EGF) family members such as transforming growth factor alpha (TGF-α) or amphiregulin (AR) play an important role in breast carcinogenesis (Dickson et al., 1994). AR contains the characteristic EGF-like motif and exerts its activity by interacting with the EGF receptor (Shoyab et al., 1989). AR was originally isolated from conditioned media of the human breast carcinoma cell line, MCF-7, that had been treated with TPA (Shoyab et al., 1988). AR is synthesized as a 252 aminoacid transmembrane precursor and is secreted as a monomeric peptide containing either 78 or 84 aminoacid residues following proteolytic cleavage of the precursor (Shoyab et al., 1989). The mature forms of AR or its precursor can be modified by glycosylation, and possibly by phosphorylation and sulfation. Various isoforms of AR that differ in the degree of glycosylation and peptide core length, have been found in the conditioned media of cells that secrete this growth factor (Johnson et al., 1993). In the amino-terminal domain of AR, there are two stretches of basic, hydrophilic resi-

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dues which resemble putative nuclear targeting sequences and heparin-binding domains (Plowman et al., 1990). AR can be detected in the cytoplasm and in the nucleus of a variety of breast and colon cancer cell lines and in primary human breast, colon, pancreatic, and ovarian carcinomas using immunocytochemistry (Johnson et al., 1991, 1992; Saeki et al., 1992; Normanno et al., 1993, 1994; Ebert et al., 1994 ; Qi et al., 1994). In contrast to other members of the EGF family but similar to heparin-binding EGF-like growth factor (HB-EGF) (Besner et al., 1992), AR can bind to heparin and can have its mitogenic activity altered (Cook et al., 1991). Whereas the biological activity of HB-EGF is sometimes potentiated by heparin, the activity of AR is severely attenuated (Cook et al., 1995a). The basic regions present in the amino-terminal domain of the AR molecule are not only responsible for heparin-binding but are also crucial for its secretion and for its biological activity (Thorne and Plowman, 1994). We have previously demonstrated that TPA and 17-β-estradiol (E_2) can rapidly increase the expression of AR in several different human breast cancer cells by enhancing transcription of this gene and that TPA can further stabilize AR mRNA (Martínez-Lacaci et al., 1995). In this report, we demonstrate that E_2 and TPA can differentially modify the post-translational glycosylation of AR and its subsequent subcellular localization. Furthermore, in the presence of TPA a novel AR-like molecule, presumably the AR precursor, is secreted to the conditioned media.

MATERIALS AND METHODS

Cell culture

Human breast carcinoma MCF-7 cells were grown in Improved Minimal Essential Medium (IMEM) supplemented with 5% FCS. After 2–4 days, media were replaced with phenol red-free IMEM containing 5% charcoal-treated calf serum (CCS) to remove endogenous steroids (Berthois et al., 1986). Cells were maintained under these conditions for 2–4 days. Subsequently, media were replaced with phenol red-free IMEM containing insulin (5 μg/ml), transferrin (5 μg/ml), and selenious acid (5 ng/ml) (ITS, Collaborative Research, Waltham, MA) and cells were treated with the different drugs and hormones, and harvested at the times indicated. Alternatively, conditioned media were collected. The spontaneously immortalized human mammary epithelial MCF-10A cells were grown in DMEM/HAMF12 supplemented with 5% horse serum, 10 U/ml penicillin-10 g/ml streptomycin, 0.5 g/ml hydrocortisone, 5 g/ml insulin, 0.1 g/ml cholera toxin, and 20 ng/ml EGF. For the growth assays, MCF-10A cells were plated in the absence of EGF.

Growth assays

MCF-10A cells were seeded in 96-well plates as described above at a density of 2,000 cells/well. After 24 hr, cells were treated in triplicates with various concentrations of recombinant EGF (GIBCO, Bethesda Research Laboratories), recombinant AR (generously supplied by Dr. Stewart Thompson, Berlex Biosciences, Richmond, CA), semi-purified AR protein secreted isoforms, and/or the 225 IgG antibody, which blocks the EGF receptor (Gill et al., 1984). After 4 days, cells were fixed with 25% crystal violet-25%methanol, washed, and dried. After 24 hr, cells were destained with 0.1 M sodium citrate-50% ethanol and absorbance at 540 nm was determined.

Western blot analysis

Serum-free conditioned media containing ITS in phenol red-free IMEM were collected from human breast cancer cells treated with different agents, concentrated and 100 μg aliquots were analyzed by Western blot using the affinity-purified polyclonal rabbit antibody AR-Ab2 (2 μg/ml) directed against residues 26–44 of AR (Johnson et al., 1992, 1993). Alternatively, cells were lysed as previously described (Martínez-Lacaci et al., 1995) or subfractionated and proteins were subjected to Western blot analysis using the same antibody as previously described (Martínez-Lacaci et al., 1995).

Subcellular fractionation of MCF-7 cells

Cells were grown as previously described and treated with TPA or E_2 for 24 hr. Subsequently, cells were washed with phosphate-buffered saline (PBS), resuspended in a buffer containing 10 mM Tris pH 7.4, 15 mM NaCl, 1.5 mM $MgCl_2$, 60 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 5% sucrose, 0.5% β-mercaptoethanol, 1% Nonidet P-40, 0.5% deoxycholate; proteinase inhibitors were added: aprotinin (40 μg/ml), pepstatin A (5 μg/ml), leupeptin (25 μg/ml), and phenylmethylsulfonyl fluoride (0.3 mg/ml) and samples were kept on ice. Cells were homogenized using a Dounce homogenizer, loaded on a sucrose cushion, and centrifuged at 600g for 10 min ar 4°C. Supernatant was saved as the cytosolic fraction and the pellet was resuspended in the same buffer with the exception of EDTA, EGTA, sucrose, and Nonidet P-40, centrifuged again, and kept as the nuclear fraction. Both fractions were sonicated, concentrated, and the protein concentration in the samples was determined.

Digestion of proteins with glycosidases

Fifty microgram aliquots of protein from conditioned media were dried in a Speed-Vac concentrator (Savant), dissolved in 50 μl of a buffer containing 0.2 M sodium phosphate pH 8.6, 25 mM EDTA, 1.25% Nonidet P-40, 0.5% SDS and 1% β-mercaptoethanol, and treated with 20 U/ml of *N*-glycosidase F or 1 mU/ml of *O*-glycosidase for 16 hr at 37°C. Alternatively, samples were resuspended in 50 μl of PBS and treated with 40 mU/ml of endoglycosidase H for 16 hr at 37°C.

Detection of carbohydrates

In order to characterize the carbohydrate nature of the glycosylated AR form(s), dialyzed, concentrated conditioned media were applied to a variety of lectin affinity columns (EY laboratories, Inc., San Mateo, CA) equilibrated with either Tris-buffered saline (TBS) or PBS. Proteins were eluted with the appropriate carbohydrate and protein concentration was monitored by determining the optical density (O.D.) at 280 nm. The immobilized lectins used were *canavalia ensiformis* (Con A), *lens culinaris* (LcH), *ricinus communis* I (RCA-I), *triticum vulgare* (WGA), *ulex europaeus* (UEA-I), and *arachis hypogea* (PNA). Glycoproteins were eluted from the different columns using the following carbohydrates: 0.1 M methyl α-D-mannopyranoside for Con A, 0.1 M D-mannose for LcH, 0.1 M α-lactose for RCA and

PNA, 0.1 M α-acetyl-D-glucosamine for WGA, 0.05 M α-L-fucose for UEA, and 0.1 M α-lactose for PNA. Alternatively, carbohydrates were detected as described in the literature of the digoxigenin (DIG) glycan differentiation kit (Boehringer Mannheim, Indianapolis, IN). Briefly, 100 μg aliquots of protein from conditioned media were electrophoresed on a 10% tricine SDS-PAGE gel, transferred to a nitrocellulose filter, blocked with manufacturer's blocking reagent, and incubated with different DIG-labeled lectins: *galanthus nivalis* agglutinin (GNA), *sambucus nigra* agglutinin (SNA), *maackia amuurensis* agglutinin (MAA), peanut agglutinin (PA), and *datura stramonium* agglutinin (DSA). Subsequently, filters were washed, incubated with anti-digoxigenin Fab fragments coupled to alkaline phosphatase, washed, and stained with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indol-phosphate. Positive controls used were the following: carboxypeptidase Y (for GNA), transferrin (for SNA), fetuin (for SNA, MAA and DSA), and asialofetuin (for PNA and DSA).

Separation of AR secreted protein isoforms

MCF-7 cells were grown and treated with TPA for 72 hr as previously described. Subsequently, approximately 300 ml of serum-free conditioned media containing ITS were collected and stored at −70°C. Proteinase inhibitors were added: aprotinin (40 μg/ml), pepstatin A (5 μg/ml), leupeptin (25 μg/ml), and phenylmethylsulfonyl fluoride (0.3 mg/ml). Subsequently, proteins were precipitated with 70% ammonium sulfate and dialyzed at 4°C against PBS. Subsequently, approximately 2 ml of dialyzed, concentrated conditioned media [in 1 M sodium chloride-PBS (pH 7.4)] were applied to a Sephacryl S-200 gel chromatography column (Pharmacia Biotech, Piscataway, NJ) previously equilibrated with 1 M NaCl-PBS and proteins were eluted in the same buffer at a rate of 0.2 ml/min. The O.D. at 280 nm of the obtained fractions was measured and fractions were stored at -20°C. Alternatively, dialyzed, concentrated conditioned media were applied to a heparin affinity column (Bio-Rad, Richmond, CA) equilibrated with PBS and proteins were eluted with a stepwise gradient of 0.05–2 M NaCl. The O.D. at 280 nm of the fractions was determined and fractions were stored at −20°C.

Immunocytochemistry

MCF-7 cells were plated in eight-chamber Lab-Tek slides and treated with either TPA (100 nM) or E_2 (1 nM) in IMEM containing 5% CCS for 24 hr. Subsequently, cells were fixed in 4% formaldehyde-PBS for 30 min at 20°C, washed twice with PBS and incubated with 10% goat serum diluted in PBS containing 0.1% BSA for 45 min. Subsequently, slides were incubated with 605 Ab or AR-Ab1 (10 μg/ml), both of them directed against residues 8–26 of AR (Johnson et al., 1991) or with preimmune serum (604) for 1 hr and washed twice with PBS for 10 min. Alternatively, 605 and AR-Ab1 antibodies were preabsorbed with peptide P_1 (AR 8-26) for 2 hr at 37°C. Then, slides were incubated with biotinylated goat antibody (1:200) (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) for 1 hr, washed, incubated with Vectastain ABC reagents for 10 min, washed, and incubated for 1–2 min in diaminobenzidine/urea hydrogen peroxide (Sigma, St Louis, MO). Slides were rinsed in water, incubated with ethanol for 10 min, incubated with xylene for 10 min, and mounted (Normanno et al., 1993).

Reagents

The antibodies 605, AR-Ab1, and AR-Ab2 were obtained from Dr. Gibbes Johnson (Division of Cytokine Biology, Food and Drug Administration, Bethesda, MD) and they were generated, purified, and characterized as previously described (Johnson et al., 1991, 1992, 1993). The antibody 225 was generously obtained from Dr. John Mendelsohn (Laboratory of Receptor Biology, Memorial Sloan-Kettering Cancer Center, New York, NY) and it has previously been described (Gill et al., 1984). Bryostatin-1 was obtained through the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, NIH, Bethesda, MD. H-7 was purchased from Seikagaku America, Rockville, MD. Tunicamycin and 4-methylumbelliferyl β-D-xyloside were purchased from Sigma, St. Louis, MO. *N*-glycosidase F, *O*-glycosidase, endoglycosidase H, neuraminidase, and digoxigenin-labeled lectins were purchased from Boehringer Mannheim, Indianapolis, IN.

RESULTS

Effects of TPA and E_2 on AR protein

MCF-7 cells were treated with TPA (100 nM) and E_2 (1 nM) for 24 hr and AR protein levels were analyzed by Western blot in total cellular lysates as well as in the cytosolic and nuclear fractions. Additionally, levels of secreted AR protein were analyzed after 72 hr of treatment with TPA or E_2. (Fig. 1). TPA induced the expression and secretion of several distinct immunoreactive species of AR. An ≈ 55–60 kDa immunoreactive isoform of AR was the most predominant. However, this form was not present either in cell lysates or in the cytosolic or nuclear fractions. On the other hand, in the presence of E_2, there was a slight induction of AR protein levels mainly in the nuclear fraction. Moreover, it is very interesting to note that only the smaller AR forms were located in the nucleus compared to the cytosol.

Blockade of TPA effect on AR secreted proteins

MCF-7 cells were treated with TPA in the presence or absence of PKC inhibitors (bryostatin-1 and H-7) for 48 hr and serum-free conditioned media were analyzed by Western blot for AR secretion (Fig. 2). Bryostatin-1 was able to abolish the induction of the ≈ 55–60 kDa species to a greater extent than other immunoreactive AR isoforms. H-7 also reduced the TPA induction of this species. Collectively, these data indicated that the induction of the secreted ≈ 55–60 kDa form is a PKC-related process.

Characterization of the ≈ 55–60 kDa band

To more fully define the relationship of this novel ≈ 55–60 kDa species to other low molecular mass forms of AR, TPA-treated MCF-7 cells were treated with tunicamycin (2 μg/ml), which prevents *N*-glycosylation of glycoproteins, for 48 hr. Conditioned media were collected and subjected to Western blot analysis (Fig. 3A). Alternatively, 50 μg-aliquots of conditioned media from TPA-treated MCF-7 cells were incubated with *N*-gly-

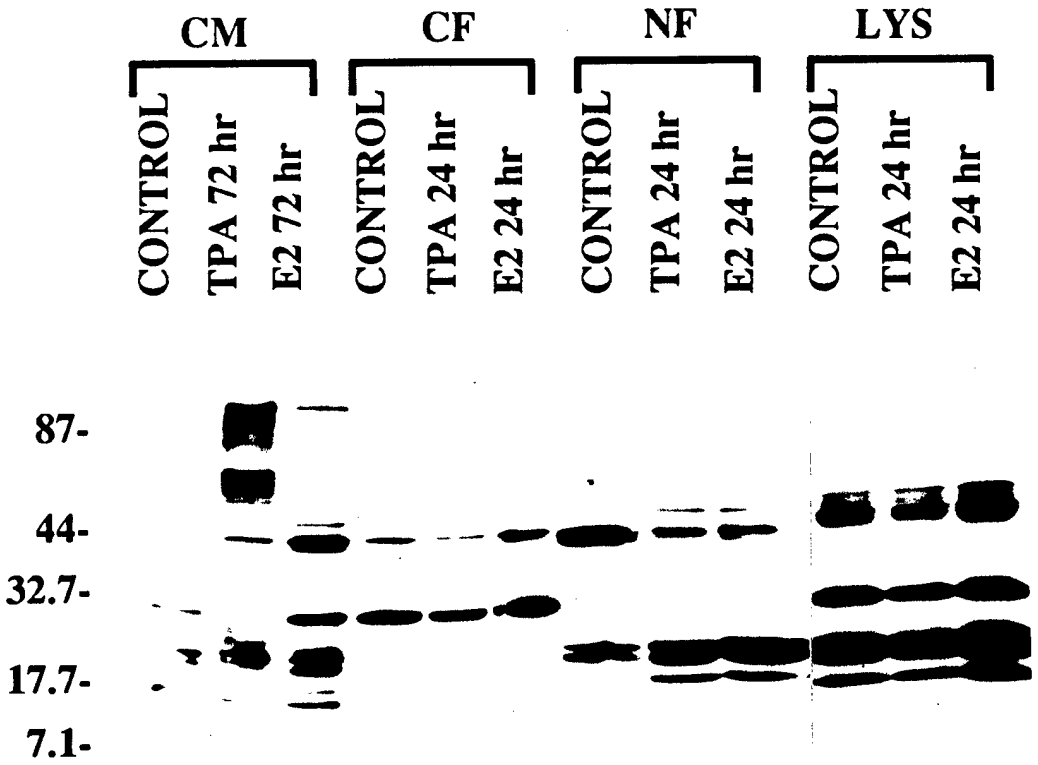

Fig. 1. Effects of TPA and E_2 on AR protein levels. MCF-7 cells were grown as described in Materials and Methods and treated with TPA (100 nM) or E_2 (1 nM) for several times. After 72 hr of treatment, serum-free conditioned media (CM) were collected and analyzed by Western blot. After 24 hr of teatment, cells were subfractionated into nuclear fraction (NF) or cytosolic fraction (CF) or lysed (LYS) as described in Materials and Methods and analyzed by Western blot. Sizes are indicated in kilodaltons to the left.

cosidase F and subsequently processed for Western blot analysis (Fig. 3B). After this treatment, the ≈ 55–60 kDa band disappeared and it was shifted down to ≈ 40 kDa, which corresponds with the size of the peptide core of the AR precursor (L. Wong and G.R. Johnson, unpublished observations). In addition, a new band of ≈ 14 kDa appeared which probably corresponds to mature deglycosylated AR (Shoyab et al., 1988). Similar results were found after treating the cells with tunicamycin at the same time as TPA (Fig. 3A). In contrast, the immunoreactive isoforms of AR were not altered after the *O*-glycosidase treatment, suggesting that the oligosaccharides are not *O*-linked (data not shown). MCF-7 cells were also treated with 4-methylumbelliferyl β-D-xyloside (0.5 mM), an inhibitor of glycosaminoglycans (GAG) additions to core proteins, in the presence or absence of TPA for 48 hr to determine whether the ≈ 55–60 kDa species represents an AR precursor form with GAG attached. Indeed, a slight reduction of the ≈ 55–60 kDa isoform was obtained after this treatment (Fig. 3C).

Carbohydrate composition of immunoreactive AR isoforms

Conditioned media were applied to different lectin columns and the eluted fractions subjected to Western blot using the AR-Ab 2 antibody in order to analyze the carbohydrate composition of the ≈ 55–60 kDa AR species. Various immunoreactive AR isoforms were able to bind to Con A and LcH, suggesting a high mannose component. In addition, glycosylated AR was also able to bind to WGA, which has affinity for *N*-acetylglucosamine residues and for sialic acid (Table 1). To further confirm these results, conditioned media from TPA-treated MCF-7 cells were electrophoresed on an SDS-PAGE gel, transferred to a nitrocellulose filter and incubated with several digoxigenin-labeled lectins. Two bands of ≈ 55–60 kDa produced a positive signal with GNA, which is specific for terminally linked mannose residues. These bands were absent when proteins from conditioned media samples were prior treated with either *N*-glycosidase F or with endoglycosidase H. In addition, two bands became apparent when proteins from conditioned media were immunoprecipitated with the AR-Ab 2 antibody and subsequently incubated with GNA, confirming that at least some of the ≈ 55–60 kDa immunoreactive AR isoforms contain mannose residues (data not shown). In addition, some bands within this region but of a slightly different size than the ones that reacted with GNA reacted with SNA and MAA which are specific for sialic acid. These data suggest that the broad ≈ 55–60 kDa band is a complex of AR-related isoforms that contain different amounts of mannose, GAG and terminal sialic acid (Table 1).

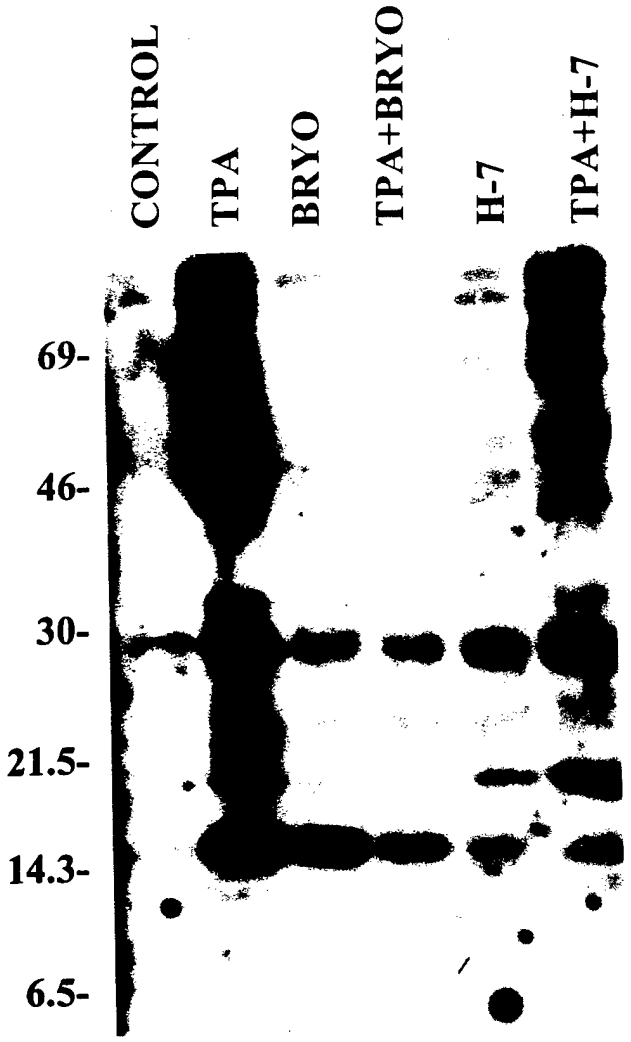

Fig. 2. Blockade of TPA effects on AR secreted proteins. MCF-7 cells were grown as described in Materials and Methods, treated with bryostatin-1 (BRYO) (100 nM) or H-7 (40 μM) in the presence or absence of TPA (100 nM) for 48 hr, CM were collected and subjected to Western blot analysis. Sizes are indicated in kilodaltons to the left.

Heparin-binding activity of glycosylated AR protein

To ascertain whether the ≈ 55–60 form(s) bind to heparin, conditioned media samples were applied to a heparin affinity column. It was observed that all the immunoreactive AR protein isoforms were able to bind to heparin, but that the ≈ 55–60 kDa species, which resolved as two separate bands, eluted with a lower concentration of NaCl than the other isoforms (Fig. 4).

Size fractionation of conditioned media

To more fully characterize the size and bioactivity of the different immunoreactive AR species, serum-free conditioned media from TPA-treated MCF-7 cells were concentrated and subjected to gel filtration chromatography (Sephacryl S-200) equilibrated with 1 M NaCl-PBS in order to separate all the AR isoforms. Fractions were collected and analyzed by Western blot using the AR Ab-2 antibody (Figs. 5, 6). The heterogeneous ≈ 55–60 kDa band was separated into two peaks, indicating that there are at least two different forms present. The ≈ 60, ≈ 55, ≈ 28, and ≈ 10 kDa protein species were pooled and their concentrations were estimated after Western blot by comparison to purified recombinant AR (rAR). Different concentrations of rAR along with aliquots from all the AR isoforms obtained from the gel filtration column were analyzed by Western blot analysis and quantified by scanning densitometry (data not shown). Fractions 22–23, 17–21, and 17–20, derived from three different runs of the Sephacryl S-200 column contained the 60 kDa form; fractions 24–26, 22–26, and 21–25 from the same runs contained the ≈ 55 kDa form. Fractions 21–29 from a different run contained the ≈ 28 kDa form. From this last run, fractions 50–53 were found to contain the ≈ 18 kDa form and fractions 54–58 contained the ≈ 10 kDa form.

Bioactivity of semi-purified AR secreted protein forms

To determine whether the AR protein isoforms eluted from the gel exclusion chromatography were biologically active, MCF-10A cells were treated with various concentrations of the isolated AR species, rAR or EGF for four days (Fig. 7). The 60, 55, and 28 kDa forms appeared to be equipotent to rAR, the 28 kDa being the most potent species at 10 ng/ml. In contrast, the 10 and 18 kDa forms exibited a much weaker mitogenic effect. Additionally, concentration-dependent cell growth assays were carried out with the different AR immunoreactive isoforms in comparison to the effect of rAR on MCF-10 A cells (Fig. 8). To demonstrate that growth stimulatory effects of these AR isoforms were mediated through the EGF receptor, MCF-10A cells were treated with either rAR or with fractions containing the 60 kDa and 28 kDa forms (10 ng/ml) in the presence or absence of the anti-EGF receptor blocking 225 IgG antibody (50 μg/ml) (Gill et al., 1984). The growth stimulatory action of each of the pooled fractions containing the different AR isoforms was effectively blocked by the anti-EGF receptor antibody (Fig. 9).

Subcellular detection of AR protein

MCF-7 cells were grown in eight-chamber slides, treated with TPA or E_2 for 24 hr and processed for immunocytochemistry in duplicates. Intracellular reactivity with the AR-Ab1 antibody was considerably more intense in E_2-treated cells (Fig. 10E) than in TPA-treated cells (Fig. 10D) or in non-treated cells (Fig. 10C). In addition, in the E_2-treated cells there were many nuclei which stained with the antibody (Fig. 10F). Preimmune serum (604) (Fig. 10A) or preabsorption of the AR-Ab1 with the peptide immunogen (P_1) (Fig. 10B) showed no immunoreactivity, demonstrating the specificity of the AR-Ab1 antibody for detecting AR protein. Additionally, when cells were grown, treated with TPA or E_2 for 24 hr and subfractionated there was a preferential distribution of the smaller AR isoforms to the nucleus, with an increase of these species after TPA or E_2 treatment (Fig. 1). Corroborating the immunocytochemical analysis (Table 2) the nuclear accumulation of AR protein species after E_2 treatment was higher than after TPA treatment.

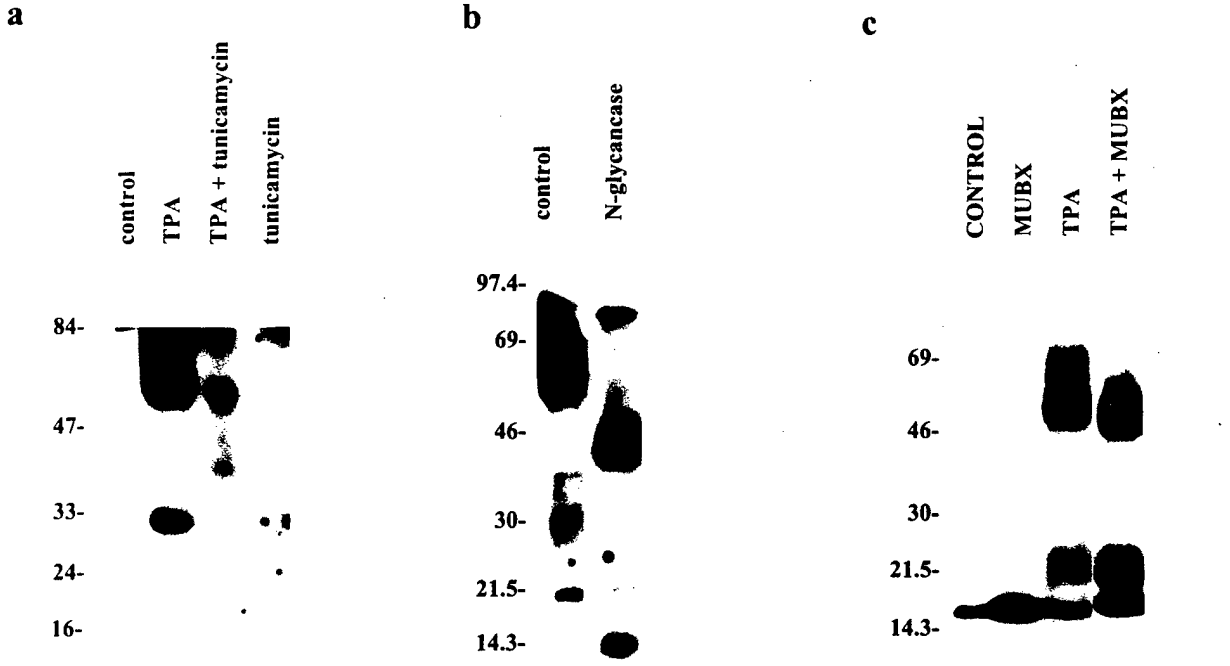

Fig. 3. Characterization of AR secreted proteins. **A:** Effect of tunicamycin on AR secreted proteins. MCF-7 cells were grown and treated with tunicamycin (2 μg/ml) in the presence or absence of TPA (100 nM) for 48 hr. Subsequently, CM were collected and 100 μg of protein were subjected to Western blot analysis. Sizes are indicated in kilodalton to the left. **B:** Digestion of AR proteins with glycosidases. MCF-7 cells were grown, treated with TPA (100 nM) fo 48 hr and 50 μg of proteins from CM were treated with *N* glycosidade F (20 U/ml) as described in Materials and Methods and analyzed by Western blot. **C:** Effect of 4-methylumbelliferyl -β-D-xyloside. MCF-7 cells were treated with 4-methylumbelliferyl -β-D-xyloside (MUBX) (0.5 mM) in the presence or absence of TPA (100 nM) for 48 hr; CM were collected and subjected to Western blot analysis.

TABLE 1. Carbohydrate Analysis of Immunoreactive AR Secreted Isoforms[1]

LECTIN (origin)	Abbreviation	Sugar specificity	Detection	Reaction
Canavalia ensiformis	Con A	Branched mannose	Column	++
Lens culinaris	LcH	D-mannose, D-glucose	Column	++
Ricinus comunis I	RCA-I	Lactose, Galactose	Column	−
Triticum vulgare	WGA	*N*-AcGlucosamine, Sialic acid	Column	+
Ulex europaeus I	UEA-I	Fucose	Column	−
Arachis hypogea	PNA	β-galactose	Column	−
Galanthus nivalis	GNA	Terminal mannose	DIG-labeled	++
Sambucus nigra	SNA	Sialic acid	DIG-labeled	+
Maackia amurensis	MAA	Sialic acid	DIG-labeled	+
Arachis hypogea	PNA	Galactose	DIG-labeled	−
Datura stramonium	DSA	Galactose	DIG-labeled	−

[1]MCF-7 cells were grown, treated with TPA (100 nM) for 72 hr; CM were collected as described in Materials and Methods and analyzed by lectin chromatography followed by Western blot analysis with the AR-Ab2. Alternatively, CM were subjected to Western blot analysis using digoxigenin (DIG)-labeled lectins as described in Materials and Methods. ++, strong reaction; +, moderate reaction; −, negative reaction.

DISCUSSION

Expression of AR protein in human breast cancer cells

The expression of several growth factors in the EGF family such as EGF, TGF-α, AR, and HB-EGF can be regulated by either estrogen and/or progesterone in human breast cancer cells or in rodent or primate uterus (Martínez-Lacaci and Dickson, 1996). We have previously demonstrated that TPA (100 nM) and E_2 (1 nM) can stimulate the expression of immunoreactive AR protein after treatment of MCF-7 cells (Martínez-Lacaci et al., 1995) and MDA-MB-231 cells (data not shown). Whereas both E_2 and TPA can enhance the

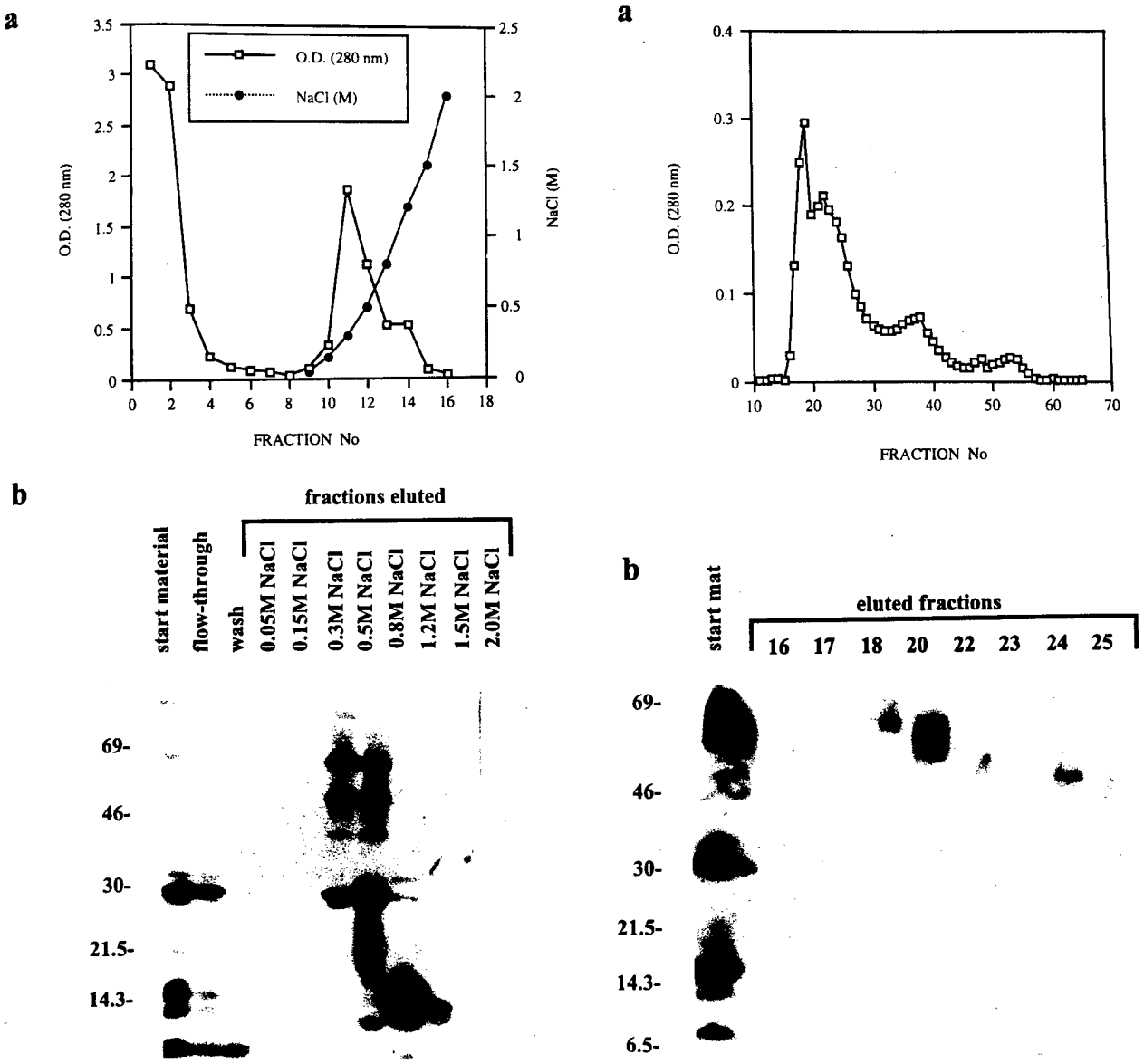

Fig. 4. Heparin binding activity of AR secreted proteins. MCF-7 cells were grown, treated with TPA and CM were collected and applied to heparin affinity column chromatography as described in Materials and Methods. Fractions were eluted from the column with a NaCl step-wise gradient and analyzed by Western blot. **A:** Graphic representation of the O.D. values (280 nm) of the different fractions collected from the column. **B:** Western blot of the different fractions eluted from the heparin chromatography column. Sizes are indicated in kilodaltons to the left.

Fig. 5. Separation of AR secreted proteins (60, 55 kDa forms). MCF-7 cells were grown, treated with TPA, CM were collected and applied to a gel filtration chromatography column (Sephacryl S-200), fractions were collected and subjected to Western blot analysis. Molecular mass standards were eluted from the column as follows: 2,000 kDa in fraction No (FN) 18, 200 kDa in FN 21, 150 kDa in FN 22, 66 kDa in FN 25, 29 kDa in FN 33, and 12.4 kDa in FN 37. **A:** Graphic representation of the O.D. values (280 nm) of the fractions collected. **B:** Western blot analysis of the fractions collected. Sizes are indicated in kilodaltons to the left.

rate of transcription, only TPA can stimulate stability of AR mRNA and has an appreciable effect upon stimulating secretion of AR (Martínez-Lacaci et al., 1995). The most predominant protein is a heterogenous species of ≈ 55–60 kDa (Fig. 1) that is significantly stimulated with TPA within 16 hr (data not shown). This species can be inhibited by treatment with bryostatin-1 (Pettit et al., 1982) and also with H-7 (Kawamoto and Hidaka, 1984), indicating that this induction is mediated by PKC (Fig. 2).

Nature of the ≈ 55–60 kDa AR isoform

The predicted amino-acid sequence of the AR precursor reveals three putative *N*-linked glycosylation sites and five potential GAG attachment sites (Plowman et al., 1990). A protein that migrates as ≈ 43 kDa in SDS-PAGE gels can be generated by in vitro transcription and translation of the full-length AR cDNA and is

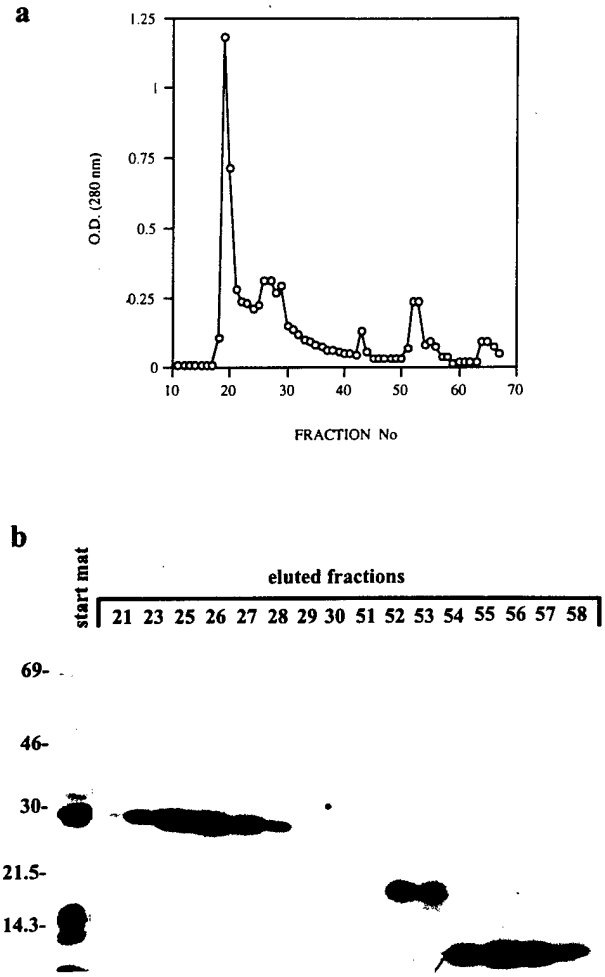

Fig. 6. Separation of AR secreted proteins (28, 18, and 10 kDa forms). MCF-7 cells were grown, treated with TPA, conditioned media were collected and applied to a gel filtration chromatography column (Sephacryl S-200) equilibrated with 1 M NaCl-PBS and fractions were collected. Molecular mass standards were eluted from the column as indicated in the legend of Figure 5. **A:** Graphic representation of the O.D. values (280 nm) of the fractions collected. **B:** Western blot analysis of the fractions collected. Sizes are indicated in kilodaltons to the left.

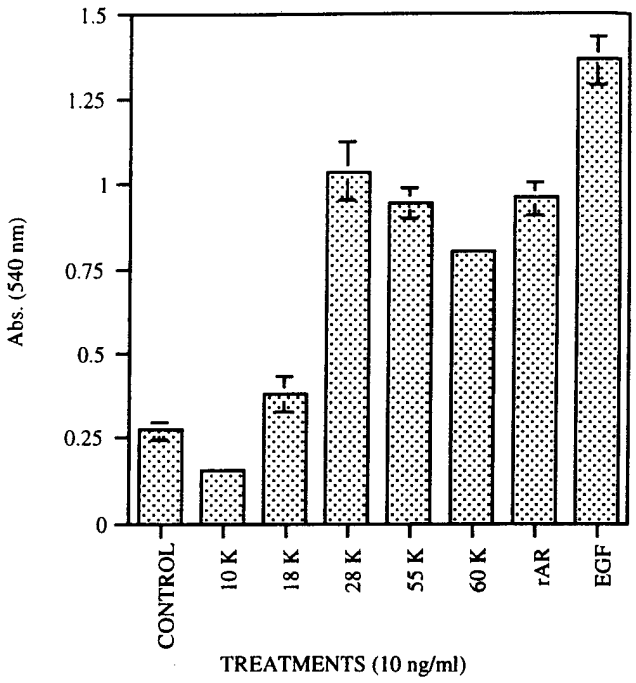

Fig. 7. Biological activity of separated AR isoforms. **A:** MCF-10 A cells were grown and treated in triplicates with 10 ng/ml of the different semi-purified AR protein isoforms, rAR or EGF for four days as described in Materials and Methods. Cells were stained with crystal violet, destained and absorbance (540 nm) was determined as indicated in Materials and Methods. Error bars are the standard error of the mean.

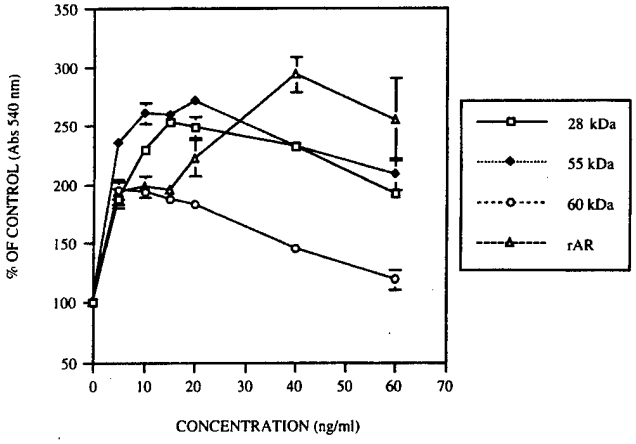

Fig. 8. Concentration-response of the biological effects of separated AR isoforms. MCF-10A cells were grown and treated in triplicates with different concentrations (5, 10, 15, 20, 40, and 60 ng/ml) of semi-purified AR isoforms or rAR for 4 days, stained with crystal violet, destained, and absorbance (540 nm) was determined as described in Materials and Methods. Error bars are the standard error of the mean and are the average of five different experiments.

thought to represent the unglycosylated form of the AR precursor (L. Wong and G.R. Johnson, unpublished observations). We observed that the ≈ 55–60 kDa species is shifted to a species of ≈ 40 kDa after tunicamycin or *N*-glycosidase F treatment (Fig. 3A,B), strongly suggesting that this species represents a modified AR precursor form with *N*-linked sugars. Conversely, treatment with *O*-glycosidase had no effect (data not shown). Additionally, the ≈ 55–60 kDa band can be significantly reduced after treating the cells with 4-methylumbelliferyl -β-D-xyloside, which prevents docking of GAG chains to core proteins (Fig. 3C). Since immunoreactive proteins in this region are present in a relatively broad band, it is likely to represent a heterogenous group of proteins which varies in the degree and/or type of glycosylation.

Nature of smaller AR-related proteins

As suggested previously, the ≈ 28 and ≈ 18 kDa proteins that react with the AR-Ab2 antibody are likely

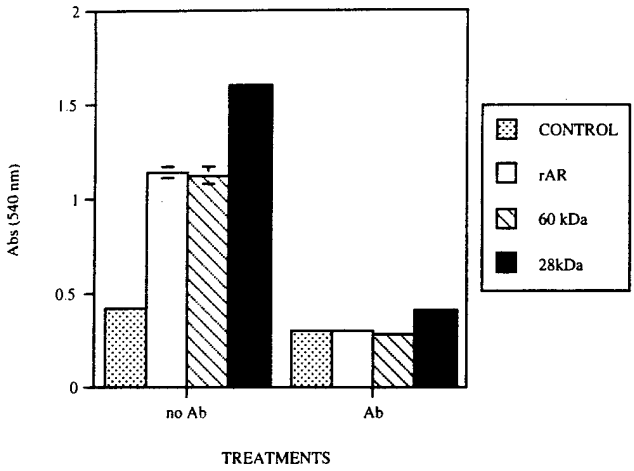

Fig. 9. Blockade of the EGF receptor of MCF-10A cells. MCF-10A cells were grown and treated in triplicates with 28 and 60 kDa AR isoforms or rAR (10 ng/ml) for 4 days in the presence or absence of the 225 monoclonal anti-EGF receptor antibody (50 μg/ml), stained with crystal violet, destained and absorbance (540 nm) was determined as described in Materials and Methods. Error bars indicate the standard error of the mean.

to represent mature forms of AR. The ≈ 10 kDa band represents a minor, proteolytically-processed form that is not always detected. A new band of ≈ 14 kDa appears after the *N*-glycosidase F or tunicamycin treatment (Fig. 3B). This species may represent a deglycosylated mature form of AR which has been previously described (Shoyab et al., 1988). In contrast to the lower molecular mass immunoreactive AR isoforms, the ≈ 55–60 kDa immunoreactive AR form is not detected in cell lysates (Fig. 1). This observation suggests that after TPA treatment, most of the synthesized AR is rapidly processed to a soluble form(s) and then released into the culture media. It is possible that this results in further processing at the surface or outside the cell.

Carbohydrate analysis of AR isoforms

Conditioned media were applied to diverse lectin affinity columns and subjected to lectin blotting in order to analyze the carbohydrate composition of the *N*-linked oligosaccharides. Immunoreactive AR was first bound and eluted from Con A and LcH affinity columns (Table 1). These two lectins selectively bind to mannose residues, which suggests that the high molecular mass AR isoform contains *N*-linked mannose sugar residues. The presence of *N*-linked sugar residues was confirmed using WGA affinity column, which binds to *N*-acetylglucosamine, β_{1-4}-linked oligosaccharides and sialic acid (Table 1). Further confirmation of this result was obtained by immunoprecipitation of conditioned media from TPA-treated cells with the AR-Ab2 antibody followed by lectin blotting with GNA, a lectin specific for terminally linked mannose residues (data not shown). Finally, in the region corresponding to the ≈ 55–60 kDa immunoreactive AR isoforms, some bands appeared that reacted on lectin blotting with SNA and MAA, which are specific for sialic acid (Table 1). This type of site heterogeneity, consisting of variable *N*-linked sugars sharing a common peptide is reported to be common (Rademacher et al., 1988). Thus, the ≈ 55–60 kDa species appears to represent a heterogenous group of *N*-glycosylated proteins.

In cancer, a correlation has been previously noted between alterations in cell-surface carbohydrates and metastasis (Ishikawa et al., 1988). Although we observed that MCF-7 secrete both *O*-linked and *N*-linked glycoproteins, immunoreactive AR and glycoproteins within the ≈ 55–60 kDa region were mainly composed of *N*-linked species containing untrimmed mannose residues, some of which appeared to contain sialic acid or GAGs. At present, the significance of terminal mannose *N*-linked glycoproteins in breast cancer is unknown. The addition of carbohydrates is thought to facilitate protein folding and protein stabilization. *N*-glycans may be also used as protein sorting signals for endoplasmic reticulum to Golgi and Golgi to cell surface transport and for apical surface routing of epithelial cell proteins for secretion (Fiedler and Simons, 1995). AR precursor may acquire *N*-linked carbohydrates for its targeting for cellular secretion. Subsequently, deglycosylation and further proteolytic processing may be required for its conversion into mature AR forms. PKC may be involved in several of these post-translational modifications.

The mechanism of AR precursor processing is unknown. However, it has been shown that the TGF-α membrane-bound precursor is palmitoylated and that secreted larger species are *N*-glycosylated (Bringman et al., 1987). These are presumably derived from the same precursor. Furthermore, the cleavage of the TGF-α membrane-bound precursor (Pandiella and Massagué, 1991) and its secretion (Ramesh and Levine, 1995) are regulated by phorbol esters that activate PKC. In addition, processing and release of the HB-EGF membrane-bound precursor is also induced by TPA (Raab et al., 1994; Goishi et al., 1995). *neu* differentiation factor (NDF)/heregulin is another EGF-related peptide whose processing and cleavage from the membrane can be regulated by TPA as well (Burgess et al., 1995). Hence, TPA may be also involved in processing of AR precursor by increasing the rate of secretion, proteolysis or cleavage from the cell surface. Additionally, glycoproteins containing high mannose residues may have other functions based on their ability to bind to the mannose 6 phosphate/insulin growth factor-II (M6P/IGF-II) receptor (Morgan et al., 1987; Lobel et al., 1988). It is not known whether the high molecular mass AR-related forms are able to bind to the M6P/IGF-II receptor.

Heparin binding activity of AR-related isoforms

To determine if the ≈ 55–60 kDa isoform could bind to heparin, serum-free conditioned media from TPA-treated MCF-7 cells were applied to a heparin affinity column (Fig. 4). All the immunoreactive AR forms were able to bind heparin, including the heavily glycosylated forms. However, the ≈ 55–60 kDa species eluted with a lower concentration of NaCl than the smaller forms suggesting that it may have a lower affinity for heparin. It has been shown that heparin and heparan sulfate can inhibit the growth stimulatory effects of AR (Cook et al., 1991; Li et al., 1992), whereas HB-EGF can be stimulated in some cases by virtue of the leucine$_{76}$ in its carboxy terminal domain (Cook et al., 1995a). AR

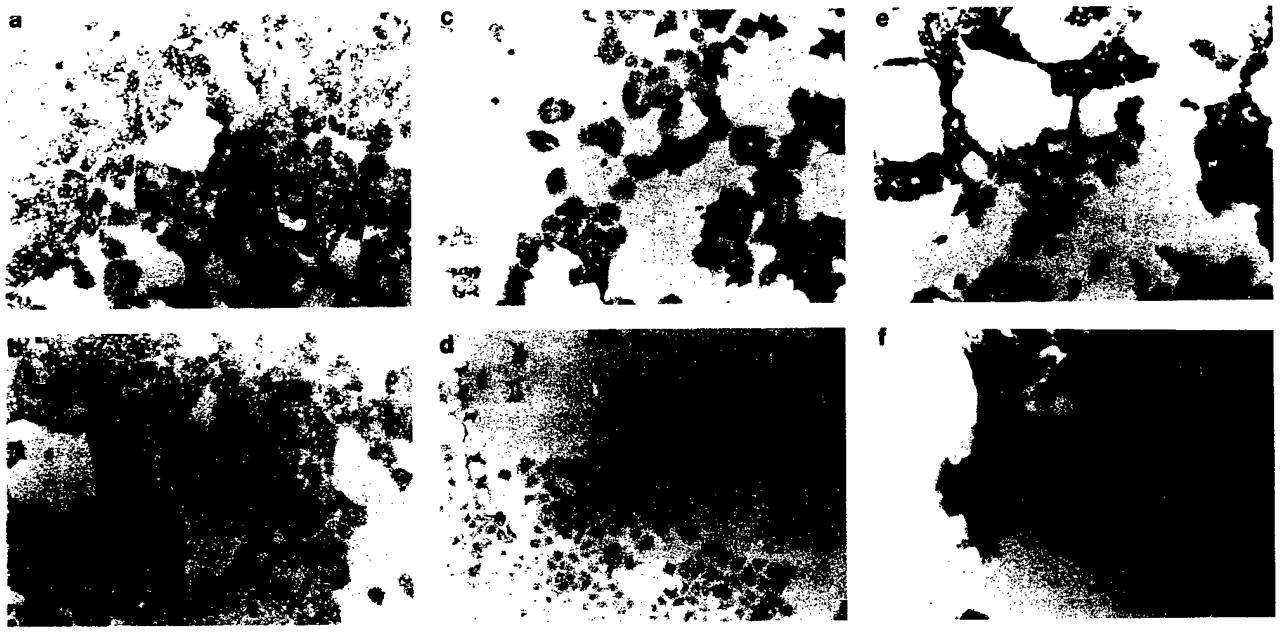

Fig. 10. Subcellular detection of AR protein. MCF-7 cells were grown in eight-chamber slides, treated with TPA or E_2 and processed for immunocytochemistry as described in Materials and Methods. **a:** Cells incubated with preimmune serum (604). **b:** Cells incubated with AR-Ab1 preabsorbed with peptide immunogen (P_1). **c:** Untreated cells incubated with AR-Ab1. **d:** TPA-treated cells incubated with AR-Ab1. **e:** E_2-treated cells incubated with AR-Ab1 (4× magnification). **f:** E_2-treated cells incubated with AR-Ab1 (10× magnification).

requires the presence of membrane proteoglycans such as heparan sulfate in order to exert its bioactivity (Piepkorn et al., 1994). However, HB-EGF mitogenic activity is not affected by heparin agonists or inhibitors of proteoglycan sulfation (Cook et al., 1995b). In this regard, soluble GAGs may compete with membrane-anchored proteoglycans for AR binding, thereby attenuating its mitogenic activity. Furthermore, it has been shown that heparan sulfate is necessary for AR to stimulate growth through the EGF receptor (Johnson and Wong, 1994; Cook et al., 1995b). This suggests that membrane-anchored proteoglycans may increase the affinity or prolong the duration of binding of AR to the EGF receptor and form a ternary complex, similar to FGF and its receptor interaction (Yayon et al., 1991; Kan et al., 1993).

Biological activity of AR-related isoforms

The biological role of glycosylation of AR is unclear at present. However, it is thought that glycosylation of proteins increase their resistance to protease digestion and that carbohydrate additions may influence thermal protein stability, rate of proteolysis, and solubility (Rademacher et al., 1988). Glycosylation of cytokines usually does not interfere with biological activity. In this respect, mature AR is known to be *N*-glycosylated and removal of the sugar residues does not compromise its bioactivity (Shoyab et al., 1988). In order to determine the biological activity of the ≈ 55–60 kDa immunoreactive AR species, serum-free conditioned media from TPA-treated MCF-7 cells were fractionated using gel filtration chromatography and subjected to Western blot analysis (Figs. 5, 6). The ≈ 60, ≈ 55, ≈ 28, ≈ 18, and ≈ 10 kDa protein species obtained were assessed for their ability to stimulate growth of MCF-10A human mammary epithelial cells (Fig. 7) that normally require exogenous EGF or AR for proliferation (Normanno et al., 1994). The ≈ 60, ≈ 55, and ≈ 28 kDa AR protein forms were able to induce MCF-10A growth in the absence of EGF to the same extent as rAR, with the ≈ 28 kDa species being the most potent form. Concentration-dependent growth studies showed that the maximal effect was obtained with 10–20 ng/ml of ≈ 28, ≈ 55, or ≈ 60 kDa forms (Fig. 8); these growth effects were equivalent to those observed with rAR. The growth stimulatory effects of the AR secreted isoforms were abolished when the 225 monoclonal anti-EGF receptor antibody was added to the cells in a 500–700-fold excess (Fig. 9). These data demonstrate that the glycosylated, putative AR precursor is able to stimulate MCF-10A cell growth by interacting with the EGF receptor as has been described for other smaller, processed AR forms (Johnson et al., 1993). Moreover, the immunoreactive AR isoforms (28, 55, and 60 kDa) were able to induce autophosphorylation of the EGF receptor in MCF-10A cells (data not shown).

Subcellular localization of AR

The subcellular distribution of AR protein was ascertained by immunocytochemistry of MCF-7 cells (Fig. 10). Surprisingly, the staining was more intense after E_2 treatment. In many cells staining was localized into the nucleus (Fig. 10e,f). Conversely, MCF-7 cells exhibited diminished staining for AR after TPA treatment (Fig. 10d). Moreover, there was a preferential localization of the smaller AR protein isoforms to the nucleus after TPA and even more after E_2 treatment. (Fig. 1). These data suggest that the mechanism

TABLE 2. Analysis of the Immunocytochemical Data[1]

Treatments	% of Nuclei stained	SEM (n = 4)
Control	4.17	1.46
E_2	30.01*	3.45
TPA	8.70*	2.64

[1]Cells were treated and analyzed by immunocytochemistry as shown in Figure 10. Cells and the nuclei that were stained were counted under a phase contrast microscope. The same number of cells (≈ 500) per slide and treatment were counted and referred as percentage. n represents the number of slides analyzed; *($P < 0.05$) comparing E_2- and TPA-treated values to control.

whereby AR is secreted into the media is mediated or modulated by PKC. The ≈ 55–60 kDa glycosylated putative AR precursor is secreted into the media and apparently excluded from the nucleus, since it was not detected either in whole cell lysates or in the cytosolic or nuclear fraction (Fig. 1). The role of growth factors in the nucleus is not well understood. Nuclear receptors for growth factors may exist in the inner nuclear membrane. Their tyrosine kinase activity may thus phosphorylate nuclear proteins upon binding of the ligand. Alternatively, growth factors may somehow interact with chromatin and ultimately regulate DNA replication or transcription (Burwen and Jones, 1987; Levine and Prystowsky, 1995). It has been recently shown that EGF can induce translocation of the EGF receptor into the nucleus of human squamous carcinoma cells and is associated with tyrosine phosphorylation of nuclear proteins (Holt et al., 1994). This effect is regulated by serum-derived factors in normal cells (Holt et al., 1995). EGF receptor has also been found in the nucleus and nuclear membranes of human choriocarcinoma cells and normal placenta cells (Cao et al., 1995). In addition, it has also been demonstrated that the amino-terminal nuclear targeting signals of rat AR are required for this protein to function as a mitogen (Kimura, 1993) and that AR can bind to nuclear phosphoproteins (Modrell et al., 1992). The nuclear targeting signals of AR coincide with the putative heparin binding regions. Since both the AR precursor and mature AR can be heavily glycosylated in the presence of TPA, these basic regions may be masked due to steric hindrance of the carbohydrate side chains thereby impeding nuclear translocation of these species. This may contribute to the paucity of nuclear AR immunoreactivity that is observed in MCF-7 cells after TPA treatment.

To conclude, TPA and E_2 can induce AR protein expression through distinct pathways, in addition to the previously described differential effects on its mRNA accumulation (Martínez-Lacaci et al., 1995). TPA may induce AR expression and protein glycosylation via PKC-dependent steps, which may result in enhanced entry of AR isoform(s) into the secretory pathway. Conversely, this may act to preclude AR from nuclear translocation. Once outside the cell, AR may undergo processing and exert an autocrine or paracrine action by interacting with the EGF receptor of the same or neighboring cells, respectively. E_2 may also enhance AR expression but may promote routing of AR to the nucleus by virtue of the nuclear targeting sequences, which may be freely accessible due to less glycosylation. Once in the nucleus, AR may trigger a mitogenic or other signal by interacting with DNA sequences, chromatin or DNA binding proteins. Although the PKC and E_2 pathways both induce AR, mechanisms of AR induction, processing and localization, mitogenic and morphological effects are different. Further studies are required to more fully establish the molecular basis for the differential effects of these two pathways on AR.

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MOLECULAR CARCINOGENESIS 15:44–56 (1996)

Detection of Amphiregulin and Cripto-1 in Mammary Tumors From Transgenic Mice

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Epidermal growth factor family members are widely expressed in human breast cancer and are thought to play an important dual role in mammary gland development and tumorigenesis. Overexpression of two relatively new members of this family, amphiregulin (AR) and Cripto-1 (CR-1), has been previously shown to transform immortalized human and mouse mammary epithelial cells. Here, we extend these results and address the dysregulated expression of AR and CR-1 in many types of transgenic neoplastic mouse mammary tissues. Transgenic mouse strains overexpressing the oncogenes transforming growth factor-α, *neu*, *int*-3, polyoma virus middle T antigen, and simian virus 40 large T antigen have been previously shown to develop spontaneous mammary neoplasia. These models were each examined for mammary-tumor expression of AR and CR-1 by reverse transcription–polymerase chain reaction, western blot, and immunocytochemical analyses. Mammary tumors from each source expressed AR and CR-1. Western blot analysis revealed that, in all mammary tumors, AR and CR-1 protein species were processed differently than in virgin and lactating mouse mammary tissue. In addition, immunohistochemical detection of AR and CR-1 in tumor tissue revealed different patterns of growth-factor localization in different types of transgenic mouse mammary–derived tumors. These findings are consistent with the possibility of widespread roles for AR and CR-1 in the promotion and/or progression stages of mouse mammary tumorigenesis.

Key words: Growth factors, transgenic mouse, mammary cancer, amphiregulin, Cripto-1

INTRODUCTION

The transgenic rodent mammary gland model can be used to measure the effects of promotion- and progression-factor gene dosage on epithelial cancers [1]. A substantial amount of data have accrued suggesting that overexpression of oncogenes and proto-oncogenes such as c-Ha-*ras*, c-Ki-*ras*, *myc*, *wnt*-1, and *wnt*-2 in transgenic mice can play a crucial role in the formation of mammary tumors in mice [2–4]. Transgenic mice have also provided a suitable means for analyzing the effects of gene dosage in vivo. We have focused on five different transgenic animal models that spontaneously produce mammary tumors by different oncogenic mechanisms to examine the generality of growth-factor expression in the onset and progression of the disease.

Transgenic animals with the rat prostatic steroid-binding protein promoter that induces the viral simian virus 40 large T antigen (SV40 Lt) initially develop mammary hyperplasia by 3 mo of age and develop multifocal mammary adenocarcinoma with occasional metastasis to the lung by 4 mo [5]. Transgenic animals that overexpress polyomavirus middle T antigen (poly V mt) by means of the murine mammary tumor virus (MMTV) promoter develop mammary hyperplasia by 3 wk of age and develop multifocal mammary adenocarcinomas by 2 mo [6]. Transgenic animals with the MMTV promoter–activated, *Notch*-related growth factor *int*-3 produce poorly differentiated mammary adenocarcinomas between the ages of 2 and 7 mo [7]. Transgenic animals with the metallothionein-1 promoter–activated human transforming growth factor-α (*TGFα*) gene product produce hyperplastic stomach and salivary tissue as well as mammary carcinoma and liver carcinoma [8]. Transgenic animals expressing the MMTV-activated c-*neu* receptor oncogene develop either multiple hyperplastic and dysplastic nodules (in virgin mice) after 5 mo or adenocarcinomas with the potential to metastasize to the lungs (in multi-

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Abbreviations: SV40 Lt, SV40 large T antigen; AR, amphiregulin; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGFα, transforming growth factor-α; CR-1, cripto-1; TMT, transgenic mammary tumor; poly V mt, polyomavirus middle T antigen; SDS, sodium dodecyl sulfate; MMTV, murine mammary tumor virus; RT, reverse transcriptase; PCR, polymerase chain reaction; TPA, 12-O-tetradecanoylphorbol-13-acetate.

parous mice) [9]. As these transgenic strains overexpress either growth factors or receptors, we wanted to determine if two newly described epidermal growth factor (EGF)–like growth factors were also modulated by transgene activation.

Human amphiregulin (AR) is a member of the EGF family. AR was initially isolated from the human breast adenocarcinoma cell line MCF-7 after treatment with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [10,11]. The secreted AR protein is 78–84 amino acids long and has a six-cysteine motif analogous to EGF and TGFα [12]. Secreted AR can bind to the EGF receptor (EGFR) [12,13] and activate tyrosine phosphorylation of both the EGFR and $p185^{erbB2}$ through EGFR-dependent transphosphorylation. In addition, AR requires a putative secretory domain for secretion and requires heparin sulfate glycosaminoglycans for mitogenic activity in human mammary epithelial cells [14,15]. Biologically active AR, like EGF, can stimulate the growth of primary and immortalized human mammary epithelial cells 184A1N4 and MCF-10A cells [12,16–18]. Reduction of *AR* mRNA levels by specific antisense oligonucleotides or by addition of exogenous heparin, heparin sulfate, or chondroitin sulfate to human mammary epithelial cells leads to a reduction in cell proliferation in vitro [14,16,17].

In the mammary glands of virgin 4- to 8-wk-old C57BL/6 and FVB female mice, immunohistochemical analysis has revealed AR expression in the myoepithelial cells, luminal epithelial cells, and cap cells. AR was also detected in mammary gland tissue of uniparous pregnant and lactating mice, where the protein levels were up to 2.7-fold higher than in virgin adult mammary tissue [19]. Approximately 80% of human primary breast carcinomas as well as several estrogen-responsive and -nonresponsive human breast cancer cell lines overexpress *AR* mRNA [20–22]. AR is an estrogen-inducible protein, and its expression is also enhanced after transformation of mammary epithelial cells by point-mutated c-Ha-*ras* oncogenes or by overexpression of c-*erbB2* [18,23].

The human Cripto-1 (CR-1) protein was initially isolated and cloned from the undifferentiated human embryonal carcinoma cell line NTERA 2 clone D1, and the mouse *CR-1* gene was cloned from the F9 mouse embryonal carcinoma cell line [24]. The mouse *CR-1* gene encodes a 171-amino-acid protein that exhibits 93% amino-acid homology to human CR-1. Like other members of the EGF family, CR-1 has a partial EGF-like cysteine motif [25]. Biologically active CR-1 can act as a potent mitogen on MDA-MB-453 and SK-BR-3 human breast cancer cells and on 184A1N4 human mammary epithelial cells in vitro [26]. In the mammary glands of virgin 4- to 8-wk-old C57BL/6 and FVB female mice, CR-1 was often observed by immunolocalization in the ductal lumen, luminal epithelial cells, and myoepithelial cells of the developing epithelial ducts. CR-1 was also detected in tissues of uniparous pregnant and lactating mice, where the protein levels were up to two-fold higher than in virgin tissue [19]. A similar increase was also noted for AR during pregnancy and lactation. Mammary epithelial cells overexpressing CR-1 may also have a selective growth advantage in foreign environments as overexpression of human CR-1 in either NIH/3T3 cells or in NOG-8 untransformed mouse mammary epithelial cells leads to their ability to form clones in soft agar [24,27]. In addition, overexpression of CR-1 protein has been detected in 80% of breast-carcinoma biopsy samples and in several estrogen-responsive and -nonresponsive human breast cancer cell lines [21,22].

In addition, immortalized untransformed and tumorigenic mammary epithelial cells may express other EGF family members such as heparin-binding EGF, betacellulin, and TGFα, which also bind and activate the EGFR [28]. Moreover, EGFR shares high homology with other transmembrane proteins (c-*erbB2*, c-*erbB3*, and c-*erbB4*) that are found in mammary tumors and that interact either directly or indirectly with other EGF family members called the heregulins [2,29].

Many researchers have attempted to identify the critical changes in expression of EGF family members associated with neoplastic tissue. To date, the distribution and levels of expression of AR and CR-1 in human breast-tumor biopsy samples and human breast-cancer cell lines have been described. We analyzed the modulatory effects of these proteins and their mRNAs in five transgenic mouse strains in vivo and attempted to correlate the human data with the mouse models of disease.

MATERIALS AND METHODS

Cell Lines and Mammary Tumor Tissue

Mouse F9 teratocarcinoma embryonal carcinoma cells were obtained from Dr. Graziella Persico (International Institute of Genetics and Biophysics, Naples, Italy) and were maintained on plastic dishes in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Mammary tumor tissues were surgically removed from the transgenic mice and either frozen in liquid nitrogen or fixed in 4% formalin.

Reverse Transcriptase–Polymerase Chain Reaction

Mammary tumor tissue was isolated from mice of each transgenic strain, frozen in liquid nitrogen, and stored at –80°C. Total RNA was isolated from tissues, digested with DNase, and extracted by the guanidine isothiocyanate–RNAzol B method (Cinna/Biotecx Laboratories, Houston, TX). One microgram of total RNA was incubated with 100 pM oligo-dT12-18 (Pharmacia Biotech, Piscataway, NJ) at 65°C for 2 min in 10.5 μL of diethyl pyrocarbonate–treated

water. After the reaction mixture was cooled to room temperature, 20 μL of 1× reverse transcription buffer (2 μL of 10× reverse transcription buffer (Biolabs, Beverly, MA), 5 mM dNTPs, 10 mM dithiothreitol, 20 U RNAsin (Promega, Madison, WI) and 200 U of Moloney murine leukemia virus reverse transcriptase (RT) (Biolabs) were added and incubated at 37°C for 1 h. A total of 35 polymerase chain reaction (PCR) amplification cycles were performed with half of the reverse-transcribed RNA. The PCR reaction mixture contained 50 mM KCl, 10 mM Tris, pH 8.4 (25°C); 1.5 mM $MgCl_2$; 10 mM each primer; 100 mM each dNTP; and 2.5 U of TaqI polymerase (Perkin Elmer, Norwalk, CT) in a final volume of 50 μL. The primers 5′-CTGTTGCTGCTGGTCTTAGG-3′ (sense) and 5′-AGAGTTCACTGCCAGAAGGC-3′ (antisense) were used to amplify a 172-bp fragment of the mouse *AR* gene [19]. To detect mouse *CR-1* transcripts, primers were designed to amplify an approximately 372-bp fragment of the mouse *CR-1* gene as previously described [19,25]. As an internal control, glyceraldehyde-3-phosphate-dehydrogenase primers were used to detect a 983-bp fragment (Clontech Labs, Palo Alto, CA). The fragments were then separated on a 1.2% agarose gel containing ethidium bromide.

Western Blot Analysis

Mammary tumor tissue samples (six from each transgenic strain) were homogenized in 1 mL of hypotonic buffer (20 mM HEPES, pH 7.4; 1 mM EDTA; 1 mM $MgCl_2$; 1 mM phenylmethylsulfonyl fluoride, and 20 mg/mL aprotinin). The samples (20 μg) were boiled and resolved on a 4–15% sodium dodecyl sulfate (SDS)–polyacrylamide gel. AR and CR-1 proteins were detected as previously described [19]. Briefly, equivalent loading of protein samples was confirmed by staining a parallel gel with Coomassie blue and visualizing the protein under light. The gel was then electrophoretically transferred to nitrocellulose, and nonspecific hybridization was blocked by preincubating the blots in phosphate-buffered saline containing 5% (w/v) nonfat dried milk and 0.2% (v/v) Nonidet P-40 (Sigma Chemical Co., St. Louis, MO) for 1 h. AR was detected by using anti-AR antibody Ab-2 (1 μg/mL) for 1 h or blocked by preabsorbing AR Ab-2 against a fivefold excess of the synthetic peptide 26–44. The control and CR-1 proteins were detected by using anti–CR-1 antibody CR67 (1 μg/mL) for 1 h or were preabsorbed against the peptide 97–113. Keratin expression was detected by using for 1 h an antikeratin antiserum (diluted 1:1000) (BioGenex Labs, San Ramon, CA) that recognizes an epitope common to cytokeratins 1–19.

The blots were incubated for 1 h with horseradish peroxidase–conjugated goat anti-mouse immunoglobin (diluted 1:1000) (Sigma Chemical Co.), and the proteins were visualized by using ECL western blotting detection reagents (Amersham Corp., Arlington Heights, IL).

Immunocytochemistry

Mammary tumor tissue samples (six from each transgenic strain) were either embedded in paraffin or OCT embedding medium and stored at 4°C or snap frozen at –80°C. Sections (5- to 10-μm thick) from embedded blocks of tissue were fixed in either xylene or 100% methanol. Endogenous peroxidase activity was inactivated with 0.03% H_2O_2 in methanol. Nonspecific binding was blocked with 5% normal goat serum, and then the sections were incubated with 1 μg/mL anti-AR antibody or with 0.5 μg/mL anti–CR-1 antibody at 4°C for 12 h. The primary antibodies used were of rabbit origin. To detect AR protein, an anti-AR IgG (Ab-2) was used. Ab-2 was affinity-purified by using immobilized AR 26–44 peptide [13,19]. To detect CR-1 protein, an anti–CR-1 IgG (CR67) was used. CR67 was affinity-purified by using immobilized CR-1 97–113 peptide [19].

Antigen/antibody complexes were detected by incubating sections for 1 h with biotinylated goat anti-rabbit antibody (1:1000), then with avidin-biotin complex conjugated to horseradish peroxidase (1 h), and finally with 3,3′-diaminobenzidine-4-HCl for visualization (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). The slides were then lightly counterstained with Gill's hematoxylin and photographed.

RESULTS

AR and *CR-1* mRNA Expression in Mammary Tumors From All Five Transgenic Strains

Previous in vivo studies of FVB CD-1 and C57BL/6 virgin, pregnant, and lactating mice have revealed both AR and CR-1 in the mammary gland [19]. To explore the relationship between neoplastic and untransformed mammary tissue, we first examined the presence of *AR* and *CR-1* mRNAs in transgenic mammary tumors (TMTs) by RT-PCR. The reason for using RT-PCR was to confirm initially the expression of these genes, as previous reports of detection of *EGF* and *TGFα* mRNA in inbred C57BL/6 and FVB mice by northern analysis and RNase protection analysis noted inherent problems with detection, sensitivity, and determining transcription rates [30]. The procedures previously reported for mRNA detection of *AR* and *CR-1* genes formed the framework for our experiments [19].

In three separate studies using RNAs prepared from different mammary tumors derived from each transgenic strain, a 172-bp *AR* product was generated. As a positive control, total RNA extracts from F9 teratocarcinoma cells and from the mammary glands of 14.0-d lactating and 10-wk-old virgin C57BL/6 mice were used (Figure 1C) [19]. As a negative control, total RNA from 10-wk-old virgin mammary glands was incubated with TaqI polymerase in the absence of RT or without TaqI polymerase in the presence of RT, with or without all other RT-PCR

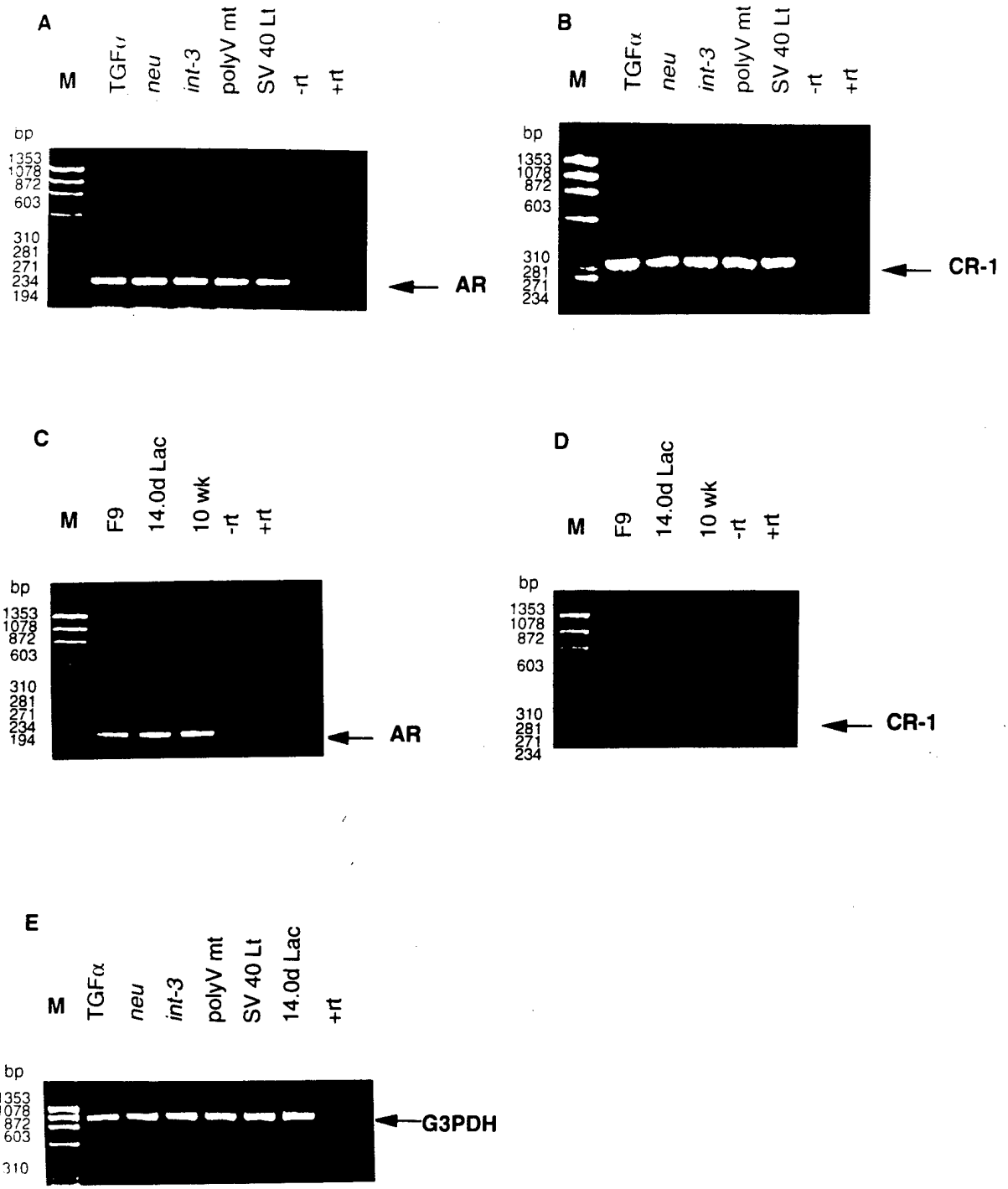

Figure 1. (A) RT-PCR detection of *AR* mRNA in mammary tumors from transgenic mice overexpressing *TGFα, neu, int-3,* polyV mt, and SV40 Lt; 10-wk-old virgin gland (10 wk); 14.0-d lactating gland (14.0d Lac); and F9 cells. A band of the expected 172-bp size (arrow) was amplified in each experimental sample. Lanes +rt and –rt, total RNA from 10-wk-old virgin glands incubated with TaqI polymerase in the absence of RT (–rt) or without TaqI polymerase in the presence of RT (+rt). Lane M, molecular-size markers. (B) RT-PCR detection of *CR-1* mRNA in mammary tumors from transgenic mice overexpressing *TGFα, neu, int-3,* poly V mt, and SV40 Lt; 10-wk-old virgin gland (10 wk); 14.0-d lactating gland (14.0d Lac); and F9 cells. A band of the expected 334-bp size (arrow) was amplified in each experimental sample. Lanes +rt and –rt, total RNA from F9 cells incubated with TaqI polymerase in the absence of RT (–rt) or without TaqI polymerase in the presence of RT (+rt). Lane M, molecular size markers. (C) AR expression from total RNA of 10-wk-old virgin mammary glands, lactating glands, and F9 cells. Total RNA of 10-wk-old virgin mammary glands was subjected to PCR in the absence of RT as a negative control. The arrow indicates a 172-bp cDNA product in 10-wk-old virgin glands, lactating glands, and F9 cells. Lanes +rt and –rt, total RNA from 10-wk-old virgin glands incubated with TaqI polymerase in the absence of RT (–rt) or without TaqI polymerase in the presence of RT (+rt). Lane M, molecular-size markers. (D) RT-PCR of total RNA from F9 cells, 10-wk-old virgin glands, and 14.0-d-old lactating glands. The arrow indicates the 334-bp transcripts. Lanes +rt and –rt, total RNA from F9 cells incubated with TaqI polymerase in the absence of RT (–rt) or without TaqI polymerase in the presence of RT (+rt). Lane M, molecular-size markers. (E) RT-PCR of the expected 983-bp product (arrow) of the *G3PDH* gene detected in all samples. Lane +rt, total RNA from TGFα TMTs incubated without TaqI polymerase in the presence of RT. Lane M, molecular-size markers.

reaction ingredients; this produced no visible product (Figure 1A and C). The internal control gene *G3PDH* (Figure 1E) was also detected in each mammary tissue.

CR-1 expression was also evaluated in mammary tumors derived from each transgenic strain. We detected a 334-bp fragment spanning the *CR-1* cDNA in mammary tumors derived from each of the transgenic strains (Figure 1B). As a positive control, total RNA extracts from F9 teratocarcinoma cells and from mammary glands of 14.0-d lactating and 10-wk-old virgin C57BL/6 mice were used (Figure 1D) [19]. As a negative control, total RNA from F9 cells was incubated with TaqI polymerase in the absence of RT or without TaqI polymerase in the presence of RT; this produced no visible product (Figure 1B and D).

Detection of Multiple Species of AR and CR-1 in Transgenic Mice

In previous studies, the expression patterns of the growth factors EGF, TGFα, AR, and CR-1 increased with lobular-alveolar development and growth of epithelial ductal structures [19,30,31]. In the study reported here, all transgenic strains shared a common inbred FVB genetic background, but the onset of initial tumor formation was sporadic. Quantitation of protein expression levels in tumor versus adjacent uninvolved tissue posed a problem because in each transgenic strain the mammary glands were atypical at a very early stage in maturation, even though the epithelial ductal network had not completely filled the glandular fat pad. This phenomenon masked our results when comparing protein signals in adjacent uninvolved/non-neoplastic tissue and hyperplastic neoplastic tissue. Therefore, we followed previously published procedures for detecting and quantitating AR and CR-1 protein species. We also compared each TMT's signal intensity and species with those of 14.0-d lactating mammary gland, and we further compared these TMTs with each other to determine differences, if any, in isoforms and intensity [19].

Extracts from mammary tumors derived from each transgenic strain were probed by western blotting with anti-AR (Ab-2) antibodies for AR expression. A separate gel was loaded with equal amounts of protein and probed with Ab-2 antibodies (preincubated for 5 h with a tenfold excess of peptide 26–44) (Figure 2C). The 17-kDa *int-3* band in TMT extracts was a nonspecific band (Figure 2C, arrow). Our results suggest that AR proteins of various sizes were expressed in mammary tumors from each transgenic strain (Figure 2A). Each arrow in Figure 2A indicates an immunoreactive AR species. When compared with previous observations in normal virgin and uniparous lactating mammary gland, each TMT's AR signal was relatively stronger. In contrast to a previous study [19], our experiments did not detect a signal in the virgin gland. This may have been because less sample was loaded in our study. In sum, after densitometric scanning of seven different blots and standardizing them against 14.0-d lactating gland, a 0.16-fold decrease or a 5.9-fold increase in AR signal intensity in TMTs was apparent. Table 1 summarizes the densitometric scanning of the blot seen in Figure 2A. In TGFα TMTs, a 30-kDa species was observed (Figure 2A, lane 1); all other AR isoforms from TMTs are listed in Table 2. Furthermore, in *int-3* TMTs, a smaller 14-kDa species was detected (data not shown). This smaller species was also previously detected in a biologically active form in MCF-10A human immortalized mammary epithelial cells after treatment with TPA [32]. Keratin expression was also analyzed in each TMT; no significant changes in anti-keratin signal were observed when 20 μg of protein from each TMT was loaded onto the gels (data not shown).

Like the detection of AR in pregnant and lactating mouse mammary gland tissue, the anti–CR-1 signal was considerably stronger at this stage than in normal virgin and uniparous lactating mouse mammary gland tissue (Figure 2B). A separate gel was loaded with equal amounts of protein and probed with CR67 antibody (preincubated for 5 h with a tenfold excess of peptide 93–118) (Figure 2D). The band larger than 200 kDa in TGFα, *neu*, and *int-3* TMT extracts was a nonspecific band (Figure 2D, arrow). Further, a prominent 45- to 50-kDa CR-1 signal was evident in transgenic TGFα mammary tissue. A complete comparison of the fold changes in the levels of CR-1 is presented in Table 1 and a representative gel in Figure 2B. For comparison of CR-1 expression in normal virgin mice, 20 μg of total protein was loaded on the gel and could not be detected by audioradiography. However, much like AR, various CR-1 isoforms could be observed in each of the TMTs. A 14-kDa CR-1 species was also detected in previously described F9 cells [26]. In all other TMTs, CR-1 proteins were processed relatively the same as summarized in Table 2. Finally, in *int-3* TMTs, 28-kDa and 26-kDa isoforms not present in any other transgenic strain were also detected.

Figure 2. (A) Western blot detection of AR protein in TMTs, 6-wk-old virgin glands (6 wk), 14.0-d lactating glands (14.0d Lac), and F9 cells. The arrows indicate 32-, 30-, 26-, and 21-kDa species. (B) Western blot detection of CR-1 protein in TMTs, 6-wk-old virgin glands (6 wk), 14.0-d lactating glands (14.0d Lac), and F9 cells. The arrows indicate 60-, 50-, 45-, 32-, 28-, 26-, 24-, 22-, 20-, and 14-kDa species. (C) Western blot detection of AR protein in TMTs, 6-wk-old virgin glands (6 wk), 14.0-d lactating glands (14.0d Lac), and F9 cells after the anti-AR antibody was preincubated for 5 h with a tenfold excess of peptide 26–44. The arrow indicates the 17-kDa species. (D) Western blot detection of CR-1 protein in TMTs, 6-wk-old virgin glands (6 wk), 14.0-d-lactating glands (14.0d Lac), and F9 cells after anti–CR-1 was preincubated for 5 h with a tenfold excess of peptide 93–118. The arrow indicates >220 kDa species. All lanes in all panels were loaded with 20 μg of total protein as described in Materials and Methods.

A

M
TGFα
neu
int-3
polyV mt
SV 40 Lt
6 wk
14 0d Lac
F9
30K
21.5K
AR

B

M
TGFα
neu
int-3
polyV mt
SV 40 Lt
6 wk
14.0d Lac
F9
66K
46 K
30K
21.5K
14.3K
CR-1

C

M
TGFα
neu
int-3
polyV mt
SV 40 Lt
6 wk
14.0d Lac
F9
220 K
97 K
66K
46 K
30K
21.5K
14.3K

D

M
TGFα
neu
int-3
polyV mt
SV 40 Lt
6 wk
14.0d Lac
F9
220 K
97 K
66K
46 K
30K
21.5K
14.3K

Table 1. Expression Levels of Selected AR and CR-1 Proteins in TMTs Versus 14.0-d-Old Lactating Tissue*

Transgenic strains	AR species		CR-1 species	
	32 kDa	26 kDa	32 kDa	24 kDa
TGFα	–	1.7	–	–
neu	2.77	5.01	7.52	–
int-3	2.91	5.986	2.95	2.01†
Poly V mt	−0.16	4.99	1.424	–
SV40 Lt	−0.35	3.58	2.167	–

*All species were scanned by densitometry and standardized against 20 μg of 14.0-d-lactating mammary tissue. The values are fold increases or decreases.
†The 23-kDa CR-1 species was not detected in TGFα-TMT or 14.0-d-lactating tissue. This value represents the *int-3* signal intensity in relation to those of *neu*, poly V mt, and SV40 Lt TMTs.

Table 2. Detection of Various AR and CR-1 Species in Transgenic Mammary Tumors

Transgenic strain	AR species	CR-1 species (kDa)
TGFα	30,* 26	60, 50,† 45,† 14
neu	32, 26, 21	60, 32, 24, 20
int-3	32, 26, 21	60, 32, 28, 26, 24
poly V mt	32, 26	60, 32, 24, 20
SV40 Lt	32, 26, 21	60, 32, 24

*30-kDa species detected only in TGFα TMTs.
†The 50- and 45-kDa species were detected only in TGFα TMTs.

Immunolocalization of AR and CR-1 Proteins in Neoplastic Tissue

Specific immunostaining of AR protein was clearly observed in tissue sections from mammary tumors derived from each transgenic strain (Figures 3A (TGFα), 3C (*int-3*), 4A (*neu*), 4C (*neu* lung metastasis), 5A (poly V mt), and 5C (SV40 Lt)). When signal strength was compared among the TMTs, the AR staining intensity did not significantly change. Likewise, when sections were reacted with anti-AR antibody preabsorbed against the peptide immunogen, the sections were negative (see insets in corners of Figures 3A and C, 4A, and 5A and C). In addition, each TMT section was scored individually for AR and CR-1 immunoreactivity by morphological identification of epithelial structures and signal intensity. AR immunolocalization was detected in each TMT and tissue sections from lung metastasis of *neu* mice. The percentages of stained cells are summarized in Table 3. In all TMTs, approximately 50% of three randomly chosen fields of tumor cells (<1000 cells/field) were positive for AR immunoreactivity. In addition, in poly V mt tumor sections, a nuclear specific reactivity was observed in 40% of the cells (Figure 5A). Also, in these same sections, little if any cytoplasmic or membrane-associated AR was observed (Figure 5A).

To further investigate whether CR-1 immunostaining could be observed in parallel sections of TMTs, sections were visualized with anti–CR-1 antibody (Figures 3B (*TGFα*), 3D (*int-3*), 4B (*neu*), 4D (*neu* lung metastasis), 5B (poly V mt), and 5D (SV 40 Lt)). Parallel control sections from each TMT were also stained with preabsorbed anti–CR-1 antibody. Negative control sections for all TMTs are shown in the right hand corners of Figures 3B and D, 4B, and 5B and D. CR-1 immunostaining was characterized by the same method as AR detection. Similarly, CR-1 immunolocalization was detected in each TMT and in sections from lung metastases of *neu* mice, and when compared among TMTs, CR-1 signal strength did not considerably change. The percentages of stained cells are summarized in Table 3. In all TMTs, approximately 50% of three randomly chosen fields of tumor cells (< 1000 cells/field) were positive for CR-1 immunoreactivity.

DISCUSSION

Our current hypothesis that increased expression of the EGF family of growth factors in the mammary gland favors promotion or progression of a tumorigenic phenotype is largely based on in vitro data. In such studies, isolated tissue cell lines were subjected to hormones and growth factors in a highly artificial environment not completely representative of the in vivo counterpart. The availability of transgenic mouse models has begun to shed more definitive light on the biological roles of these growth factors [33].

AR Expression and Complexity of Isoforms in Normal Virgin, Lactating, and Neoplastic Mammary Tissue

Mouse mammary glands of virgin female 4- to 8-wk-old C57BL/6 and FVB mice are known to express AR in the myoepithelial cells, luminal epithelial cells, and epithelial cap cells. AR is also detected in the mammary gland tissue of uniparous pregnant and lactating mice, in which the immunoreactive protein levels increase up to 2.7-fold [19].

Our studies indicate that AR was expressed in all of the solid tumors isolated from *TGFα, neu, int-3,* poly V mt, and SV40 Lt transgenic strains and appeared to undergo posttranslational processing in a strain-specific fashion. The differences in AR isoforms and overall signal intensity detected by SDS–polyacrylamide gel electrophoresis in vivo suggest there is a temporal increase and shift of AR processing in conjunction with oncogene-induced neoplasia. To date, these observations have been restricted to transgenic mouse mammary tumor tissue and have not been extended to human breast cancer cell lines or human primary breast cancer tissue [21–23].

A Diversity of Functions for AR Isoforms?

Mammary tumors from transgenic mouse strains expressing EGF family members provided interesting

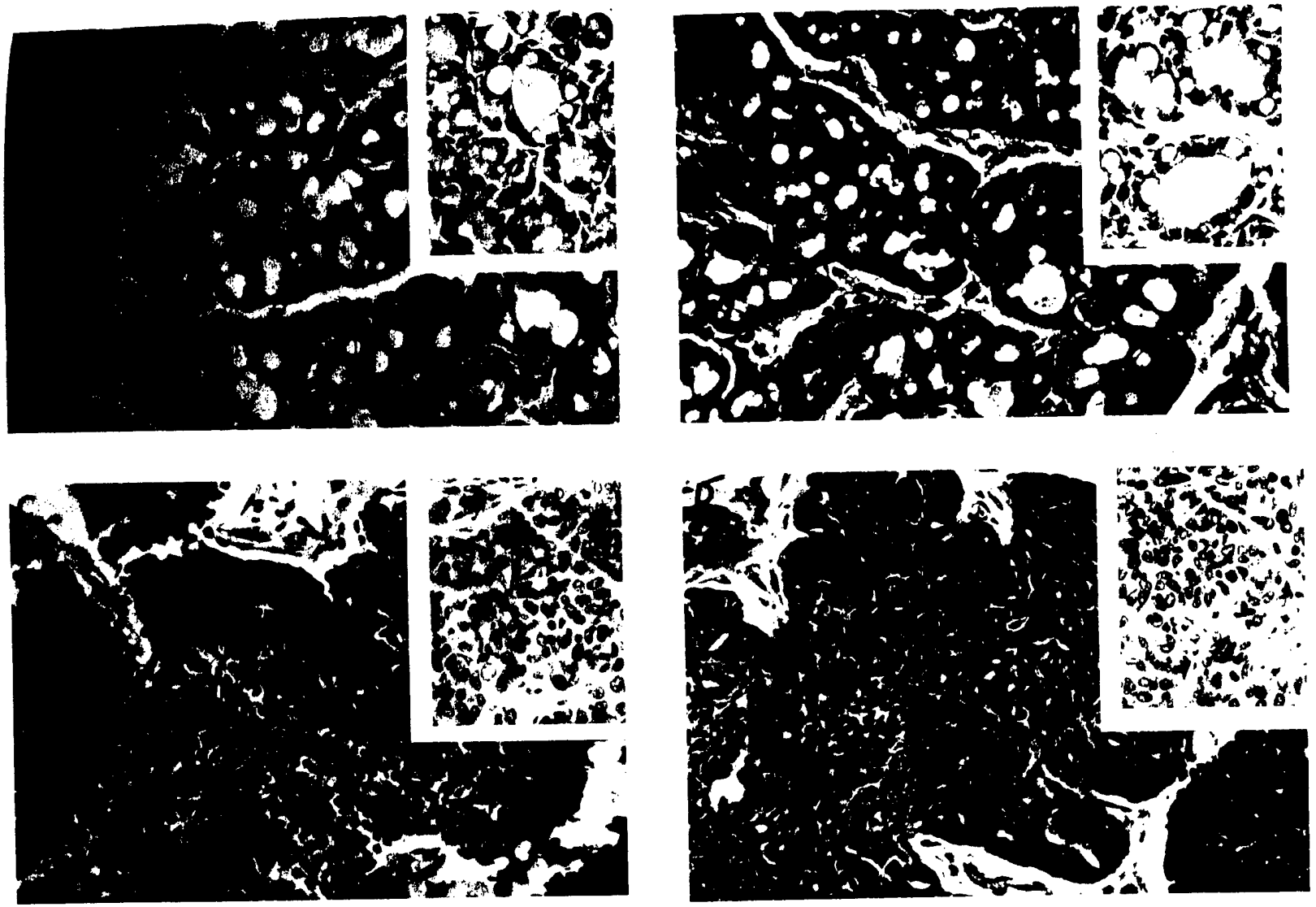

Figure 3. Immunoperoxidase detection of AR (A and C) and CR-1 (B and D) in TMTs overexpressing TGFα (A and B) and *int-3* (B and D). Staining for nuclear and cell-associated AR was abolished by preincubating the antibody with soluble AR peptide immunogen (A and C, insets). CR-1 staining was abolished by preincubating the antibody with soluble CR-1 peptide immunogen (B and D, insets). Magnification ×400.

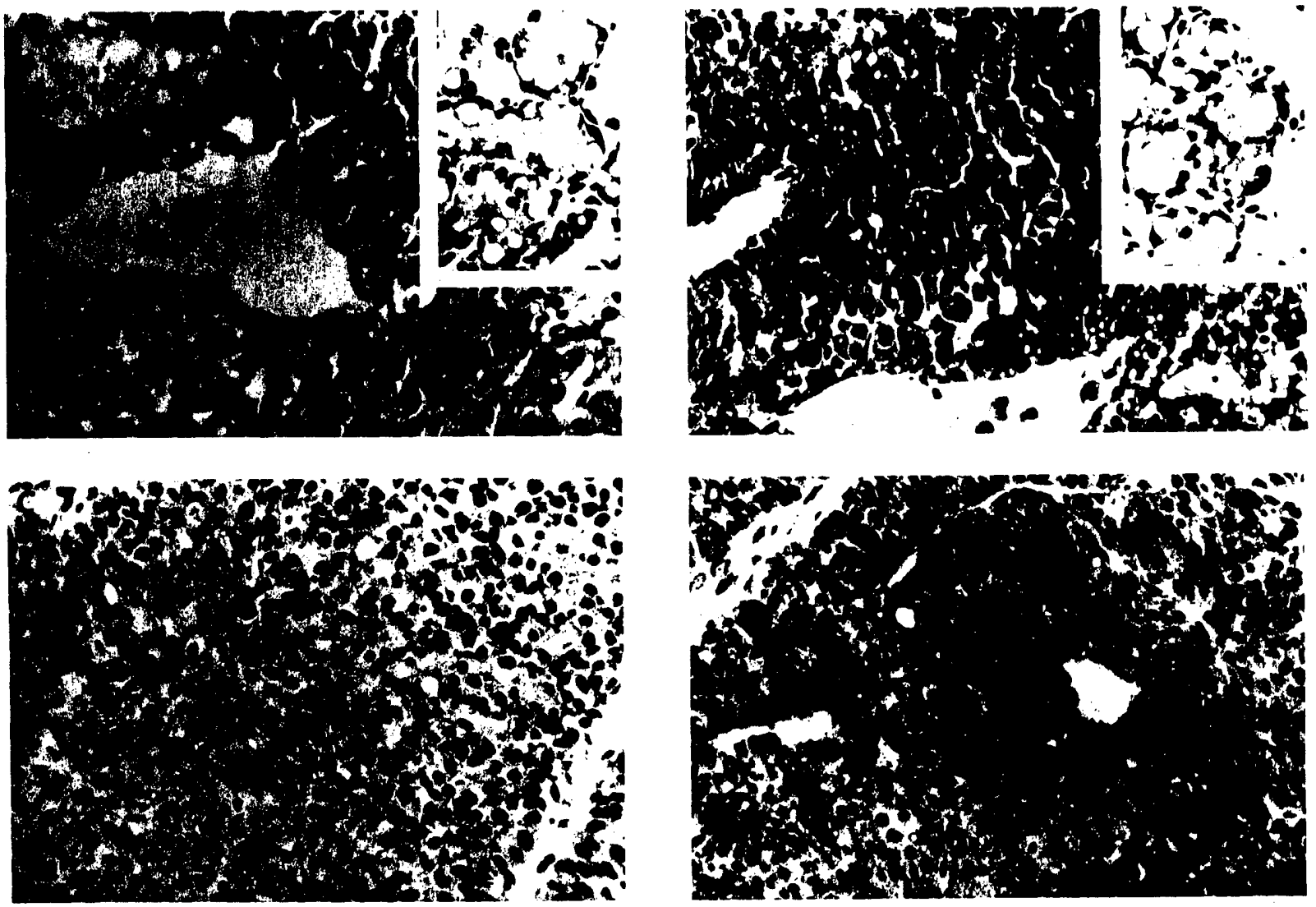

Figure 4. Immunoperoxidase detection of AR (A and C) and CR-1 (B and D) in TMTs overexpressing *neu* (A and B) and in a *neu* TMT lung metastasis (C and D). Staining for nuclear and cell-associated AR was abolished by preincubating the antibody with soluble AR peptide immunogen (A, inset). CR-1 detection was abolished by preincubating the antibody with soluble CR-1 peptide immunogen (B, inset). Magnification ×400.

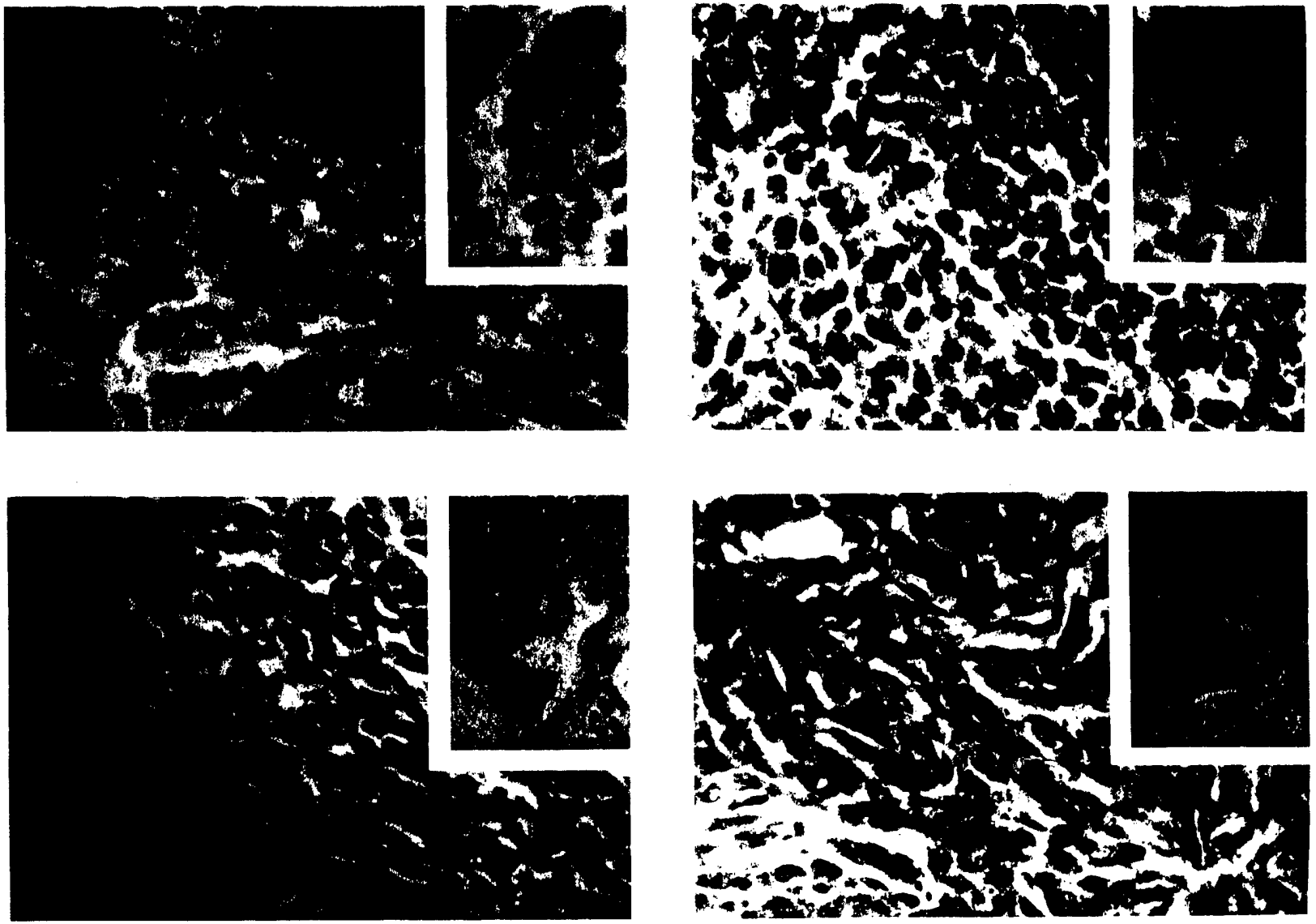

Figure 5. Immunoperoxidase detection of AR (A and C) and CR-1 (B and D) in TMTs overexpressing poly V mt (A and B) and SV40 Lt (C and D). Staining for nuclear and cell-associated AR was abolished by preincubating the antibody with soluble AR peptide immunogen (A and C, insets). CR-1 detection was abolished by preincubating the antibody with soluble CR-1 peptide immunogen (B and D, insets). Magnification ×400.

Table 3. Immunohistochemical Analysis of AR and CR-1 Proteins

Transgenic strain	AR protein staining (% cells)				CR-1 protein staining (% cells)			
	Strong	Weak	Negative	ND*	Strong	Weak	Negative	ND*
TGFα	8.0 ± 1.0	34.3 ± 3.2	19.0 ± 1.3	38.8	17.4 ± 4.5	37.8 ± 4.5	12.6 ± 1.7	32.2
neu	19.1 ± 2.2	27.5 ± 3.2	17.5 ± 4.0	35.9	4.8 ± 1.7	30.0 ± 2.8	27.1 ± 1.6	38.1
int-3	12.0 ± 1.1	42.0 ± 2.2	46.0 ± 2.0	0	7.9 ± 2.3	42.2 ± 1.6	16.5 ± 1.4	33.4
poly V mt*	12.9 ± 2.3	29.8 ± 1.5	15.5 ± 2.4	41.8	5.3 ± 2.1	34.4 ± 3.0	11.8 ± 1.9	48.5
SV40 Lt	19.0 ± 1.3	50.0 ± 1.3	27.3 ± 2.0	0.7	12.1 ± 1.8	62.1 ± 2.1	13.6 ± 2.2	12.2

*40% of tumor cells were positive for nuclear staining.
*ND, percentage of cells not determined to be either weak or negative.

protein patterns as detected by SDS–polyacrylamide gel electrophoresis. Specifically, in the *TGFα* transgenic tumors, we observed a unique 30-kDa AR isoform, and in *int-3* TMT tissue, AR expression was increased sixfold over that in lactating mammary tissue. This may indicate that, in mammary epithelial cells, AR not only is autoinduced by EGF family members in vivo but also undergoes EGF family transgene–selective processing [19]. Although the biological activity of AR isoforms has not been proven, our previous data have suggested that at least some AR isoforms may have biological activity. Johnson and colleagues [32] observed that TPA-treated MCF-10A immortalized human mammary epithelial cells secreted native AR (18 kDa) as well as 16-kDa and 9-kDa isoforms; all three forms were biologically active. These same forms are known to induce DNA synthesis as well as autophosphorylate and activate the EGFR tyrosine kinase [13,32]. Furthermore, Lacaci and colleagues [23] isolated a 50-kDa isoform from TPA-treated MCF-7 cells that was biologically active in MCF-10A cells. These isoforms may have individual activities in autocrine, paracrine, juxtacrine, and intracrine signaling. For example, the extracellular milieu may modulate AR activity as AR contains two strongly basic domains in the NH_2-terminal region that are thought to act as binding sites for heparin sulfate glycosaminoglycans. This class of matrix molecules strongly modulates AR-dependent EGFR signaling and AR-dependent proliferation in keratinocytes and mammary epithelial cells in vitro [14,17,34]. AR also contains a DNA-binding, nuclear-targeting sequence similar to that found in the SV40 Lt antigen that may allow AR to act in an intracrine fashion to promote mitogenesis [34,35]. Taken together, these findings indicate the potential for a diverse array of isoforms of AR to assist in the initiation or progression of mammary tumors in multiple strains of transgenic mice.

Strain Specificity of AR Immunolocalization

Immunohistochemical analysis has suggested subcellular localization of AR is quite different in the transgenic strains we tested. AR immunolocalization in poly V mt TMTs presented a striking variation: sections of these tumors demonstrated highly specific nuclear staining in 40% of the tumor cells scored. This discrepancy is at odds with earlier proposals that AR nuclear localization correlates with lower risk in human breast cancer [20,36]. Because poly V mt transgenic mice present the earliest, most prominent, and most consistent invasive tumors of all the strains used in our study, it is clear that AR localization may not be a general predictor of tumor aggressiveness [20,36].

CR-1 Expression in Normal Virgin, Lactating, and Neoplastic Mammary Tissue

In the mammary glands of virgin 4- to 8-wk-old C57BL/6 and FVB female mice, CR-1 is expressed by luminal epithelial cells and myoepithelial cells and in the lumen of the developing ducts. CR-1 is also detected in uniparous pregnant and lactating tissue, where its protein levels can increase up to twofold compared with those of virgin tissue [19].

We also reported that CR-1 is expressed in mammary tumors derived from each of the transgenic strains studied (*TGFα, neu, int-3,* poly V mt, and SV40 Lt). This finding correlates with the results of human breast cancer tissue studies in which 80% of human primary breast carcinomas and several estrogen-responsive and -nonresponsive human breast cancer cell lines overexpressed *CR-1* mRNA and protein [21,22].

The expression patterns of CR-1 in TMTs, as determined by western blot analysis, indicated that, like AR, CR-1 underwent extensive posttranslational processing in the *TGFα, neu, int-3,* poly V mt, and SV40 Lt transgenic strains. Moreover, CR-1 protein levels increased in TMTs compared with lactating tissue. A prominent 45- to 51-kDa immunoreactive CR-1 species was detected in TGFα TMTs. This again suggests that, in mouse mammary epithelial cells, overexpression of an EGF family member can induce expression of another member of the family. Furthermore, the expression and localization of CR-1 protein was not strain specific. CR-1 was detected in all TMT sections analyzed for CR-1 immunostaining as well as in sections from lung metastases of *neu* transgenic mice.

Functions of CR-1 Isoforms?

The biological activity of CR-1 has been established. A CR-1 peptide spanning amino acids 93–118 of the human mature protein can stimulate the pro-

liferation of human immortalized mammary epithelial cells [26]. It is not yet known what biological activities might be associated with the diversity of CR-1 isoforms we detected. The nature of the receptor for CR-1 and the primary cellular targets are also not known. A possible scenario for this protein in tumorigenesis could involve induction of a distinct program of gene expression in the adjacent stroma. An alteration in the modulation of signal transduction pathways regulating malignancy-associated genes such as those encoding c-Ha-*ras*, *Krev-1*, TIMP-1, nm23Hl, MMP-9, osteopontin, and MMP-3 may also contribute to the role of CR-1 in tumor initiation or tumor progression [37].

AR and CR-1 as Markers of Oncogenic Conversion

Recently, Morrison and colleagues [4] reported that six genes—*k*-casein, transferrin, cellular binding protein I, *WDNM1*, *brp-39*, and *mat-8*—are differentially expressed in the mammary tissue of transgenic *neu* mice, c-Ha-*ras* mice, c-*myc* mice, and *int-2* mice as well as in virgin, pregnant, lactating, and regressed mouse mammary tissue. They suggest that by transgenic oncogene conversion, expression of these genes may indicate a common cellular lineage and/or common signal transduction pathways.

AR and CR-1 were also expressed in all of the transgenic strains we studied. Furthermore, these proteins undergo either preferential processing or immunolocalization (AR) in a strain-specific fashion and may be used as markers of oncogenic conversion. More important, the isoforms and patterns of localization of AR and CR-1 may provide additional insights into the action of oncogenes and/or proto-oncogenes on the growth-factor activities that contribute to tumorigenic pathways within the mammary gland.

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Induction of Ductal Morphogenesis and Lobular Hyperplasia by Amphiregulin in the Mouse Mammary Gland[1]

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Abstract

As the juvenile mouse mammary gland matures, it undergoes extensive epithelial proliferation, leading to a network of ductal branching that transverses the organ. Recent evidence suggests that the epidermal growth factor-related peptide amphiregulin (AR) may play multiple roles in the proliferation, differentiation, and neoplastic conversion of the mouse mammary gland. Using a dual approach of recombinant AR in slow-release pellets and retroviral expression of AR, we explored the roles of this growth factor in the mouse mammary gland *in vivo*. We first noted that recombinant AR can reestablish longitudinal ductal proliferation in growth quiescent mammary glands of ovariectomized mice. Furthermore, retrovirally transduced mammary transplants overexpressing AR developed into hyperplastic tertiary ducts and hyperplastic lobules with increased lateral branching, apparent 9 weeks after transplantation into cleared mammary fat pads. This is the first study to demonstrate that AR can reestablish the early developmental activity of ductal mammary epithelium and induce hyperplasia *in vivo*. These data, coupled with previous findings that demonstrated nearly universal overexpression of AR in human breast cancer and rodent mammary tumorigenesis, suggest that AR may be an important intermediary in glandular maturation and early malignant progression.

Introduction

The mouse mammary gland undergoes morphogenesis and differentiation during postnatal development (1, 2). At 4 weeks of age, ovarian hormone levels increase, which in turn signal club-shaped epithelial structures (terminal end buds) to appear and grow distally from the nipple region to fill the fat pad. During this rapid phase of growth, an extensive network of epithelial ductal branching is developed within the parenchyma (1). Another round of epithelial expansion and maturation begins slightly before implantation of the fertilized egg and ends just before parturition in the pregnant mouse. Rapid proliferation and differentiation of milk-synthesizing secretory epithelium are evident during this period (2).

Serum- and tissue-derived growth factors are thought to play an inductive role in the modeling of the mammary gland (2). Members of the TGF[3]-β family, fatty acid-binding proteins, mammary-derived growth inhibitor, Insulin-like growth factors, fibroblast growth factors, and EGF-like family members TGFα, cripto-1, and AR may act as local mitogens or inhibitors in human and mouse mammary epithelial cells. Developmental studies suggest that the expression of these genes are modulated during various stages of growth and maturation of the mouse mammary gland (2–9).

We have concentrated our efforts on AR expression and its biological role in the mammary gland. Human AR is a heparin-binding 78–84 amino acid growth factor that was initially isolated from the human breast cancer cell line MCF-7 (10). Structurally, AR has a six-cysteine motif, similarly found in most members of the EGF-like family (11). AR binds, activates, and autophosphorylates the EGFR and transphosphorylates p185^{erbB2} through an EGFR-dependent mechanism (10, 12).

Biologically active AR can act as a potent mitogen for nontransformed immortalized human mammary epithelial cells (11, 13, 14). A reduction in AR mRNA levels by antisense oligonucleotides reduces the proliferative rate of the immortalized, nontransformed, human mammary epithelial cell line 184 A1N4 (13). Moreover, the biological activity and receptor signaling of AR can also be modulated in mammary epithelial cells by its interaction with heparin and heparin sulfate glycoaminoglycans *in vitro* (14).

In vivo, AR protein is expressed during all stages of the developing mouse mammary gland. AR protein is expressed by the cap cells of the terminal end bud, myoepithelial cells, and luminal epithelial cells, and it is secreted into the mammary ductal lumen of 4–13-week-old C57 Bl and FVB mice (7). The levels of AR protein are also elevated in the mammary gland following pregnancy and lactation. Mechanistically, AR is a protein kinase C-, progesterone-, and estrogen-inducible protein, and its expression is modulated in several estrogen-responsive and

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[3] The abbreviations used are: TGF, transforming growth factor; EGF, epidermal growth factor; EGFR, EGF receptor; AR, amphiregulin; OVX, ovariectomized; BrdUrd, 5′-bromo-2′-deoxyuridine; TEB, terminal end bud; MMTV, mouse mammary tumor virus; S-GAG, sulfated glycoaminoglycan; HS-GAG, heparan sulfate-GAG.

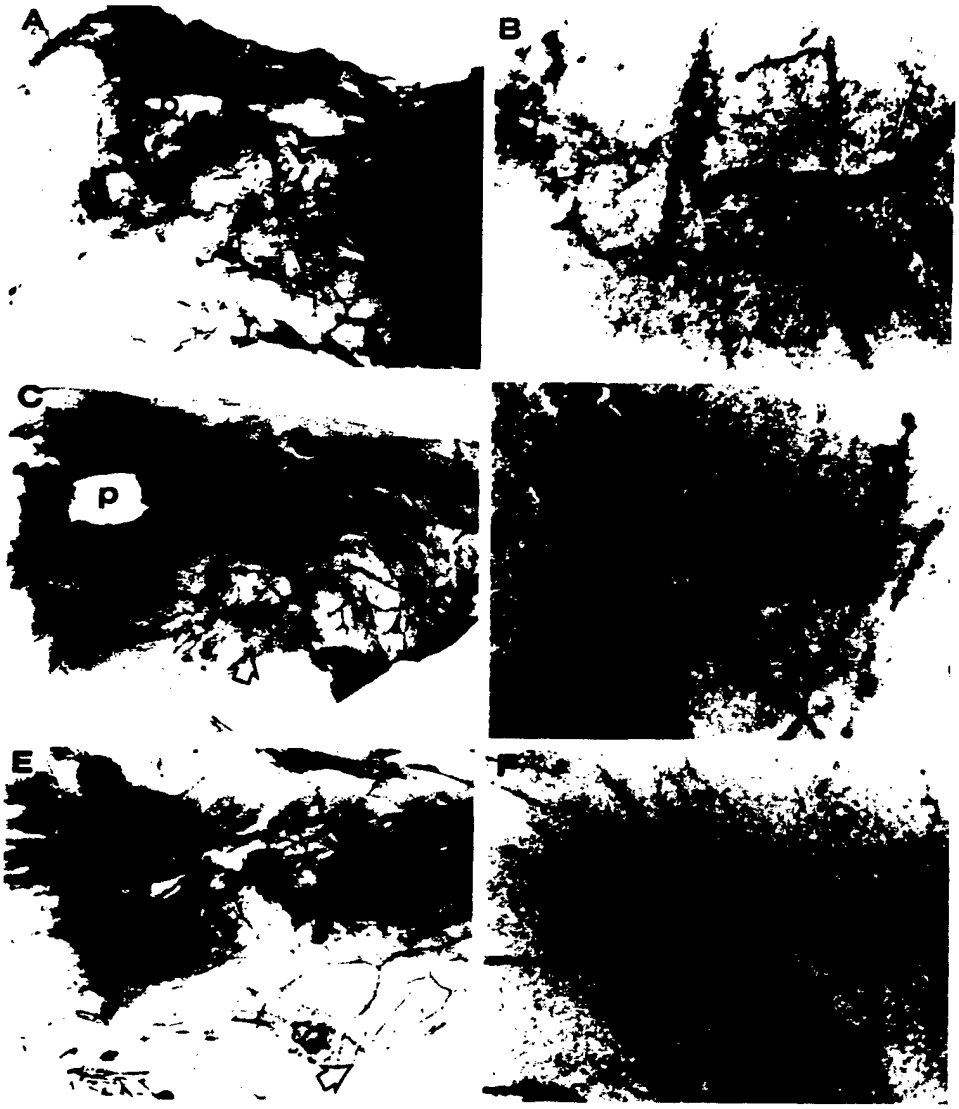


Fig. 1. Whole-mount analysis of OVX mammary glands that received BSA, EGF, and AR Elvax pellets. Elvax pellets containing recombinant EGF (5 μg/pellet; *C* and *D*; positive control) and recombinant AR (5 μg/pellet; *E* and *F*) were implanted into the no. 4 mammary gland of 5-week-old OVX mice. Elvax pellets containing BSA (5 μg/pellet; *A* and *B*; negative control) were implanted into the contralateral no. 4 mammary gland. In each repeated experiment (three times), both EGF and AR stimulated ductal morphogenesis and initiated the reappearance of TEBs. *Open arrows* in *A*, *C*, and *E* indicate areas enlarged in *B*, *D*, and *F*, respectively. *P*, pellet; *Nd*, normal mammary duct. *A*, *C*, and *E*, ×15. *B*, *D*, and *F*, ×80.

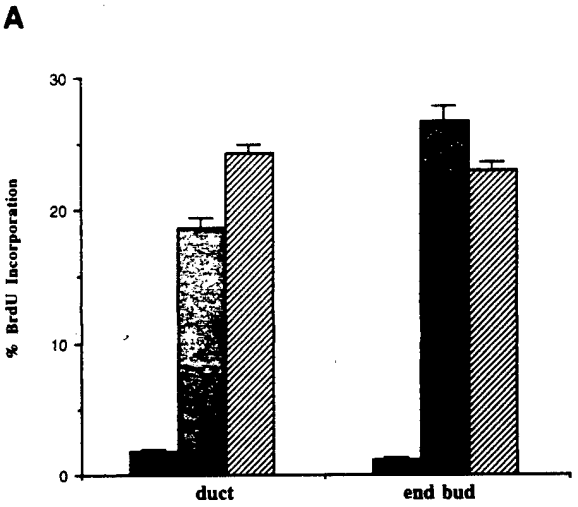
estrogen nonresponsive human breast cancer cell lines (15). TGF-α, *neu*, *int-3*, polyoma middle T antigen, and SV40 large T antigen transgenic mice that spontaneously produce mammary epithelial tumors all preferentially process several unique isoforms of AR protein. In addition, point-mutated c-*Ha-ras* or c-*erbB2* transformed mammary epithelial cells, and approximately 80% of human primary breast carcinomas overexpress AR protein and mRNA (16–20).

Because previous reports indicate that dysregulated expression of AR may be a component of mammary tumorigenesis *in vitro*, we examined the short-term and long-term consequences of AR protein and gene overexpression. In this report, we have introduced a combination biogenetic approach (retrovirus-slow release pellet) to efficiently explore the early developmental morphological aberrations within a genetically manipulated mouse mammary epithelial population.

Results

Exogenous AR and Ductal Morphogenesis. Previous reports have demonstrated Elvax pellets that contain either recombinant EGF or TGF-α are able to stimulate ductal morphogenesis in the mammary glands of OVX mice. To compare the morphogenic effects of AR Elvax pellets to EGF or TGF-α pellets, we repeated this approach in both C57 Bl6 and FVB mouse strains.

Our results indicate that in C57 Bl mice, AR reestablished the early developmental appearance that constituted the formation of terminal end buds and ductal morphogenesis, all of which occurred in close proximity of the pellet (Fig. 1, *E* and *F*). We also observed that in the AR group, the formation and migration of the secondary ducts closely resembled EGF implants, which served as our positive controls [Fig. 1, *C* (EGF) and *E* (AR)]. Furthermore, to determine if this effect could be reproduced in another inbred mouse strain, this method was carried out in FVB OVX female mice. We con-



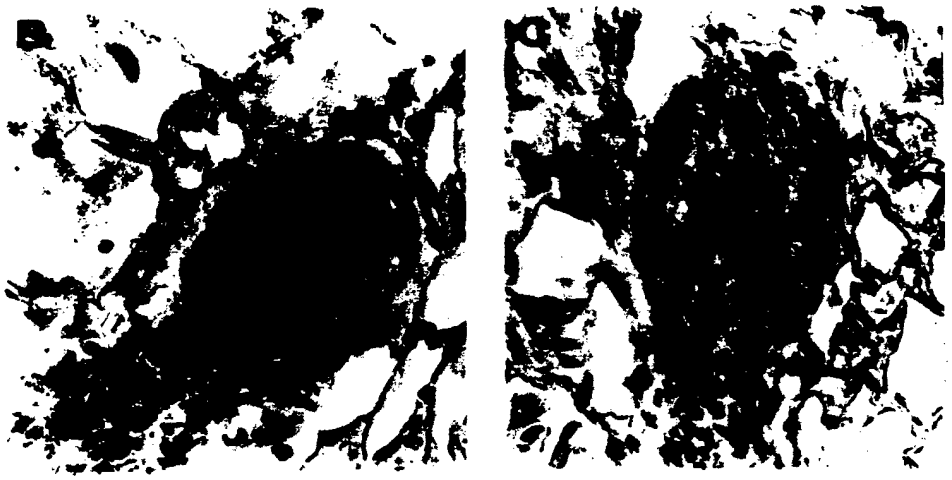


Fig. 2. Percentages of BrdUrd incorporation and immunohistochemical localization of anti-BrdUrd in EGF and AR pellet-implanted mammary glands. The percentages of labeled cells for BrdUrd incorporation were derived according to "Materials and Methods." *A*, a histogram of anti-BrdUrd-labeled cells in the mammary ductal and end bud compartments. ■, BSA treatment; □, EGF treatment; ▨, AR treatment. *Bars*, SE. *B* and *C*, 5-μm cross-sections of TEBs that have reappeared after EGF and AR treatment. *Arrowheads*, cap cells positive for anti-BrdUrd localization; *large arrows*, luminal epithelial cells positive for anti-BrdUrd localization. *B* and *C*, ×1000.

clude that the appearance of terminal end buds was fewer in approximate number as well as the relative size of these structures in the C57 Bl6 EGF/AR groups. These results suggest that AR bioactivity in the OVX mammary gland varies with mouse strains (data not shown).

Our next approach was to analyze the amount of DNA synthesis that occurred after implantation of the AR pellet. In the sectioned glands from the C57 Bl6 AR implant groups, an increase in DNA synthesis was detected in the epithelium of the end bud (22.8%) and epithelial cells that comprise the mature duct (24.2%) compared to the control BSA-treated group (2%; Fig. 2*A*). The percentage of DNA synthesis detected in our study was comparable to mammary glands treated with EGF, as reported previously (Ref. 3; Fig. 2, *B* and *C*). In addition, a small percentage of stromal cells (fibroblasts and adipocytes) were also positive for DNA synthesis (data not shown). Our data indicate that in addition to EGF and TGF-α, AR can also stimulate the reappearance, migration, and DNA synthesis of mammary epithelium in an ovarian hormone-free environment.

Effects of AR Expression in the Virgin Mammary Gland. Our group has recently identified the cellular lineages that express AR in the mouse mammary gland. In both ductal and lobular compartments, AR is expressed by the myoepithelial cells, cap cells of the terminal end bud, and luminal epithelial cells in 4-week-old to 12-week-old mammary glands (7). This diverse expression pattern led us to postulate that transducing mammary epithelial cells with an AR expression vector would perturb development and significantly alter the architecture of the mammary gland. In photos from Fig. 3, *C–E*, we demonstrate that deregulated expression of the *AR* gene in mouse mammary epithelial cells results in an atypical appearance of repopulated mammary fat pads (mammary outgrowths) 12 weeks following transplantation. However, AR-transduced epithelium did not completely fill the entire fat pad area. We presume that in the migrating ductal network, AR overexpression was responsible for this effect because 95% of all AR outgrowths resulted in this appearance.

In 12 of 18 outgrowths that were visually hyperplastic, the outgrowths appeared to be quite similar to those observed

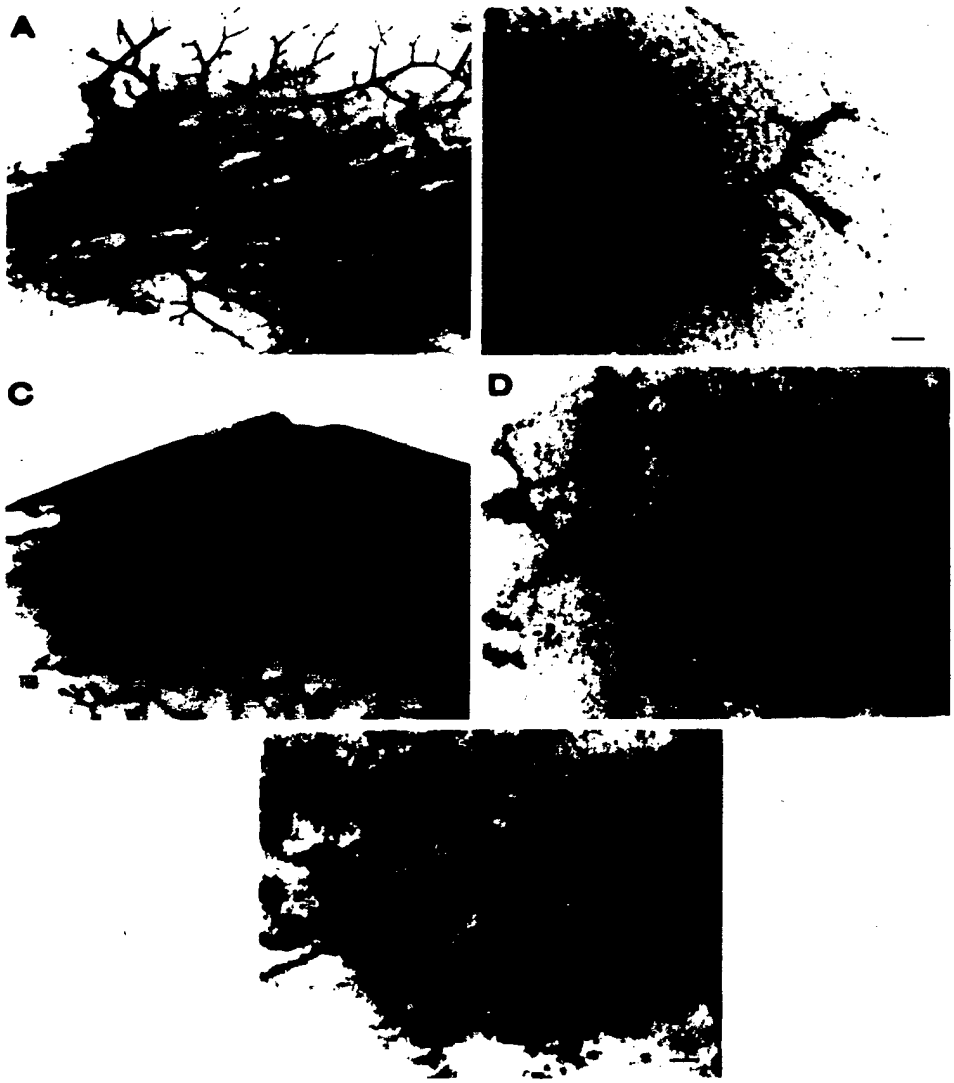


Fig. 3. Whole-mount analysis of intact control pNO4- and AR-transduced mammary outgrowths 12 weeks after transplantation into the no. 4 mammary gland of 3-week-old intact mice. Mammary glands were excised, fixed, and stained according to "Materials and Methods." *A* and *B*, pNO4-transduced outgrowths that show normal terminal ducts (*Nd*) with no apparent mammary aberrations. *C* and *E*, AR-transduced outgrowths that show persistent TEBs (*C*) and at least three types of limited hyperplasia (*H*) (benign, papillary, and hyperplastic lobules). *D*, high magnification of an enlarged area in *C* demonstrating focal areas of lobular hyperplasia. *Bars: A* and *C*, 200 μm; *B*, *D*, and *E*, 25 μm.

previously in the TGF-α transgenic mammary gland (21, 22). In addition, when several outgrowths were excised, sectioned, and visualized under high power magnification for pathological aberrations, we observed limited lobular hyperplasia (which is composed of greatly enlarged and dilated ductules or acini that contain proliferations of the cribriform type in Fig. 4, *C* and *D*), intraductal hyperplasia (represented as luminal epithelial proliferations within the duct), and ductal hyperplasia (indicated by hyperproliferative ducts in Fig. 3*C*). In addition, several fields as represented in Fig. 4*C* contained benign hyperplasia (enlarged ductules with columnar cells) and mild atypia not shown is these photos. We also observed a mammary tumor (intraductal papilloma) in 1 of the 12 nulliparous mice that arose 7 months following transplantation (data not shown). In contrast, no tumors arose in mice following a single round of mating. Mammary gland aberrations found in AR-transduced outgrowths are summarized in Table 2.

Upon further examination of AR mice that had hyperplastic outgrowths, an extensive array of hyperplastic lobular buds and tertiary ducts were easily identified under low magnification (Fig. 3*C*). We then compared these hyperplastic structures to their normal counterparts. Briefly, photos were taken from a total of 20 random fields of five AR hyperplastic outgrowths and five control pNO4 outgrowths, exposed on 5 $\times$ 7 prints, and measured for ductal distance, width, and the number of secretory lobules. Our results suggest that at high magnification, tertiary ducts from AR outgrowths were slightly more narrow (20 $\pm$ 5μm) compared to control tertiary ducts (30 $\pm$ 5 μm; Fig. 3, *B* and *D*). Furthermore, AR outgrowths (Fig. 3*D*) contained a significantly high number of hyperplastic secretory lobules (20 $\pm$ 12) and secondary ducts (>9) not visually found in control pNO4 outgrowths (Fig. 3*A*). A comparative quantitative analysis of the width, length, distance, and the number of ducts and secretory lobules from control outgrowths and AR-transduced outgrowths are summarized in Table 1. In sum, these data demonstrate that overexpression of the *AR* gene can induce an architectural switch within the ductal network and pro-

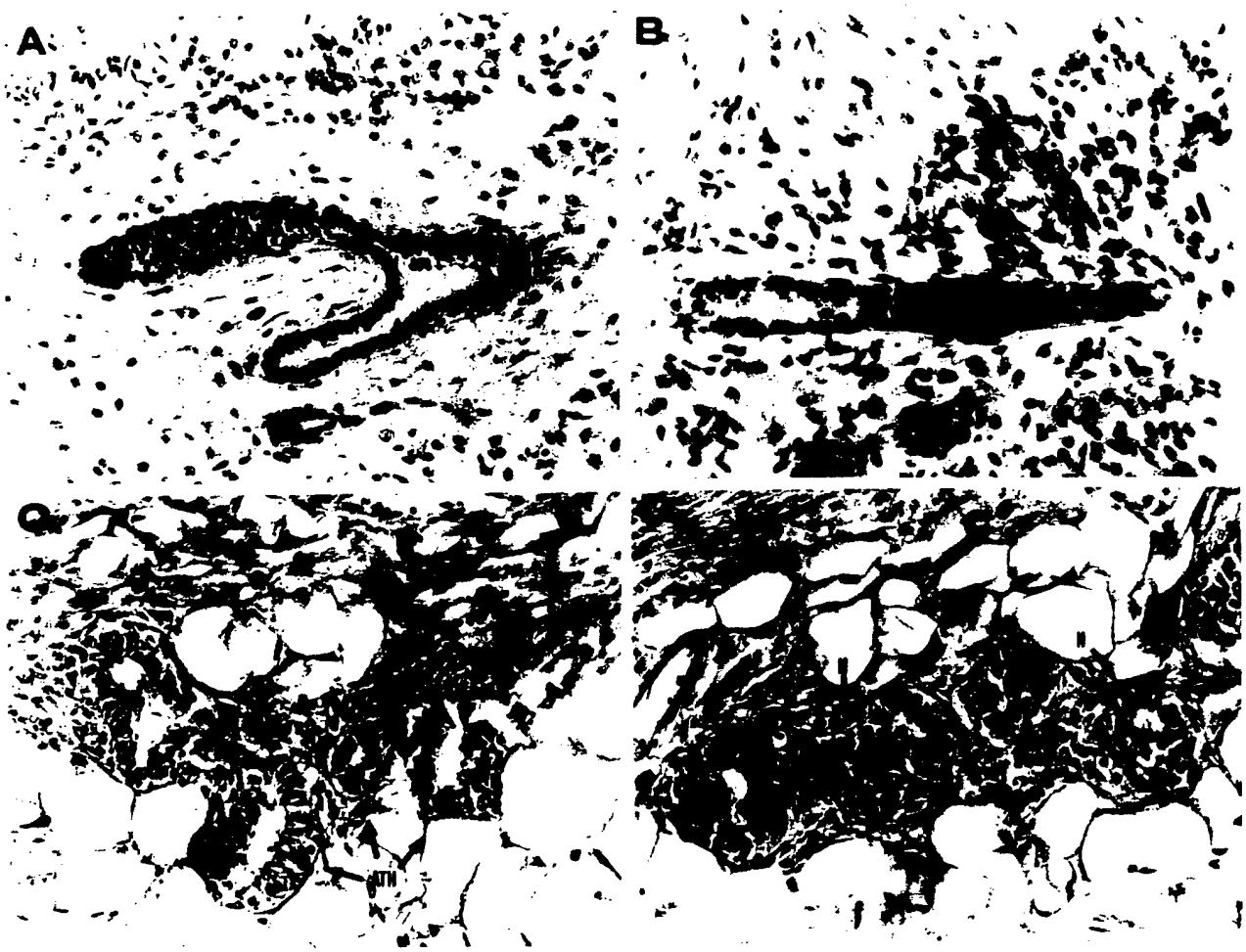


Fig. 4. Cross-section analysis of intact pNO4- and AR-transduced mammary outgrowths. Paraffin-embedded mammary glands were sectioned at 5 mm and stained according to "Materials and Methods." *A* and *B*, control pNO4 normal end bud (*A*) and normal mammary duct (*B*). *C* and *D*, AR atypical hyperplastic lobules (*ATH*) and hyperplastic lobules (*H*). *A–D*, ×400.

Table 1 Morphometric characteristics of AR outgrowths[a]

Parameter	Control	Primary outgrowth
Number of branches from secondary duct	>4	>9
Distance between ducts (secondary to tertiary)[b]	850 ± 300	250 ± 100
Length of tertiary duct[b]	240 ± 100	130 ± 35
Width of tertiary duct[b]	30 ± 5	20 ± 5
Number of hyperplastic lobules per tertiary duct	0	20 ± 12

[a] The table is a representation of three repeat experiments of primary cultures that were transduced then transplanted according to "Materials and Methods." The table reports average duct length ± SD. Measurements represent 10 random fields of 20 mammary ducts (either secondary to tertiary) from 5 mammary glands that were examined by whole-mount analysis, sectioned, and stained with H&E.
[b] Measurement in micrometers.

mote both ductal and lobular hyperplasia in the mouse mammary gland.

AR Serial Transplants into Normal and OVX Cleared Fat Pads. Previous studies have shown that nontransgenic and transgenic, serially transplanted mammary gland outgrowths can regenerate focal growth and undergo differentiation quite similar to their normal counterparts (22, 23). In this context, we investigated whether AR-transduced mammary tissue has the capacity to develop, migrate, and form a similar atypical appearance in normal hormonally intact or OVX mice. Therefore, we serially transplanted portions (2×10^5 cells) of 8–14-week-old AR-transduced outgrowths into 3-week-old hormonally intact female and 5-week-old OVX syngeneic hosts.

Our observations, as illustrated in Figs. 5 and 6, and summarized in Table 2, suggest that of the 10 mice that received serial transplants, 9 contained gross pathological abnormalities comparable to those observed in the donors. However, our most interesting observation occurred in the OVX mammary gland transplants. Nine months post-transplantation, we still observed focal hyperplastic mammary lesions, persistent TEBs and regional loss of ductal spacing (Fig. 6, *D* and *E*). These results indicate that even in ovarian-compromised mammary tissue, AR overexpression can facilitate the propagation of hyperplastic mam-

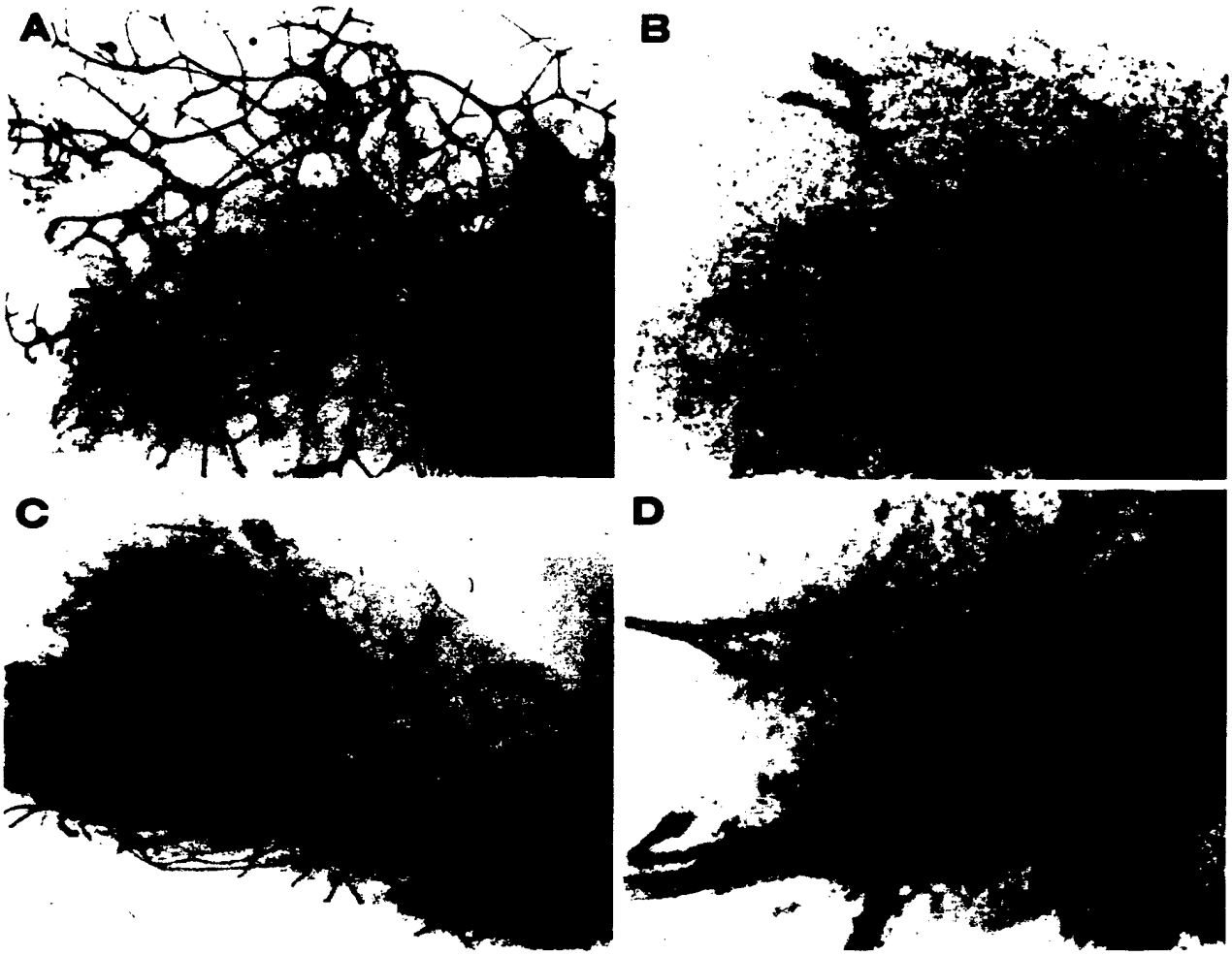


Fig. 5. Whole-mount analysis of 12-week-old control pNO4- and AR-transduced outgrowths serially transplanted into hormonally intact 3-week-old cleared mammary fat pads. Pooled portions from intact pNO4 and AR transgenic donors were transplanted according to "Materials and Methods." In each host, the outgrowth take was 100%. The growth of control pNO4-transplanted outgrowths occupied 70% of the total fat pad area, whereas AR-transplanted outgrowths filled 40% less than its control counterpart. *A* and *B*, normal transplanted outgrowth that show normal terminal ducts (*Nd*) and no apparent mammary aberrations. *C* and *D*, AR-transplanted outgrowth that show focal zones of hyperplasia (*H*). *A* and *C*, ×15; *B* and *D*, ×80.

mary epithelium and induce disregulated ductal growth patterns.

AR Outgrowths and the Regressed Mammary Gland. As mentioned earlier, serially transplanted mammary outgrowths can readily undergo functional differentiation within a syngeneic host. Recent evidence suggests that during regression of lactating transgenic TGF-α mammary epithelium, overexpression of the *TGF-α* gene can enhance the survival of secretory epithelium participating in apoptosis as well as contribute to the reorganization of the gland during involution (22). In this regard, we examined whether the effects of AR overexpression in the mammary gland could affect the architectural design of the gland during its natural course of reorganization following parturition. Briefly, AR fragments were transplanted into the fourth mammary glands of four 3-month-old nulliparous syngeneic female hosts with parallel cycling periods and allowed to repopulate the gland for 12 weeks. All mice were allowed to suckle their pups during these time periods. We then examined these outgrowths by microscopy to determine if there were any gross effects. Our results suggest that by whole-mount and cross-section analysis, the AR transgenic regressed gland had no apparent residual histological changes in epithelial cell death or gland reorganization. In addition, we did not detect the appearance of hyperplastic alveolar structures in the AR regressed gland, an observation which is not uncommon in the normal gland (data not shown; Ref. 24).

Detection of the AR Protein in Intact Outgrowths. Our laboratory reported previously that preferential processing and variable localization patterns of AR protein occur within the mammary tumors of transgenic mice overexpressing TGF-α, *int-3*, *neu*, SV40 large T antigen, and polyoma virus middle T antigen (20). To determine whether this phenomenon appeared within the AR transgenic mammary epithelium, we analyzed the distribution and expression intensity of AR protein isoforms in control and transduced mammary gland extracts by Western blot analysis, as described previously (20).

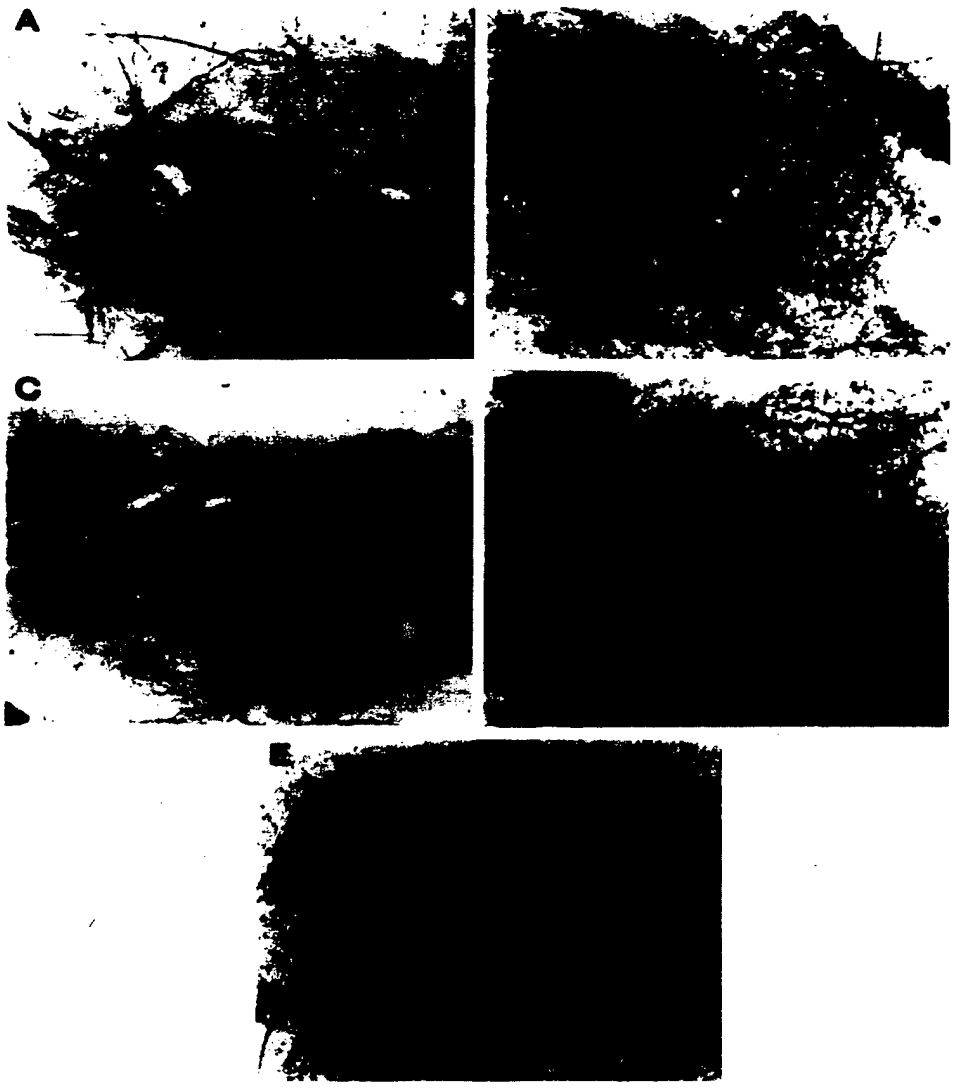


Fig. 6. Whole-mount analysis of 9-month-old control pNO4- and AR-transduced outgrowths serially transplanted into OVX 3-week-old cleared mammary fat pads. Pooled portions from intact pNO4 and AR transgenic donors were transplanted according to "Materials and Methods." The total area of growth in both control pNO4- and AR-transplanted outgrowths were equal. *A* and *B*, normal transplanted outgrowths that show normal terminal ducts (*Nd*) and no apparent mammary aberrations. *C–E*, AR-transplanted outgrowth that shows a focal region of hyperplasia (*H*), persistent TEBs (*open arrowhead*), and localized areas in which there was a loss in ductal spacing (*curved arrow*). *A* and *C*, ×15; *B*, *D*, and *E*, ×80.

Our negative controls during these experiments were mammary gland extracts from control nontransduced outgrowths, pNO4 transduced outgrowths (empty viral vector outgrowths), and empty fat pads (epithelial-cleared fat pads). For positive controls, extracts from MMTV/int-3 transgenic mammary tumors were used. These mammary tumors are poorly differentiated adenocarcinomas that spontaneously develop as early as 7 weeks of age and express various isoforms of the AR protein (M_r 32,000 and M_r 21,000; Ref. 20).

Our results when analyzed by densitometric analysis indicate that relative to the negative controls, a 6.5-fold induction of the M_r 28,000 and M_r 8,000 AR protein occurred in the AR-transduced outgrowths (Fig. 7, *arrow*). In addition, when we compared the AR isoforms of control, pNO4 outgrowths to AR outgrowths, we found the same patterns of expression but not in the MMTV/int-3 tumors. We interpret these results as an indication that the preferential processing of the AR protein in the AR transgenic mammary gland in relation to previously described oncogene-induced mammary tumor tissue (20) must be influenced by the stage of malignancy (hyperplasia *versus* adenocarinoma) as well as the type of germ-line mutation (*i.e.*, *int-3*).

Finally, as an additional means of confirming if these outgrowths indeed contained our virally inserted expression vector, we performed Southern blot analysis on *Hin*dIII/*Xho*I digested genomic DNA using a 2.7-kb *Hin*dIII/*Xho*I fragment containing the neomycin-cytomegalovirus sequence. In control pNO4 outgrowths and AR-transduced outgrowths, but not in control nontransduced outgrowths, a 2.7-kb hybridized fragment was detected (data not shown).

Discussion

To date, the limited available data are not able to specifically define the *in vivo* biological pathways by which the EGF family of growth factors mediate the neoplastic conversion of the mammary gland (21, 22, 24–26). Several EGF-related peptides have been shown to play important roles in malignant progression, cell survival, metastatic potential, and

Table 2 Pathological abnormalities observed in AR outgrowths[a]

Outgrowth	No. of transplants/take	Time course	Gross abnormalities
Primary transplants	12/18	8–36 weeks	Lobular hyperplasia Ductal hyperplasia Intraductal papilloma
Serial transplants			
intact	5/5[b]	8–12 weeks	Lobular hyperplasia Ductal hyperplasia
OVX	4/5	4–12 weeks	Lobular hyperplasia Ductal hyperplasia Persistent TEBs[c]
Mated mice	4/4	5 and 28 days[d]	None

[a] The table represents a combination of three repeat experiments from primary cultures that were transduced then transplanted according to "Materials and Methods." Outgrowths were examined by whole-mount analysis, sectioned, and stained with H&E.
[b] Animal death that contained a positive take.
[c] Serial transplant that contained persistent TEBs and loss of ductal spacing 9 months after transplantation.
[d] Represents time points that mice were sacrificed during or after nursing.

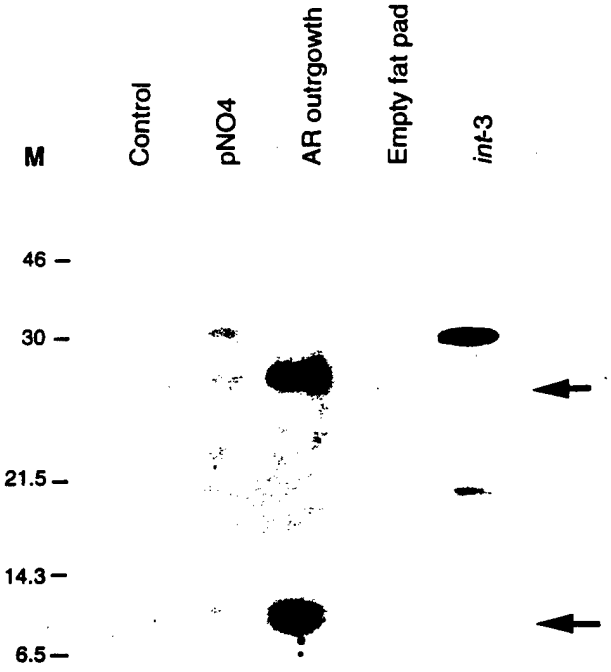


Fig. 7. Western blot detection of AR protein in control outgrowths, pNO4 (empty vector) outgrowths, AR-transduced outgrowths, empty fat pads (epithelial-cleared fat pads), and MMTV/int-3 transgenic mammary tumors. Total protein extracts were derived according to "Materials and Methods." All lanes were loaded with 50 μg of total protein. The extracts from MMTV/int-3 tumors were used to detect various AR isoforms observed previously (20). No apparent preferential processing occurred in AR-transduced outgrowths but instead showed an 6.5-fold increase in the M_r 26,000 and M_r 8,000 species. *Arrow*, M_r 26,000 and M_r 8,000 species. M_r molecular weight markers in thousands.

clonal expansion of the mammary gland (25). Recently, transgenic and gene targeting technology has shed light on this subject (21–22, 24). The *ex vivo* approach has been previously used to analyze the efficiency of retroviral transfer (β-galactosidase) or the effects of cancer-related genes such as *wnt-1*, c-Ha-*ras*, or *neu* (27–30). These reports demonstrate that when transplanted into cleared fatty stroma of a syngeneic host, cultured mammary epithelium can undergo morphogenesis and differentiation and can also express early and later stages of malignant progression.

In the current report, we have utilized two techniques, Elvax slow release pellets and retroviral transfer, to manipulate primary mouse mammary epithelium and to study the short- and long-term behavior of the epithelium in virgin, involuted, and OVX female mice. We demonstrate that the short-term introduction of recombinant AR initiates ductal morphogenesis in the absence of ovarian hormones and further note that the effects of long-term overexpression of the *AR* gene within the mammary tree induces an atypical appearance in outgrowths within at least 9 weeks of introduction. These findings are summarized in Fig. 8.

Transduced AR primary cultures that were transplanted into a total of 30 C57 Bl6 mice resulted in 12 of 18 takes that had hyperplastic-ductal-lobular structures with an increase in the tertiary ducts, which were not present in control or pNO4 transplanted cultures. When the morphometric characteristics of five AR outgrowths were examined, mammary ducts (secondary through tertiary) were significantly different than control outgrowths (Table 2). This suggests that as a localized factor, AR bioactivity, can modulate the pattern of mammary ductal growth.

The Complexity of Serial Outgrowth. Using the serial transplantation approach, we have also observed that AR outgrowths present the same morphological hyperplastic appearance in mammary fat pads. However, since these outgrowths were possibly clonal in origin, the innate ability of the cells to repopulate the transplant site was only sufficient to occupy 40% of the total volume of the fat pad. We do not think that the reduction in growth of the serially transplanted AR outgrowths was due to technical difficulties (*i.e.*, location, excision, and transplantation of an AR outgrowth) because the number of surviving transplants was 100%. On the contrary, the practice of propagating serially normal, preneoplastic, and neoplastic outgrowths has been successfully carried out for several decades. The salient observations by DeOme and later by Daniel demonstrated that through each successive transplantation, the regenerative capacity of the mammary outgrowth was reduced by about 20%, and the age of the outgrowth as well as the recipient's mesenchyme played important roles in the formation of the mammary tree [reviewed by Kenney *et al.* (23)]. Whereas in our study, AR serially transplanted outgrowths invaded as much surface mesenchymal area as control outgrowths; the phenotypic changes that accrued may have been attributed to the following factors: (*a*) not all atypical lesions in AR-transduced outgrowths contain immortalized progenitors; and (*b*) the regenerative capacity of AR-transduced secretory progenitors are greatly reduced following cell renewal. The latter argument can be supported by the observations that in the regressed AR transgenic gland, we could not detect the presence of immortalized hyperplastic alveolar nodules, and in the OVX AR transgenic gland, we identified only focal

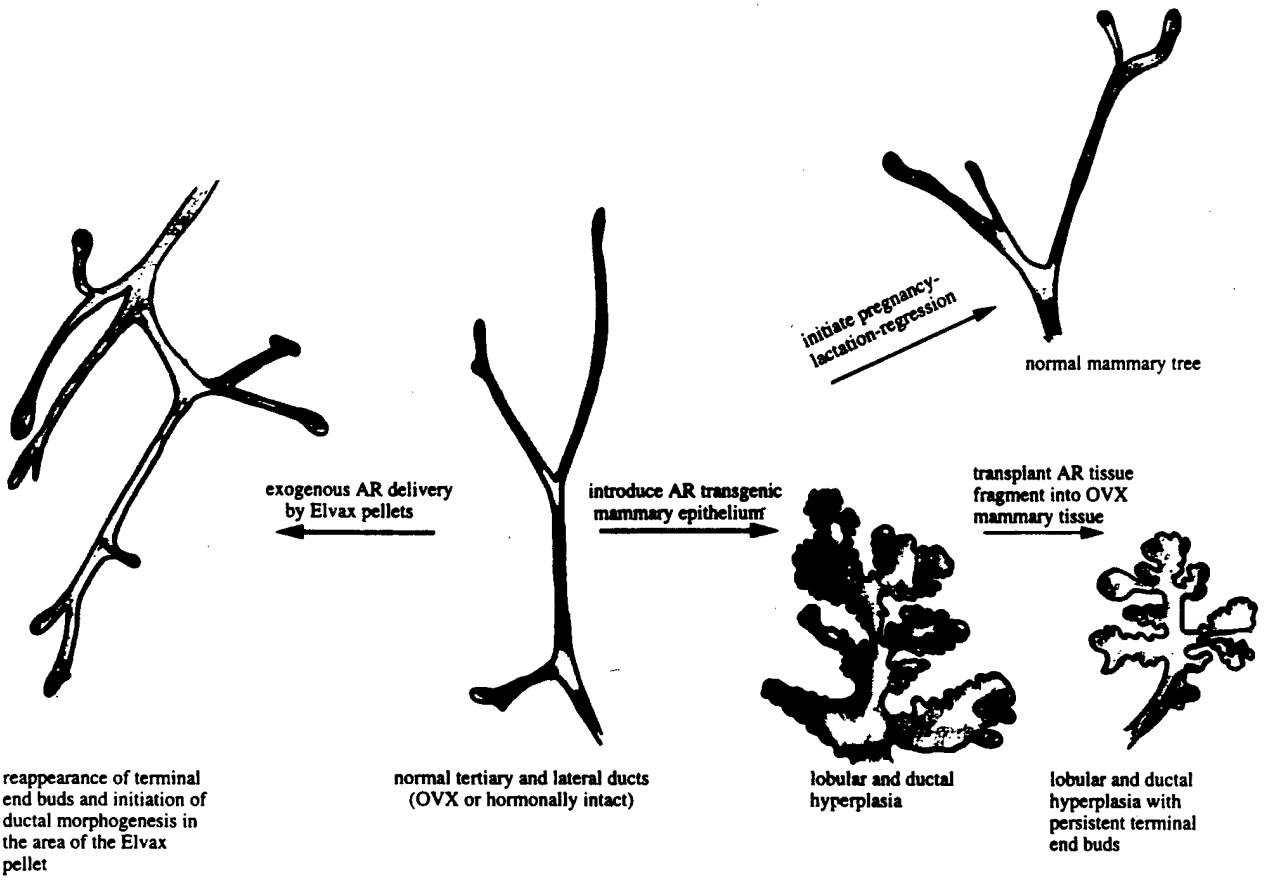


Fig. 8. Summary of AR activity in the mouse mammary gland. All images represent the activity of the *AR* gene and its recombinant protein in the context of the intact normal, OVX, regressed, and serial transplanted mammary gland *in vivo*.

areas of hyperplastic growth compared to intact AR transgenic glands. We thus believe that because less than 10% of the committed secretory progenitors may have been transduced with AR virus, the process of immortalization to malignancy and cell renewal are distinct and separate entities. The best example thus far is that of the hyperplastic alveolar nodules, which are derived from stable premalignant lesions. Medina *et al.* (31) have identified an independent hyperplastic alveolar outgrowth line that reproducibly develops hyperplastic lobular lesions when propagated by serial transplant. However, of the 12 additional independent hyperplastic outgrowth lines isolated, two immortalized outgrowth lines presented primarily ductal growth that also contained TEBs and limited lateral branching. In this regard, we also observed one potent progeny that maintained its capacity to develop persistent TEBs and dysregulated ductal growth (Fig. 6, *curved* and *open arrows*). The circumstance by which the TEBs persisted in the OVX mammary gland well after 9 months posttransplantation remains quite complex.

AR Effects on the Multipotent Stem Cell. By definition, the TEB is a bulbous-like epithelial structure that appears at puberty and is associated with the morphogenic movement of the rudimental mammary duct during the first 2 months of development. During this period, the terminal end bud primarily consists of two types of stem cell progenitors, both of which are derived from the multipotent cap cell (1, 32): the ductal progenitor, which has the capacity to form the dense branching network of epithelial ducts that traverses the fat pad and the secretory progenitor, that has the capacity to synthesize and form milk secretory structures. Together, these cells participate in the formation and function of the mammary tree. The multipotent cap cell is capable of synthesizing laminin, hyuronate, and S-GAGs (33). However, only hyuronate is confined to the region of the end bud tip (33). During the establishment of the open ductal arrangement in the nonpregnant rodent mammary gland, collagen fibrillogenesis plays a significant role in the maturation of the mammary tree (33). As a part of this process, stromal synthesis of S-GAGs forms a unique concentration gradient around the end bud flank, which may act in an inhibitory manner to discourage other ducts from approaching the same area of growth, thereby channeling the direction of terminal end bud growth (33). Recently, HS-GAGs have been implicated in suppression of AR signaling (14, 34, 35). The NH_2-terminal hydrophilic region of the human AR protein contains a lysine-arginine-rich heparin-binding region adjacent to the COOH-terminal end of the EGF-like domain (14). *In vitro,* the mitogenic activity of AR can be modulated in an inhibitory fashion by the addition of exogenous heparin (14).

Furthermore, heparin is structurally related to heparan sulfate (36). Piepkorn *et al.* (35) recently found that in mouse keratinocytes, soluble heparin-like GAGs can inhibit the mitogenic activity of AR, whereas the membrane-bound S-GAGs may bind to the cell membrane receptor or matrix and may act as mediators in AR signaling. They also suggest that during the process of keratinocyte commitment to terminal differentiation, the expression of membrane-bound endoglycosidase maybe up-regulated. This enzyme, in turn, then generates soluble heparan sulfate free chains by cleaving the membrane or matrix-bound HS-GAGs. The exogenous heparan sulfate free chains can compete with S-GAGs for AR binding and disrupt the AR signaling pathway, which results in the cessation of keratinocyte growth (35). In support of this hypothesis, Johnson and Wong (14) suggest that in mammary epithelial cells, HS-GAGs maybe covalently linked to membrane proteins, such as syndecans, that stabilize the mitogenic signaling pathway between AR and the EGFR (14). In this regard, we speculate that in the context of the normal, intact, and OVX mammary gland, the AR signaling pathway may be mediated through these mechanisms. This speculation can be supported by the recent observations in the juvenile C57 Bl6 female mammary gland. We have demonstrated that in the quiescent rudimental mammary ducts of 3-week-old C57 Bl6 female mice, AR protein expression is limited to the epithelial component. However, in the 4–8-week-old actively growing mammary gland, AR protein expression was detected in the adjacent stroma ahead of the migrating TEBs and in the stroma flanking the region of subtending duct (7). Given this scenario, we suspect that with the concurrent overexpression of AR, the appearance of abnormal developmental structures (Fig. 6, *curved arrow* and *open arrow*) may have been derived by, but not solely attributed to, a modified multipotent cap cell that is responding to: (*a*) an abundant source of AR protein complexed with HS-GAGs that maximize a constitutive EGFR autocrine signaling loop anchored on its surface at the end bud tip; and (*b*) the effect of abundant AR protein during the normal course of collagen fibrillogenesis that allows for an unfavorable S-GAG:AR ratio in the S-GAG gradient in the end bud flank, resulting in dysregulated ductal growth. To clarify these hypotheses, we are currently conducting AR and EGFR *in situ* hybridization, immunofluorescence, and S-GAG staining.

Does the Activity of AR Share EGF-like Characteristics? We suggest that the biological activity and patterns of immunolocalization of the AR protein in the developing mammary ductal compartments resemble EGF more than TGF-α. Using the slow-release Elvax pellet technique, recombinant AR stimulated a mitogenic and ductal growth pattern quite similar to that which is induced by recombinant EGF. However, when AR is overexpressed in normal mouse mammary epithelium *in vivo,* the resultant epithelium resembles that of TGF-α transgenic tissue. AR transgenic mammary tissue consistently developed a dense network of branching ducts similarly found in TGF-α transgenic pubescent mammary glands (21). Likewise, both TGF-α and AR have the capacity to stimulate lobular alveolar development in ovarian-compromised mammary tissue (6). Currently, there is no evidence of the direct consequence of EGF transgene in the context of the rodent mammary gland (25). *In vitro,* AR has the capacity to act as a potent epithelial mitogen for several human breast cancer cell lines (25, 37). Under growth factor-depleted conditions, AR can replace the growth-promoting effects of EGF (37). In addition, the proliferation of human immortalized mammary epithelial cells may rely in part upon the production of endogenous AR. Recently, our group has shown that the growth of nontransformed, immortalized mammary epithelial cell line 184 A1N4, which expresses AR mRNA, can be growth inhibited by specific antisense oligonucleotides in the presence of exogenous EGF. However, a combination of endogenous antisense TGF-α mRNA expression and addition of antisense AR oligonucleotides did not lead to a further cessation in growth when compared to the inhibitory effects of an anti-EGFR neutralizing antibody alone. This may suggest that in nontransformed mammary epithelial cells, additional endogenous non-EGF-related mitogens may act to stimulate growth (37).

What is the consequence of AR expression? In the context of the mammary gland, the biological activity of AR is equipotent to EGF, and the transgene effect parallels that of TGF-α. In the paradigm of mammary transformation, we hypothesize that the activity of AR in mammary epithelial stem cell progenitors facilitates maturation arrest and allows these cells to enter into the malignant pathway via EGF independence. This hypothesis has been conceptualized by Medina *et al.* (26), who suggest that some molecular alterations that contribute to the development of mammary preneoplasias and neoplasias may be EGF-linked. The relationship between ovarian dependence and EGF dependence (preneoplasia I) and EGF independence and tumorigenic potential (preneoplasia II) may indicate that the participants in the EGF pathway (*i.e.,* AR) play important roles in the generation of mammary preneoplasia which have measurable tumorigenic potential (26). Interestingly, we have observed previously that the *AR* gene is expressed in mammary tumors generated from TGF-α, *neu, int-3,* polyoma middle T antigen, and SV40 large T antigen transgenic strains (20). Of the three growth factor/receptor-related transgenic mammary tumors (TGF-α, *neu,* and *int-3*) the growth of both TGF-α and *int-3* transgenic mammary tumors cell lines is EGF independent *in vitro*.[4] The AR protein in these tumors undergoes a temporal increase and preferential processing in a strain-specific fashion. In the current study, we could not detect preferential processing but did identify a 6.5-fold increase in two AR isoforms from AR-transduced outgrowths compared to controls. It is very likely in this experimental model that secondary genetic germ-line events contribute to tumorigenesis and to preferential processing of the *AR* gene product. Additional studies that approach the biochemical effects of the *AR* gene *in vivo* and *in vitro* should shed further insights into the activity of this gene. Thus far, our combined approach has provided a unique and in-depth analysis into the *AR* gene and its biological activity. Our study provides additional evidence for the importance of the *AR* gene and

[4] G. Smith, personal communication.

the AR protein and its contribution during various stages of mammary development and cancer. We suggest that the data accrued here can potentially lead toward the development of diagnostic and therapeutic strategies.

Materials and Methods

Animals and Pellet/Transplant Surgery. A total of 75 (50 C57 Bl6 and 25 FVB/N; Charles River Laboratories) pathogen-free mice OVX at 5 weeks of age were used in the Elvax slow-release pellet experiment. These inbred mice were also housed for 2.5 months prior to pellet implantation to eliminate the possibility of systemic sex steroid effects on mammary ductal morphogenesis. Before the experiment, a small L-shaped incision was placed on the abdominal wall of the mouse, exposing the fourth inguinal and contralateral mammary glands (23). Next, a small pocket within the mammary tissue was placed by small forceps. An Elvax pellet containing growth factor or BSA (control) was placed within the pocket, and the incision was closed with wound clips. For each experiment (repeated three times), a total of 10 OVX mice received AR Elvax pellets (5 μg each), 10 OVX mice received EGF (5 μg each), and 5 OVX mice received BSA Elvax pellets (5 μg each).

In transplantation experiments that used retrovirally transduced AR mammary primary cultures, pNO4 (empty vector)-transduced mammary primary cultures and control nontransduced mammary primary cultures, a total of 75 3-week-old virgin female C57 Bl6 mice were used. A total of 15 13-week-old hormonally intact female C57 Bl6 mice were used to derive primary mammary epithelial cultures. In each experiment (repeated three times), a total of 10 mice received AR-transduced primary cells, 10 mice received pNO4 (empty vector)-transduced primary cells, and 5 mice received control nontransduced primary cells.

In the second set of experiments that used serially transplanted outgrowths into syngeneic hosts, a total of 15 mice were used in this study. Briefly, five hormonally intact 3-week-old female mice, four 5-week-old OVX mice, and four 3-month-old fertile female mice received a pooled fragment (2×10^5 cells) of AR hyperplastic outgrowths from living donors (a total of three). In all experiments, the fat pads of these mice were cleared of the host's epithelial rudiment. Fragments were allowed to repopulate the hosts mammary epithelium for 4–12 weeks or 12 weeks for mice that were eventually mated and sacrificed at 5 and 28 days, respectively. In all experiments, animals were supplied with food and water *ad libitum* and were housed under a 12-h light/12-h dark cycle. Upon surgery or completion of the experiment, animals were either anesthetized with metofane or euthanized with CO_2 and metofane.

Implants. Ethylene acetate copolymer (Elvax) was gift from the DuPont Chemical Co. AR was a gift from Dr. Stuart Thompson (Berlex Biosciences, Concord, CA). EGF was purchased from Collaborative Research (Waltham, MA). Briefly, 20–25 mg of BSA and 100 μg of AR and EGF were dispersed in 125 μl of Elvax dissolved in dichloromethane (20% w/v). The mixture was quick frozen, dried, and cut to weight (1.0 mg = 5 μg of growth factor or BSA). The pellets were surgically implanted into the fourth mammary gland, and the BSA pellet alone was implanted in the contralateral no. 4 mammary gland.

Retroviral Vectors. An 850-bp human AR fragment that contains the coding sequence for AR was derived from a full-length 1.4-kb human AR cDNA as described previously (15). This fragment was then introduced in the 5′ to 3′ orientation into the Moloney sarcoma virus-long terminal repeat-derived pNO4 recombinant plasmids at the 3′ end of the cytomegalovirus viral promoter (37). Transfection of the recombinant 850-bp AR sense plasmid was then carried out as described previously into the PA 317 amphotropic packaging cell line to produce amphotropic, replication-defective retrovirus stocks for AR (13, 37). Virus-containing supernatants were obtained from mass-transfected G418-resistant PA 317 cells. These supernatants were then screened for helper virus and found to be negative (38). Primary cells were infected with PA 317 retroviral vector containing supernatants (1 ml) that had been previously titered on NIH 3T3 cells at 1.5×10^5 neomycin-resistant colonies/ml. Primary cells were infected in 25-cm^2 tissue culture plates in medium containing 4 mg/ml DEAE dextran. Primary cells were later maintained in G418 for an additional 7 days before transplantation.

Primary Cultures. Primary mouse mammary epithelial cultures were obtained and transplanted as described previously (7). Briefly, the mammary glands from a total of 15 13-week-old hormonally intact female C57 Bl6 mice were used to derive primary mammary epithelial cultures. Isolated cultures used for infection were allowed to grow in Improved Modified Eagle's Medium supplemented with 10% FBS, 10 μg/ml bovine insulin, 10 μg/ml transferrin, 5 μg/ml hydrocortisone, and 10 ng/ml EGF at 37°C and 5% CO_2 for 7 days before transplanting 1×10^6 cells into the cleared fat pads of intact 3-week-old syngeneic hosts.

Histology. Transplanted or implanted (Elvax slow-release pellets) mammary glands were excised and fixed overnight in 4% formalin. Whole-mount analysis were prepared by first defatting the tissue in acetone, then hydration through alcohols, stained with Gill's hematoxylin, and dehydrated and mounted for photography. Whole glands were then embedded in paraffin and sectioned for immunohistochemical analysis (BrdUrd labeling) or morphometric characteristics by staining with H&E. Morphometric characteristics are measurements that calculate ductal width, length and degree of branching, and secretory lobules. Briefly, for the degree of branching and secretory lobules present, a total of 20 random fields from five AR-transduced and five control nontransduced outgrowths were examined under light microscopy. For morphometric measurements, a total of 20 random fields from five AR-transduced outgrowths and five control nontransduced outgrowths were photographed and exposed on 5 × 7 Kodak prints and then standardized in millimeters for the origin length of the image. Finally, the distances and widths of mammary ducts were calculated in micrometers.

Scoring of Labeled Nuclei. Briefly, 50 C57 Bl6 5-week-old OVX mice that were implanted and treated with either AR, EGF, or BSA Elvax pellets for 5 days were then injected with BrdUrd (100 mg/kg; Sigma Chemical Co.) i.p. into the lower right quadrant of the abdomen 2 h prior to sacrifice. Mammary glands were excised and embedded in paraffin and sectioned for immunocytochemistry. Sections were put through xylene and graded alcohols, and endogenous peroxidase activity was inactivated with 0.03% H_2O_2 in methanol. Nonspecific binding was blocked with 5% normal goat serum, after which the sections were incubated for 12 h with 1 μg/ml of mouse anti-BrdUrd antibody (Sigma Chemical Co.). A section of duodenum was used as a control to confirm systemic delivery of BrdUrd to tissues. Sections were then treated with 1:1000 dilution of rat antimouse antibody (Vectastain) and visualized with avidin-biotin complex conjugated to horseradish peroxidase with 3–3′-diaminobenzidine-4-HCl as the chromagen (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). The slides were then lightly counterstained with Gill's hematoxylin or carmine aluminum. Random fields surrounding the periphery of the pellet were used for BrdUrd scoring. A total of 20 fields scored in 10 sections from each treatment group contained more than 100 cells/field. Percentages of labeled cells were calculated by dividing the number of labeled nuclei by the total number of nuclei counted (8). Mammary epithelial cells that had BrdUrd incorporation were identified by dark brown to tan over purple (negative) pigment.

Western Analysis. Primary cultures or the fourth inguinal mammary gland tissue was homogenized in 1 ml of hypotonic buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM PMSF, 1% NP40, and 20 μg/ml aprotinin; Ref. 13). Samples (50 μg) were boiled and resolved on a 4/15% SDS-PAGE gel. AR was detected by an affinity-purified anti-AR Ab-2 (1 μg/ml) antibody (7). Equivalent loading of protein samples was observed by staining a parallel gel with Coomassie Blue. Specificity was confirmed by pre-absorbing AR Ab2 against a 5-fold excess of the synthetic peptide 26-44.

Southern Blot Analysis. Genomic DNA was extracted from transplanted outgrowths using the proteinase K-phenol extraction method (39). The DNA was then digested with *Hin*dIII/*Xho*I, separated by electrophoresis in 1% agarose, and transferred to nylon filters. A 2.7-kb probe containing the *neomycin* gene-cytomegalovirus promoter region of pNO4 complimentary DNA plasmid was generated by *Hin*dIII/*Xho*I restriction digestion. Southern hybridization was carried out at 68°C in hybridization buffer from Clone Tech (Palo Alto, CA) and 15 ng/ml probe labeled with [α-^{32}P]dCTP (Amersham Corp.) by random priming (Boehringer Mannheim, Indianapolis, IN). After overnight hybridization, the blots were washed in 0.2× SSC-1% SDS at 65°C for 20 min and then washed with 0.1× SSC-0.1 SDS at 65°C for 5 min. The blots were

subjected to autoradiography using Kodak films at −70°C with an intensifying screen.

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