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Cancer Treatment and Research

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Preface

In *Breast Cancer: Cellular and Molecular Biology* [Kluwer Academic Publishers, 1988], we tried to present an introduction to the emerging basic studies on steroid receptors, oncogenes, and growth factors in the regulation of normal and malignant mammary epithelium. The response to this volume was superb, indicating a tremendous interest in basic growth regulatory mechanisms governing breast cancer and controlling its malignant progression. In the two years since its publication, much new and exciting information has been published and the full interplay of regulatory mechanisms is now beginning to emerge. We have divided this book into four sections that we hope will unify important concepts and help to crystallize areas of consensus and/or disagreement among a diverse group of basic and clinical scientists working on the disease.

The first section is devoted to studies on oncogenes, antioncogenes, proliferation, and tumor prognosis. The first chapter, by Sunderland and McGuire, introduces the characteristics of breast cancer as studied by pathologists to establish prognostic outcome. Of particular interest is a new protooncogene called HER–2 (or neu), which is rapidly becoming accepted as a valuable new tumor marker of poor prognosis. The second chapter, by Lee Bookstein and Lee, introduces the best known antioncogene, the retinoblastoma antioncogene, whose expression is sometimes lost in breast cancer. Malignant progression appears to be influenced by the balance of protooncogene and antioncogene expression.

The second section focuses on growth factors and their receptors in breast cancer. Rudland and co-workers, in the first chapter, discuss the interplay of growth factors among the three major cell types in the rat mammary gland: stroma, epithelia, and myoepithelia. Growth factors modulate both proliferation and differentiation of the epithelium. In particular, TGF–α is shown to be released by myoepithelia and stimulates the epithelium. In the second chapter, Daniel and Silberstein utilize local growth factor implants in the developing mouse mammary gland to establish their effects *in vivo*. In particular, TGF–β is shown to be an important negative modulator of mammary growth. The next chapter, by Yee and co-workers, focuses on the insulin-like growth factors. These factors appear to be principally synthesized

in breast cancer stroma and they stimulate the epithelium. In the next chapter, Salomon and co-workers discuss interactions among growth factors and oncogenes in breast cancer. In particular, *ras* oncogene can both induce TGF–α and modulate the cellular response to TGF–α in breast cancer. The next chapter, by Buick and co-workers, explores the mechanism of signal transduction through the EGF receptor. In breast cancer, the EGF receptor appears to be associated with estrogen receptor negativity and poor prognosis. The final chapter, by Sonnenschein and Soto, explores interactions between growth inhibitory factors in serum and growth stimulatory effects of estrogen. Steroids, growth factors, and oncogenes form a complex web of growth modulatory influences in breast cancer.

The third section presents chapters on the mechanisms of action of estrogens and antiestrogens. The first chapter, by McCarty and McCarty, establishes the effects of estrogens and antiestrogens in modulating breast cancer expression of growth factors and proto-oncogenes. The next chapter, by Iino, Gibson, and Jordan, focuses on antiestrogens as anticarcinogenic and antitumorigenic treatment agents in breast cancer. The next chapter, by Westley and May, examines in molecular detail the genes that are expressed when estrogen stimulates breast cancer proliferation. The following chapter, by Martin et al., establishes the molecular effects of estrogen and antiestrogen on the expression of the estrogen receptor itself. The next chapter, by Arteaga and Osborne, evaluates the role of secreted growth factors in directly mediating the effects of estrogen and antiestrogen on breast cancer cell proliferation. The last chapter of the section, by Musgrove and Sutherland, examines the cell cycle effects of estrogens, antiestrogens, and other growth regulators of breast cancer.

The final section presents chapters on the interaction of the mammary stroma and epithelium in normal and malignant/metastatic processes. The first chapter, by Donjacour and Cunha, examines the ability of stromal cells to modulate the development and function of normal mammary epithelium. The next chapter, by Streuli and Bissell, focuses on the ability of the basement membrane to polarize the DNA-organizing epithelial function. The basement membrane can bind to the cell surface and transmit signals to the cell nucleus to modulate the expression of genes, such as casein. Sakakura and co-workers, in the next chapter, present data on a new component of the basement membrane surrounding precancerous and cancerous breast epithelial lesions. This component, tenascin, modulates breast epithelial cell attachment and proliferation; it may be a valuable new tumor marker. The next chapter, by Haslam, establishes the requirement for breast stromal cells to allow for estrogen stimulation of epithelial cell proliferation. Cancer may involve at least partial escape from these requirements. The final chapter, by Goldberg and Eisen, deals with the ability of breast cancer to escape from its encapsulating basement membrane. Collagenolytic enzymes are secreted, and activated, and they then serve to degrade the basement membrane and facilitate metastases.

We hope this new volume will build on the framework of the previous volume and interest students and basic and clinical researchers interested in fresh perspectives on breast cancer. Almost certainly, clues to improved prevention, prognosis, and treatment of breast cancer lie within these chapters.

M.E. Lippman
R.B. Dickson

I.

Oncogenes, Antioncogenes, and Tumor Prognosis

1. Oncogenes as clinical prognostic indicators

Margaret C. Sunderland and William L. McGuire

Introduction

Oncogenes

Recent advances in molecular biology have enabled oncologic researchers to probe the mechanisms of neoplasm at the level of individual gene expression. Current studies of oncogenesis suggest that specific genes may induce cancer and that normal resident genes, termed proto-oncogenes, required for cellular function can be converted to oncogenes by genetic mutation [1]. Alterations in the structure or expression of certain proto-oncogenes appear to occur through a variety of mechanisms, including point mutations within the gene, rearrangements within the coding sequence of the gene or within a noncoding but functionally effective segment, amplification or overexpression of the gene, and deletion of possible 'antioncogenes.' Each of these mechanisms may result in the activation of cellular proto-oncogenes and are found to be associated experimentally with human cancer [2]. While it has not been proven that these genetic anomalies are directly responsible for tumorigenesis, the frequency of their presence and/or expression in some oncologic diseases suggests a biologic importance in the transformation and growth of neoplastic cells [3, 4].

The cytogenetic analysis of human cancer cells by high-resolution banding techniques indicates that more than 90% of human malignancies carry clonal cytogenetic changes [5]. The great majority of hematologic malignancies have specific chromosomal alterations, suggesting that nonrandom chromosomal changes may be involved in the pathogenesis of certain human malignancies [6]. In the case of most solid tumors there is an extraordinary diversity of chromosomal aberrations, limiting our ability to identify and correlate consistent changes with specific tumors.

Characterization of oncogenes has been greatly facilitated by a technique developed by Southern [7]. DNA is cleaved with one or more restriction enzymes and separated according to fragment size. A replica, or 'blot' of the DNA fragments is made on a filter that can then be hybridized with a radioactive probe. The oncogene can also be characterized by the measure-

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ment of messenger RNA, using the same strategy as for DNA, or alternatively by the estimation of the oncogene's protein product. Immunohistochemical staining with antibodies directed against known sequences of the gene and the Western blot technique, which allows quantification of the antibody reaction by densitometric scanning, have been used to investigate oncogene proteins [8].

Use of these newer techniques in molecular biology has led researchers to postulate a correlation between disease prognosis and abnormalities related to oncogenes, such as gene amplification, overexpression, and loss of alleles. The possible presence of extra gene copies within a tumor genome was indicated by cytogenetic analysis, which revealed double minute chromosomes, homogeneously staining regions, and chromosome duplications. Several studies involving large numbers of patients have demonstrated a correlation between karyotype abnormalities, or DNA content, and clinical outcome [9, 10]. Approximately 70% of breast tumors have been shown to be aneuploid or to contain cytogenetic abnormalities [11].

Over the past several years there has been intense interest in finding an association between specific gene alterations and the clinical behavior of tumors. Those studies that have investigated proto-oncogene perturbations in association with known prognostic indicators in breast cancer will be highlighted in this chapter.

Prognostic factors in breast cancer

A variety of clinical and pathological variables are known to have prognostic importance in human breast cancer (table 1) [12]. The most valuable indicative factors to date have been clinical staging of tumor size per the TNM system, involvement of regional lymph nodes, and the presence or absence of distant metastases. Numerous studies, beginning with Fisher et al. in 1969, have confirmed the importance of tumor size and the extent of tumor involvement in axillary lymph nodes [13]. Recently published data from the Surveillance, Epidemiology, and End Results program of the NCI was gathered from 24,470 breast cancer cases. It showed survival rates that varied from 45% for tumors of 5 cm or more in diameter and with associated axillary lymph nodes, to 96.3% for tumors smaller than 2 cm without involved nodes [14]. Tumor diameter and lymph node status were viewed as independent but additive prognostic indicators; an increase in tumor size implied a statistical decrease in survival, regardless of lymph node status. Likewise greater lymph node involvement was associated with decreased survival, regardless of tumor size.

The expression steroid hormone receptors is also an independent prognostic factor. A recent update of the San Antonio database of 5,347 patients showed that positive estrogen receptivity predicts for longer disease-free survival and overall survival, regardless of the axillary nodal status. The median follow-up for these patients is now 50 months [15].

Table 1. Prognostic factors.

Tumor size
Axillary lymph node involvement
Steroid hormone receptors
Estrogen
Progesterone
Histopathology indices
Nuclear grade
Cellular kinetics
Thymidine-labeling index
DNA content
S-phase fraction

Cellular kinetic studies using thymidine labeling in fresh tissue or flow cytometry with paraffin-embedded tissue also demonstrated a strong association between the tumor growth rate and disease outcome [16, 17]. Recent data from studies using DNA flow cytometry demonstrated that in node-negative patients with diploid tumors and a low proliferative rate (defined as the percent of cells in S-phase), the five-year recurrence was only 10% as compared with a 27% recurrence rate for patients with a high proliferative rate [18].

Histomorphologic grading and nuclear grading have also proven valuable as prognostic indicators; unfortunately, a high rate of discordance among individual observers has prevented these assays from playing a more significant role in prognosis for the majority of breast cancer cases [19, 20].

The need for additional guidelines by which to predict the likely course and to choose the optimal treatment is nowhere more evident than in breast cancer. In many cases traditional staging and grading systems fail to provide a sufficiently accurate basis for prognostication. There continues to be a subset of patients in whom the most widely applied criteria are unable to identify those at high risk for early disease recurrence. For example, approximately 25% to 30% of patients who have no axillary nodal involvement at the time of surgery will subsequently have a disease relapse either locally or at a distant site [21]. In this group of patients, our current stag-ing system provides little information about the growth rate of the tumors or the possible presence of occult metastases in apparently early stages of the disease.

Despite the remarkable advances made during the past several years in the diagnosis and treatment of breast cancer, there is an obvious need to identify new and more accurate prognostic indicators for this very common malignancy. Treatment decisions based on stratifications for expected risk of disease recurrence and progression in breast cancer will affect the lives of thousands of women annually [21, 22]. Many researchers eager to apply the expanding molecular biology information to clinical medicine have turned to the role of oncogenes in breast cancer as potential new prognostic factors [23].

Table 2. Oncogenes and breast cancer.

HER–2/*neu*, c–*erb*B–2, *neu*
Gene amplification
Protein expression
Ha–*ras*
Gene amplification
Rare alleles
c–*myc* amplification
int–2 amplification

Numerous proto-oncogene abnormalities have been shown to occur in breast carcinoma in mice infected with mouse mammary tumor viruses, and from studies using breast cancer cell lines. Abnormalities found frequently in these models are being investigated in primary human breast cancer [24–27]. Research to date must be considered preliminary, although some well-designed studies have found clinically important associations [28–30]. The oncogenes linked to prognosis in breast cancer, HER–2/*neu*, Ha–*ras*, c–*myc*, and *int*–2 (table 2), are discussed in detail below.

The HER–2/*neu* oncogene

Gene identification/function

Growth factors and the cellular receptors for them are believed to be involved in the abnormal growth characteristics of cancer. Some of these are encoded by known proto-oncogenes [31]. One putative growth factor receptor, the HER–2/*neu* gene (also identified as c–*erb*B–2 and *neu*), is a member of the tyrosine kinase family [32]. The gene product has transmembrane topology similar to the epidermal growth factor receptor with which it is closely related [33]. Like the EGFR protein, HER–2/*neu* has an extracellular domain a transmembrane domain that includes two cysteine-rich repeat clusters, and an intracellular kinase domain. Because of the homology with epidermal growth factor receptor, the HER–2/*neu* protein is thought to be a receptor molecule, although the putative ligand has yet to be identified.

Oncogene amplification

Amplification of the HER–2/*neu* oncogene, or its overexpression via mRNA transcription, has been reported in a large number of recent studies. Publications describing gene amplification have compiled results in over 1,500 human breast carcinomas [24, 26, 27, 34–47]. By combining all the data, gene amplification was frequently found with 21% of the tumor specimens showing increased gene copy number ranging from twofold to greater than a hundredfold normal. There is a 10% to 40% variation among studies in

Table 3. HER–2/*neu* gene amplification.

Investigators	No. Total	No. Amplified (%)	No. With Positive Nodes	No. Amplified (%)
Cline et al., [26]	53	3 (15)	35	8 (23)
Biunno et al., [27]	25	2 (8)	...	...
Slamon et al., [35]	189	53 (28)	86	34 (40)
van de Vijver et al., [36]	95	15 (16)	41	6 (15)
Varley et al., [37]	37	7 (19)	26	6 (24)
Venter et al., [38]	36	12 (33)	15	3 (20)
Zhou et al., [39]	86	15 (17)	37	8 (22)
Ali et al., [40]	122	22 (10)	75	8 (11)
Berger et al., [41]	51	13 (25)	17	7 (41)
Fontaine et al., [42]	15	7 (47)	...	...
Tal et al., [43]	21	2 (10)	...	...
Zhou et al., [44]	157	17 (11)	89	19 (11)
Zeillinger et al., [45]	291	52 (18)	...	...
Lacroix et al., [46]	57	11 (19)	...	...
Slamon et al., [47]	526	146 (26)	345	101 (27)
Totals	**1761**	**377 (21)**	**766**	**200 (26)**

the reported percentage of tumor specimens demonstrating gene amplification (table 3).

Few of the reports provided comprehensive clinical data to compare with the gene copy data. Among those studies where clinicopathologic information was available at the time of diagnosis, there are many inconsistencies with respect to potentially significant correlations.

None of the investigators have found any correlation of HER–2/*neu* gene amplification with the histologic type of breast cancer. A correlation with estrogen and/or progesterone receptor status has been noted by some [35, 41, 45] but not by others [34, 36, 44, 46]. Two groups have reported an association between poor nuclear grade and increased gene copies [41, 42].

The first study that provided long-term survival data along with clinicopathologic parameters was published by Slamon and colleagues. Analyses performed on 103 tumor specimens showed that gene amplification was significantly more common in tumors taken from patients with more than three positive lymph nodes. The study was then extended to include 86 patients with positive lymph node metastases and for whom long-term clinical data was known. There was a strong association between HER–2/*neu* amplification and nodal status ($p = 0.002$), but not with other previously defined prognostic factors, including progesterone receptor status, tumor size, or patient age. For the patients with positive lymph nodes, significant correlations were found between the degree of gene amplification and both the time to relapse ($p = 0.001$) and survival ($p = 0.02$). When compared in univariate analysis to other parameters, amplification of the HER–2/*neu* oncogene was found to be superior to all other prognostic factors [35].

In this study the greatest differences in survival between patient groups

Table 4. HER–2/*neu* gene amplification in node positive patients.*

	Disease-Free Survival, *P* value		Overall Survival, *P* value	
	1987 N=86	1989 N=270	1987 N=86	1989 N=270
Positive nodes	0.001	0.0024	0.0003	0.0057
HER–2/*neu* amp	0.001	0.029	0.02	0.15
PgR status	0.23	0.28	0.47	0.72
ER status	0.96	0.041	0.93	0.062
Tumor size	0.046	0.35	0.17	0.16
Age	0.56	0.73	0.13	0.56

* Data from Slamon et al, [35] and GM Clark, personal communication.

was found for those patients with greater than five gene copies. This subset of patients had even shorter disease-free periods and survival times when compared to those patients who had no amplification, regardless of nodal status. Correlations between increasing gene copy and worse prognosis has also been corroborated by research with breast cancer cell lines and with other oncogenes. An example of this is the N–*myc* oncogene in childhood neuroblastoma [48–51].

The data for the group of 86 patients with positive lymph nodes was recently updated [GM Clark, personal communication] to include an additional 184 patients. Again, disease-free survival was decreased in those patients with HER–2/*neu* amplification, although overall survival did not remain statistically significant (table 4).

Significant correlations between gene amplification and clinical outcome has not, however, been a universal finding (table 5) [40, 44]. In the series of 122 patients reported by Ali et al., only 10% of tumor specimens exhibited gene amplification; with a follow-up time of 62 months for those patients still alive, no correlation was found for HER–2/*neu* amplification and tumor recurrence or patient survival. Similarly, Zhou et al. found no decrease in survival or early disease recurrence among a group of 157 patients whose tumors showed HER–2/*neu* gene amplification [44]. A similar 11% amplification was identified for this patient population when compared with those described by Ali et al. In contrast, Slamon found a much higher percentage of tumors (28%) to have gene amplification; the percentage was even higher (40%) in those patients with positive axillary lymph nodes.

How can these widely disparate results be explained? Some of the conflicting results may be a result of small sample sizes, differences in study design or statistical tests, or the use of different techniques to demonstrate HER–2/*neu* gene amplification. Southern blot analysis, for example, may underestimate the amount of amplification, since this technique does not reliably detect low levels of amplification. Technical difficulties in the handling of tumor specimens may also result in DNA degradation with a subsequent underestimation of gene copy [52]. Also, within many pathologic

Table 5. HER–2/*neu* gene amplification and survival.

Investigators	No.	Disease-Free Survival *P* value	Overall Survival *P* value
Varley et al., [37]	37	<0.002	...
Ali et al., [40]	122	NS	NS
Zhou et al., [44]	157	NS	NS
Clark et al.*	270†	0.029	0.15
Slamon et al., [47]	345†	0.006	0.045

*GM Clark, personal communication
†Node-positive patients only
NS = not significant

specimens, especially breast cancer, there will be a variable number of infiltrating lymphocytes, vascular cells, or stromal cells with normal genetic content. Since all the solid matrix blotting techniques involve the homogenization of tumor specimens, dilution of the tumor DNA with normal cells introduces a potential error. Variability of oncogene expression within a single tumor specimen may also confuse results [53]. Finally, since gene amplification eventually exerts its effect through protein overproduction, the direct measurement of gene product may be more relevant to malignant disease than gene copy analyses.

HER–2/neu protein overexpression

Recent research has sought to avoid the problems described above and to directly explore the role of oncogene protein expression in breast cancer. These studies have concentrated on examining the levels of the HER–2/*neu* oncogene protein product. Protein expression as determined by protein immunohistochemical staining has shown a significant, although not exact, correlation with gene copy number [38, 41, 47, 54]. Gene amplification has also been demonstrated in the absence of protein overexpression; extra gene copies may not always be actively transcribed. Overexpression of the gene product also may occur in the absence of gene amplification [41, 46]. As with gene amplification, the measurement of protein levels is hampered by technical difficulties. Since tumor homogenization is accomplished before a value is determined, protein expression is subject to the dilutional effects of the accompanying normal cells. This effect may be particularly troublesome in dealing with tumors associated with large amounts of connective tissue, which will introduce disproportionately high levels of protein in relation to the total DNA/RNA content [47]. Several studies measuring HER–2/*neu* protein expression have now been completed. The results of these investigations have been summarized in table 6.

Studies examining HER–2/*neu* overexpression have shown variable results when compared with known indicators of prognosis. In their study

Table 6. HER–2/*neu* protein expression.

Investigators	No.	% Positive staining
Berger et al., [41]	38	37
Fontaine et al., [42]	15	47
Lacroix et al., [46]	53	45
Slamon et al., [47]	37	10
Tandon et al., [54]	728	17*
Barnes et al., [55]	195	9
van de Vijver et al., [56]	89	14
Gusterson et al., [57]	103	16
Paik et al., [53A]	292	21
Wright et al., [58]	185	17
Gusterson et al., [59]	137	16
Total	**1872**	**22.6%**

*Tumor specimens with elevated protein levels by Western blot in 378 node positive patients.

of 38 patients, Berger et al. found protein expression to correlate with both poor nuclear grade and the number of positive lymph nodes [41]. HER–2/*neu* membrane staining also appeared to correlate with pathologic grade, but not nodal status or hormone receptor status in a group of 195 patients only 17 of whom had membrane staining [57]. An association between HER–2/*neu* overexpression and positive nodal status was reported by two additional groups [53, 53A], but was not found by others [46, 55–58]. There has been no positive correlation reported between hormone receptor status and protein overexpression.

To determine the prognostic status of HER–2/*neu* protein overexpression, expression needs to be analyzed in association with date on tumor recurrence and overall patient survival, rather than correlating protein levels with other known prognostic parameters. To date six such studies have been completed (table 7) [53A, 55–58]. These conflicting results illustrate the current controversy in HER–2/*neu* research.

Tandon et al. examined HER–2/*neu* in 728 primary breast cancer specimens [54]. In 378 node negative patients, protein expression failed to predict disease outcome. However, in 350 patients with involved lymph nodes, those with higher levels of protein had a significantly shorter disease-free survival ($p = 0.029$) and overall survival ($p < 0.0022$) than patients with lower protein expression. For the node positive patients, multivariate regression analyses on all clinical parameters showed that the number of positive lymph nodes remains the most powerful prognostic factor, followed by HER–2/*neu* overexpression.

Tumor specimens from women enrolled in NSABP protocol B–06 were retrospectively analyzed for HER–2/*neu* overexpression by Paik et al. [53A]. This set of patients was not subject to the complication of varying treatment

Table 7. HER–2/*neu* protein expression and survival.

Investigators	No. Total	Disease-Free Survival *P* value	Overall Survival *P* value
Tandon et al., [54]	728	0.029*	0.0022*
Wright et al., [58]	185	0.025	0.04
Paik et al., [53A]	292	NS	0.0012
van de Vijver et al., [56]	189	NS	0.04
Barnes et al., [55]	195	NS	NS
Gusterson et al., [57]	103	NS	NS

* Applies to node-positive patients only.

modalities, since uniform systemic therapy was given to everyone in the node positive group. Two hundred and ninety-two cases with full follow-up information were examined for either positive or negative immunohistochemical staining. Women with protein overexpression had a significantly decreased overall survival ($p = 0.0012$). Overexpression was also more common (29%) among tumors of poor nuclear grade than among those of good nuclear grade (12%). Yet the association of HER–2/*neu* overexpression with decreased survival was most evident among women with good nuclear grade.

A third study documenting the prognostic significance of HER–2/*neu* protein expression was reported by Wright et al. [58]. Positive immunohistochemical staining was again significantly correlated with both early disease recurrence and shorter overall survival in 185 primary breast cancer patients. The other usual prognostic factors in breast cancer were also shown to have prognostic significance, although on multivariate analysis lymph node status was more important than HER–2/*neu* protein expression in predicting disease-free survival and overall survival.

In contrast, van de Vijver et al. did not find *neu* protein expression to be an important prognostic factor for patients with stage II disease. In their study, twenty-seven patients (14%) with invasive carcinomas had HER–2/*neu* membrane staining. However, HER–2/*neu* overexpression was not associated with lymph node metastases. In the patients with protein overexpression, overall survival was decreased; but this finding did not prove significant after adjustment for tumor size. Disease-free survival was not influenced by protein overexpression. Tumor size for this group of patients was the strongest prognostic factor, followed by patient age, lymph node status, and histologic grade [56].

Barnes et al. and Gusterson et al. identified 9% of 195 breast cancers and 14% of 103 breast cancers, respectively, to have HER–2/*neu* protein overexpression. Statistical analysis of their results did not demonstrate a significant correlation between protein expression, recurrence-free survival, or overall survival [55, 57].

Of particular interest in the study by van de Vijver et al., 42% of the ductal carcinomas in situ showed positive membrane staining — a much higher percentage than was obtained for invasive tumors. The ductal carcinomas in situ showing *neu* protein overexpression had only the large-cell comedo-type of histologic appearance [56]. Another group has also shown a high percentage of ductal carcinomas in situ to have HER–2/*neu* protein overexpression. Strong positive membrane staining with the synthetic antibody 21N was found in 33 of 74 patients (44%) in the study by Gusterson et al. [59]. Among those patients for whom clinical data was available, protein expression did not have prognostic significance.

Ductal carcinoma in situ is an increasingly frequent diagnosis as smaller tumors are now found by improved detection with screening mammography [60]. Not all of these tumors progress to become invasive cancer presumably because they have not acquired the necessary biological profile for malignant behavior. HER–2/*neu* overexpression perhaps may be an early pathogenic factor in the development of malignant potential [61].

As with HER–2/*neu* gene amplification, the contradictory results among studies of protein expression have not been adequately explained. Smaller patient groups, unequal follow-up of patients, and the confounding effects of therapy have all been suggested as factors contributing to the observed differences. Other potential sources of error may include technical difficulties with the immunohistochemical staining, the different antibodies generated, and the use of frozen tissue. Tissue that has been fixed in formalin and embedded in paraffin will lose some antigen reactivity, especially if protein expression is only moderate. There is also the obvious problem encountered with subjective measurement of specimen staining. Only one group utilized Western blot analysis to quantitate the HER–2/*neu* protein [54]. Because this technique allows assignment of a numerical value to each specimen, subjective scoring of a tumor is minimized.

In summary, it appears that HER–2/*neu* protein expression may prove to be a useful prognostic indicator if, perhaps, only in node positive breast cancer. Additional retrospective studies and randomized prospective studies will be needed to clarify its exact indicative significance.

The Ha-*ras* proto-oncogene

Gene/protein information

The most frequently detected transformation-inducing genes in human solid tumors are members of the *ras* family of cellular oncogenes. These oncogenes are a group of related genes with an evolutionary origin that is apparently prehominid. Their protein products are localized to the inner surface of the plasma membrane and they possess high affinity guanosine triphosphate (GTP) binding properties with an intrinsic GTPase activity [62].

Three members of the *ras* family, designated Ha–, Ki– and N–*ras*, encode distinct products that are greater than 90% related at the amino acid level. The Ha–*ras* proto-oncogene is the most significantly activated *ras* gene in human breast and colon carcinomas [63, 64].

Ras genes in normal cells do not appear to have transforming ability when transfected. Transforming *ras* genes, however, have point mutations that usually involve codon 12 and, less commonly, codon 13 or 61. X-ray crystallographic data have demonstrated the mutated positions to encode amino acids residing within or in close proximity to the GTP-binding domain of the *ras* molecule. There is circumstantial evidence suggesting that mutated *ras* genes provide the affected cell with a selective growth advantage thereby contributing to the malignant potential [65].

Transforming *ras* point mutations are only infrequently detected in human carcinomas, including breast carcinomas [66]. In a recent study of over a hundred primary breast tumors, no *ras* point mutations were observed [67]. Several investigators, however, have noted the frequent overexpression of Ha–*ras* mRNA or its gene product in breast cancers. A number of different groups have also detected elevated levels of Ha–*ras* expression in dysplastic lesions of the breast [68–71].

Since extraction of RNA from tissue is a complex procedure, studies relating to *ras* overexpression have, for the most part, concentrated on the use of monoclonal antibodies raised against the *ras* gene product p21, a protein with a molecular weight of 21,000 daltons. Two specific antibodies have most commonly been used; these immunoglobulin molecules identify both the mutated and the normal forms of p21.

Despite the number of articles that have addressed qualitative and quantitative abnormalities in the proto-oncogene Ha–*ras*, few papers have described long-term follow-up of patients to investigate the role of the oncogene in predicting future clinical outcome. In a study group of 41 patients, Lundy et al. found higher p21 protein overexpression in patients with positive lymph nodes, suggesting that overexpression was associated with malignant potential [72]. The expression of p21 also correlated with tumor size but not with age or estrogen receptor status.

Querzoli and colleagues also found expression of the p21 product to be significantly associated with nodal status in their study of 142 primary breast tumors [73]. Of the 77 tumors for which hormone receptor data was available, tumors with high p21 levels contained a higher percentage of estrogen receptor positive cells ($p = 0.05$). This correlation with estrogen receptor status has also been confirmed by others [70, 72–75].

In addition to a positive correlation with estrogen hormone receptor status, Clair et al. found that 13 of 16 patients with tumors expressing low p21 levels were disease-free for more than four years after the primary treatment, whereas only 5 of 9 patients with high p21 tumors remained disease-free [75]. While small numbers of patients make statistical evaluation meaningless, a potential relationship was suggested.

Ha–ras rare alleles

The Ha–*ras* proto-oncogene is polymorphic in human cellular DNA. The gene is characterized by the presence of a closely linked 28-nucleotide sequence that is repeated a variable number of times. Using restriction fragment length polymorphism analysis, several fragments of different size that define a corresponding number of Ha–*ras* alleles can be identified by Southern analysis [76].

Krontinis et al. examined a number of carcinomas and found that cancer patients as a group had a higher frequency of rare Ha–*ras* alleles than did unaffected populations [77]. Several investigators have found this increased rare Ha–*ras* allele frequency in patients with breast cancer [78, 79], although other groups dispute these findings [80–84].

In one study the Ha–*ras* locus was examined for allelic polymorphism in 104 breast cancer patients and 56 normal controls [78]. Four common and 16 rare alleles were detected in these two populations. The common restriction fragments represented 91% of the allele pool in the unaffected population compared to only 59% in patients with breast cancer ($p < 0.001$). The frequency of rare Ha–*ras* alleles and hence genotypes composed of two rare alleles was increased in the breast cancer population ($p < 0.001$). Saglio and colleagues reported similar findings after examining peripheral leukocyte DNA samples from breast cancer patients; 34 rare alleles were found in 92 breast cancer patients and only 4 were found in 60 control samples [79]. This difference was highly significant ($p < 0.0001$).

Theillet et al. analyzed Ha–*ras* polymorphisms in normal and breast cancer DNA and observed a loss of Ha–*ras* heterozygosity in 27% of 51 breast carcinomas diagnosed as highly aggressive carcinomas [67]. A related finding was presented by Ali et al., who showed that the loss of heterozygosity for several chromosome 11 loci had a significant correlation with tumors that were steroid receptor negative and histopathologic grade III and that displayed distant metastases [83]. A correlation between tumor size, estrogen level, and a partial deletion of chromosome 11 was also reported by Mackay et al. [84]. The common location of the progesterone receptor gene, the *int*–2 oncogenes, and the Ha–*ras* oncogene on chromosome 11, plus the above data, suggests the possibility of 'suppressor genes,' or 'antioncogenes' residing on chromosome 11 in human breast cancer [85, 86].

The molecular and biologic consequences of a high frequency of rare Ha–*ras* alleles in breast cancer patients are at present unknown, and attempts to correlate the rare alleles with clinical and biochemical characteristics have been to date unrevealing. The increased frequency of these genetic abnormalities and/or the expression of specific alleles do suggest that the inheritance of these alleles may be associated with an increased risk of developing breast cancer. This may represent another potential prognostic factor for unaffected individuals in a high risk category [87].

The c–*myc* oncogene

Myc proteins/function

The oncogene v–*myc* was first identified as the transforming gene of an avian myelocytomatosis virus. C–*myc* is the cellular homologue of this gene; its nuclear location is outside the nucleolus, alongside small nuclear ribonucleoprotein particles [88]. This oncogene has the ability to enhance the transcription of several different genes and to bind DNA without sequence specificity. Cell culture results suggest that c–*myc* may act as a common intracellular transducer for a variety of unrelated growth factors whose effects are to promote the transition of cells from the quiescent to the proliferative state (G_0 to G_1) [89]. The constant mitotic stimulus provided by a high level of c–*myc* expression may thus circumvent or greatly reduce the requirement for exogenous growth factors.

Oncogene amplification/tumorigenesis

DNA rearrangements of the c–*myc* oncogene have been identified in a number of hematologic malignancies. For example, in Burkitts lymphoma, c–*myc* on chromosome 8q24 is commonly translocated adjacent to the immunoglobulin heavy chain locus on chromosome 14q32 [90]. Other hematologic malignancies have also been associated with translocations involving the *myc* locus [91].

In contrast to the types of c–*myc* rearrangements seen with hematologic malignancies, the major abnormality associated with solid tumors is gene amplification rather than disruption by translocation, although a few cases of c–*myc* alteration at the 3′end have been described for breast carcinomas. There are many examples of c–*myc* amplification in a variety of carcinomas, including mammary carcinoma [92–94]. In some cases where gene amplification has been observed, there is concomitant elevation in the expression of the gene as determined by measures of messenger RNA or protein levels.

The largest patient study involving the c–*myc* oncogene was published by Escot et al. in 1986 [95]. The genomic organization at the c–*myc* locus was analyzed in 121 human primary breast carcinomas. The oncogene was found to be amplified 2-fold to 15-fold in 31% of the tumors. A significant correlation was observed between patients more than 50 years of age and the presence of an altered oncogene ($p < 0.02$). Amplification did not correlate with other known prognostic variables, such as nodal status or receptor status. A similar percentage of patients with amplification of c–*myc* (41%) was reported by Bonilla et al. for their group of 48 tumors; amplification, however, did not appear to correlate with the clinical stage of the patient [96]. Cline et al. found c–*myc* overexpression in only 16% of 53 primary breast cancers [26]. Again, no correlation was indicated between c–*myc*

amplification and the stage of disease or the presence of involved lymph nodes.

Varley and associates also showed amplification of c–*myc* in 8 of 37 primary breast tumors [37]. There was no correlation between the histologic grade, TNM staging, or the estrogen receptor status of the patient and c–*myc* amplification. There was, however, a significant correlation between a genetically altered c–*myc* gene and a poor prognosis, as measured by early recurrence of the disease or death ($p < 0.02$). In this study the altered c–*myc* gene appeared to be a more significant indicator of poor prognosis than was either the estrogen receptor status or the extent of tumor at the time of surgery.

At present, although abnormalities have been described and some correlative information gathered, it does not appear that c–*myc* expression in breast cancer will have much prognostic use. It certainly does not play the same prognostic role for breast cancer that the related oncogene N–*myc* does for childhood neuroblastoma. Amplification of N–*myc* in that disease is a powerful prognostic indicator of aggressive tumor behavior and early death [51].

Other proto-oncogenes

The oncogene *int*–2 is believed to be a modulator of cell growth, although little is known about its specific function or gene product. The *int* gene family becomes activated in mouse model systems after integration of an MMTV protovirus into adjacent chromosomal DNA. In one clinical study primary breast cancers in 107 patients were examined for amplification of the *int*–2 gene. Sixteen patients showed gene amplification ranging from 2-fold to 15-fold [97]. Amplification had a highly significant association ($p < 2\times10^{-6}$) with tumors from patients who subsequently suffered a disease relapse.

A larger percentage was identified in a study by Varley et al. in which 9 of 40 samples (23%) were shown to have amplification of the *int*–2 sequences [98]. Zhou identified 4 of 46 infiltrating ductal cancers with 7-fold to 25-fold amplification of the gene. All tumors with gene amplification, along with two of eight squamous cell tumors of the head and neck, had lymph nodes involved with tumor at the time of diagnosis [99].

Mutational inactivation of the retinoblastoma gene, a recessive cancer gene implicated in the genesis of retinoblastoma, has also been identified in other human neoplasms, including breast cancer and small cell lung cancer [100, 101]. One group studying 41 primary breast tumors found 7% to have structural changes of the retinoblastoma gene [100].

Other abnormal proto-oncogenes in breast carcinomas have been identified as research in the active field of molecular genetics continues. Numerous studies are beginning to catalog oncogene changes much as we have done for

other chromosomal abnormalities in malignancies [102], although few retrospective analyses in breast cancer patients for these oncogenes have been completed to determine their role in predicting tumor behavior and clinical outcome.

Conclusion

Treatment decisions for newly identified breast cancer patients are typically based on assessment of their clinicopathologic parameters at the time of diagnosis. Much progress has been made to aid the clinician in reaching therapeutic decisions for individual patients. However, much remains to be accomplished in accurately determining the clinical course of disease for all affected women. Breast cancer patients are a clinically heterogeneous group. There will always be a need to identify new prognostic markers in order to separate patients with a good prognosis from those who may be deserving of more aggressive or experimental therapy to prolong survival.

The continued study of oncogene/proto-oncogenes may ultimately provide the clinician with powerful diagnostic, prognostic, and therapeutic tools. With the possible exception of HER–2/*neu* protein overexpression, identification of oncogenes as prognostic indicators remains, as yet, of unproven clinical value. Further studies reviewing large databases of patients with well-documented clinical, pathologic, and molecular genetic information must be completed if progress in this area is to continue. In the future, broad prospective studies using oncogene information to aid in the assignment of disease stage and thus therapeutic decisions will ultimately define the appropriate prognostic role for oncogenes in breast carcinomas.

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2. Role of the retinoblastoma gene in the oncogenesis of human breast carcinoma

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Overview

The notion that genetic alterations are involved in cancer has gained support from many studies. Certain genetic alterations in tumors have been precisely defined. First, the activated proto-oncogenes were isolated from tumors by their abilities to transform nonneoplastic cells in culture [1]. To date many genes potentially involved in cancer have been isolated based on the sequence homology to oncogenes of retroviruses [for review see 2]. In contrast to the activated oncogenes in tumor cells, a different class of cancer genes has been suggested by somatic cell hybrid studies. Fusion cells made between tumor cells and normal fibroblasts, lymphocytes, or keratinocytes are often nontumorigenic, an effect contributed by chromosomes [3–5]. A correlation exists between suppression of tumorigenicity and the retention of certain chromosomes in the fused cells [6]. For example, the introduction of chromosome 11, but not chromosome X nor 13, suppresses the tumorigenic phenotype of a Wilm's tumor cell line, indicating the tumor suppressor gene for pediatric nephroblastoma is located on chromosome 11 [7]. These genes are actively expressed in normal cells and are mutationally inactivated in tumor cells. They are termed 'tumor suppressor genes', since the presence of one or more normal alleles is thought to prevent the tumor phenotype [for review see 8, 9]. A complementary line of evidence comes from cytogenetic observations in which a specific chromosomal deletion occurs in somatic or tumor cells from patients with retinoblastoma, Wilm's tumor, and bilateral acoustic neurofibromatosis [10–12]. This suggests the existence of a tumor suppressor gene in normal cells in specific chromosomal regions. Additional support for tumor suppressor genes is derived from the isolation of DNA fragments capable of reverting the transformed phenotypes of cultured cells [13]. A recent study led to the identification of the Krev–1 gene that converts the H–*ras* transformed cells into flat revertants upon transfection. Sequence analysis indicates that significant amino acid homology exists between Krev–1 and H–*ras* [14].

The list of potential tumor suppressor genes has grown rapidly in the past few years due to advances in their assay and to the availability of

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probes at different chromosomal regions. However, cloning of the individual genes has proven to be a challenging task, as is evident with the example of the retinoblastoma gene. Rapid progress has been made since the isolation of the candidate retinoblastoma gene. A brief review of retinoblastoma and the characterization of the retinoblastoma (RB) gene, its gene product, and the possible role of RB gene inactivation in breast carcinoma are presented here. Reviews on the related topics have appeared recently [12, 15, 16].

Retinoblastoma: A model for heritable cancer predisposition

Families with aggregated occurrence of adult cancers have been reported by Li and Fraumeni [17], suggesting the involvement of genes in cancer predisposition. Follow up of familial adenomatous polyposis (FAP) patients shows the development of colon cancer later in life [18]. This progression from benign tumor in FAP to malignant carcinoma can be traced [18–20]. In pediatric cancers, including retinoblastoma, neuroblastoma, and Wilm's tumor, strong predisposition to cancer is observed in some families. Based on these clinical observations, the hypothesis of a heritable cancer predisposition was proposed [21].

Retinoblastoma, a highly malignant but readily treatable cancer of early childhood that arises in the developing retina, offers a clear example of heritable cancer predisposition. The tumor occurs in about 1 in 20,000 live births [22]. Two forms of retinoblastoma are distinguished on a genetic basis [11]. Roughly 40% of all cases are hereditary and the predisposition to retinoblastoma is transmitted as an autosomal-dominant cancer susceptibility trait, each offspring of a carrier parent has a 50% chance of inheriting the trait, and 90% of carriers will develop retinoblastoma [11]. The majority of cases arise as new mutation without prior family history. Multiple or bilateral retinal tumors and early onset are indicative of, and typical for, herditary retinoblastoma. Furthermore, RB carriers are at high risk of developing additional primary neoplasms later in life; these second cancers are generally of unusual types, such as osteosarcoma or soft-tissue sarcomas [23, 24]. In contrast, patients with nonhereditary retinoblastoma have single, unilateral retinal tumors and no increased risk of second primary cancers. However, about 15% of patients with unilateral retinoblastoma actually have the hereditary form. Because of its remarkable heritability, retinoblastoma has been a model system for the study of genetic factors in human cancer.

Using the statistical analysis of clinical data, Knudson theorized that both forms of retinoblastoma could result from the same genetic lesion and as few as two '*hits*', or mutational events, would be sufficient to lead to tumor development [21]. In patients with the heritable form of retinoblastoma, the first hit was inherited as a germinal mutation; additional mutation in the somatic cells (retinoblasts) would be sufficient for retinoblastoma for-

mation. For sporadic retinoblastoma both mutations occur in the somatic cells of patients. Comings added that two hits may serve to inactivate both alleles of a single gene that essentially functioned to suppress retinoblastoma formation [25]. This model can account for both the earlier onset and the multiplicity of tumors in predisposed individuals.

Karyotypic examination of somatic cells (fibroblasts) from patients with hereditary retinoblastoma disclosed a minor subset of cases containing visible deletions of the long arm of chromosome 13 [26, 27]. Similar deletions were also identified in retinoblastoma tumor cells [28]. Among all deletions band 13q14 was commonly involved. Therefore, the gene determining susceptibility to retinoblastoma was assigned to this region. Studies of a large retinoblastoma pedigree also supported this notion, in that normal individuals carried a balanced translocation involving 13q14, while those with retinoblastoma had only one 13q14 region [29].

Although the inheritance pattern of retinoblastoma predisposition follows that of an autosomal-dominant Mendelian trait [11], the mutated RB allele appears to be recessive at the cellular level as suggested by restriction fragment length polymorphism (RFLP) analysis of the tumor and somatic DNA from the same patient. Loss of heterozygosity of chromosmoe 13q is frequently observed in tumor DNA [30, 31]. In light of Knudson's 'two hit' hypothesis, loss of heterozygosity was interpretated as the second hit that revealed mutations of the other RB allele (first hit). According to this hypothesis the mutated RB allele would be 'recessive' to the normal allele. Additional evidence of the 'recessive' nature of the mutated RB gene comes from a study by Dryja et al. Homozygous deletions were found in two retinoblastomas, using an anonymous probe mapped to 13q14, H3–8, but not for other probes mapped to the same chromosomal band [32]. The recessive nature of the retinoblastoma gene played a key role in identifying the RB gene [see below].

Isolation and characterization of the retinoblastoma (RB) gene and identification of its gene product

Isolation of RB gene candidates

With the assignment of the RB gene to chromosome 13q14, molecular cloning of the candidate gene was undertaken. Since nothing was known a priori about the RB gene product, candidate genes were to be identified solely on the basis of appropriate chromosomal location and presumed 'recessive' behavior; that is, an intact RB gene should be expressed in normal retinal tissue but not in retinoblastomas. 'Reverse genetic' cloning strategies require a collection of probes from the region of interest. Linkage analysis has placed one of the markers, esterase D, to the same chromosomal location [33, 34]. This enzyme was purified to homogeneity [35] and its

cDNA cloned [36]. This clone and other random DNA probes mapped to chromosome 13q14 were used as starting points for chromosome walk to obtain nearby genes. Genes cloned were then tested for candidacy as RB gene based on the hypothesis of recessive nature of the tumor suppressor gene.

We initiated bidirectional chromosome walking from the esterase D gene to generate overlapping genomic clones. At 20 kb intervals in walking regions, unique sequences were identified that were used as probes to isolate cDNA clones from fetal retina and placenta libraries. By alternative screening of genomic and cDNA libraries, we obtained overlapping DNA, clones covering 120 kilobases around the esterase D gene. Two cDNA clones, called SD–1 and SD–2, were isolated using probes 5′ to the esterase D gene. Chromosome walking 3′ to the esterase D gene was hampered by a 20 kb region containing highly repetitive sequences. Meanwhile, probe H3–8 was used in the second walk, since its homozygous deletion in some retinoblastoma suggests the close vicinity of this probe to retinoblastoma gene [32]. A nearby unique DNA fragment was used to identify two overlapping cDNA clones of 1.6 kb and 0.9 kb in human cDNA libraries. Additional clones were obtained by rescreening several cDNA libraries. Together these clones defined a cDNA sequence of 4,757 nucleotides.

Detection of altered expression of RB gene candidate in retinoblastoma cells

The clone obtained above detected a 4.7 kb mRNA transcript in fetal retina and placenta (figure 1). Three retinoblastomas (figure 1A, lanes 1, 2, and 5) demonstrated abnormal mRNA transcripts measuring approximately 4.0 kb. In two retinoblastomas (lanes 3 and 6), mRNA transcripts were not observed, while faint bands of about 3.8 and 4.5 kb were visible in lane 4 only after prolonged exposure. In contrast, three neuroblastomas and two medulloblastomas displayed transcripts of 4.7 kb, equivalent to those in normal tissues (figure 1B) [37]. As a control, the esterase D probe was used in the Northern hybridization; transcripts were detected in all tumor and tissue samples (figure 1, bottom panel), consistent with the known 'constitutive' expression of esterase D [35]. All esterase D mRNA transcripts had identical size (1.4 kb). When cDNA clones obtained by the first walk were used as probes in Northern hybridization, neither SD–1 nor SD–2 seemed promising as candidate RB genes because transcripts hybridizing to these clones were not detected in retina and placenta mRNA samples or in any retinoblastomas.

Alterations in gene expression were thus found in six of six retinoblastomas, but not in two normal tissues and in two other related human tumors of neurectodermal origin. This constituted the strongest evidence that the candidate gene obtained by H3–8 walking represented part of the putative RB gene [37]. Friend et al. first reported a cDNA fragment isolated by chromosome walking from H3–8 [38]. Both Friend et al. and Fung et al.

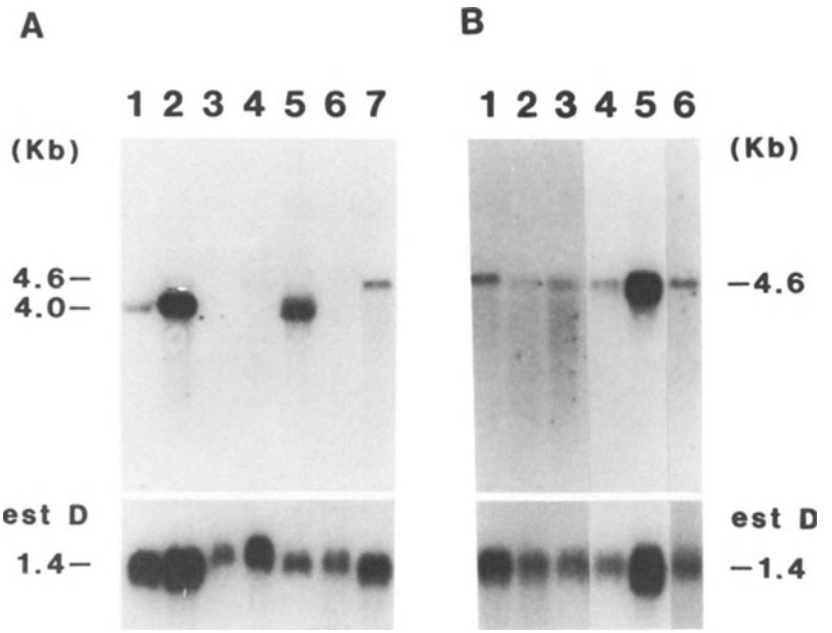

Figure 1. RNA blot analysis of RB gene transcripts in tumors and normal tissues. Two–5 μg of polyadenylated RNA prepared from retinoblastoma cell lines Y79, RB355, WERI–1, WERI–24, and WERI–27 (lanes A1–5), short-term cultured cells from a primary retinoblastoma tumor (lane A6), fetal retina (lane A7), neuroblastoma cell lines (lanes B1–3), a medulloblastoma cell line and a fresh tumor (lane B4 and 5), human placenta (lane B6) were analyzed. Filters were hybridized with ^{32}P-labeled RB–1 DNA (top panel). Tumors initially showing no signal were retested by overloading lanes with 10 μg of polyadenylated RNA and autoradiographing for up to 10 days. After this procedure, tumor 4 demonstrated an additional faint band of 3.8 kb (data not shown). Filters were then rehybridized with ^{32}P-labeled EL–22 DNA and exposed for 3 days (bottom panel). The apparent slight variation in mobility of esterase D mRNA transcripts reflects overloading. [From Lee et al., Science 235:1394–1399, 1987.]

detected a gene with properties similar to those described above: ubiquitous expression in normal tissues but absent or altered transcription in retinoblastomas [38, 39]. Based on the gene's localization at 13q14, specific inactivation in retinoblastoma, germinal mutation in bilateral retinoblastoma patients [39, 40], and the demonstration of its tumor suppression function in retinoblastoma [see below], it is concluded that the gene isolated is the retinoblastoma gene.

Genomic organization of the RB gene

At the genomic level, the RB gene contains 27 exons dispersed over a 200 kb stretch of DNA [41, 42]. The smallest exon, exon 24, contained only 31 nucleotides. Restriction and sequence analysis demonstrated that the last exon (#27) was 1,889 bp in length and included the translation stop codon

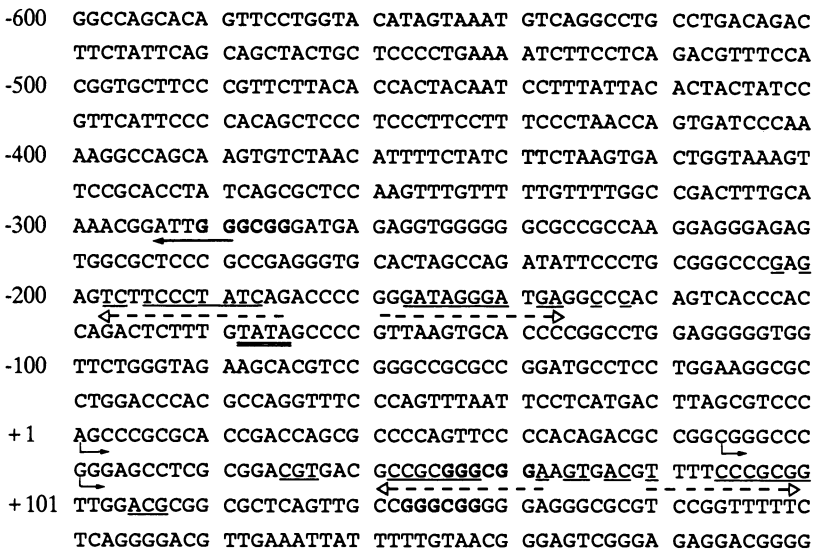

Figure 2. Nucleotide sequence of the RB promoter. Position +1 denotes the putative major transcription start site. Bent arrows indicate the positions where initiation of RB transcription is predicted based on S1-protection data. A sequence resembling the TATA element is doubly underlined, and a CCAAT sequence is designated by a solid arrow. The GGGCGG sequences (at −291, +76, and +123), corresponding to the Sp1 factor recognition motif, are shown in bold letters. Two potential stem-and-loop structures involving nucleotides −203 through −163 and +64 through +107 are designated by dashed arrows and by underlining of complementary bases. [From Hong et al., Proc Natl Acad Sci USA 86:5502–5506, 1989.]

(nucleotides 2923–5) of the longest open reading frame. Variation in intron size was also observed with the smallest, intron 15, of 80 bp and the largest, intron 17, of more than 60 kb. The untranslated region at the 3′ end is about 1.6 kb. RB cDNA diverged (as AAAAA) from the genomic sequence 16 bp after a consensus AATAAA polyadenylation signal, indicating that our longest cDNA clone was complete at the 3′ end.

To insure that the 5′ end of the RB cDNA indeed contains the first exon, we have further characterized transcriptional initiation sites by S1 nuclease mapping. Transcription of RB is initiated at multiple positions (+1, +44, and +51) (figure 2). Identification of the transcriptional initiation sites confirms the designation of the first exon and the longest reading frame [43]. The complete 5′ and 3′ ends of RB mRNA are thus confirmed. This complete cDNA yields a long open reading frame encoding a protein of 928 amino acids. The hypothetical Rb protein has a calculated molecular weight of 106 kD. There are some special features of the predicted Rb protein, including an unusual proline and alanine stretch at the N-terminal, a potential 'leucine-zipper-like' motif in exon 20 and a distinct proline-rich region in exon 23. Other than these features, the RB protein has no close relatives in the current protein sequence databases.

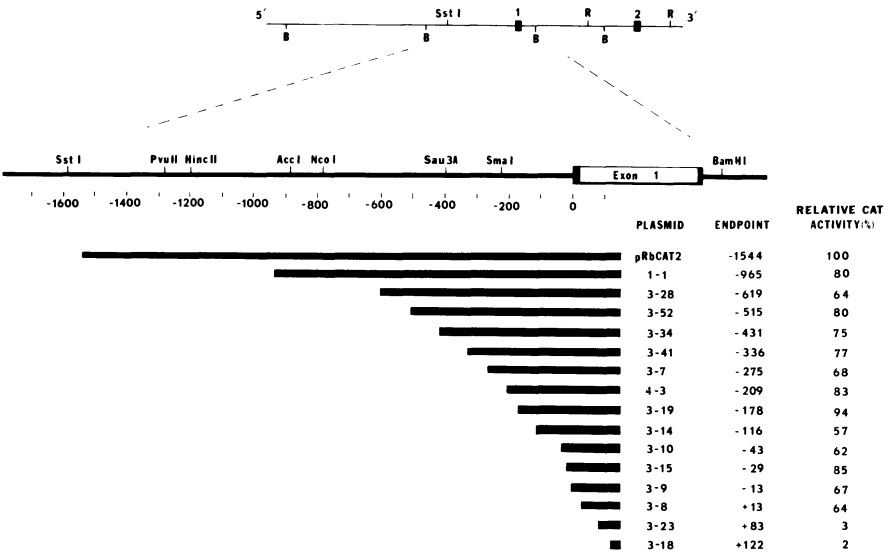

Figure 3. Deletion analysis of RB promoter region. A map of the genomic DNA corresponding to the 5′ region of RB is shown. A DNA fragment spanning the region from −1546 to +186 was isolated and fused to a bacterial CAT gene. This construct, pRbCAT2, and the 5′ deletion mutants that were generated by using pRbCAT2 were transfected into CV–1 cells, and cell extracts were assayed for CAT activity. The CAT activity values shown are expressed in percentages relative to the level exhibited by cells transfected with the original plasmid pRbCAT2. As an internal control for the transfection efficiency, a plasmid with a luciferase gene controlled by a retroviral promoter was used. [From Hong et al., Proc Natl Acad Sci USA 86:5502–5506, 1989.]

Definition of the RB gene promoter region

To identify the RB promoter region, a genomic fragment extending 5′ of exon 1 was fused to the bacterial chloramphenicol acetyltransferase (CAT) gene, which yielded a strong signal by the usual measure of promoter activity (figure 3). Deletion analysis reveals that a region as small as 70 bp is sufficient for RB-promoter activity. Sequence features of the RB promoter included a high G+C content, and a lack of CCAAT or TATA motifs. These features are typical of promoters driving so-called housekeeping genes that are ubiquitously expressed at a relatively constant level [42].

Identification of the RB gene product

The RB gene product was identified by immunoprecipitation with antibody recognizing either synthetic peptides or Trp E–Rb fusion proteins based on the predicted protein sequence data [43]. The purified polyclonal antibody (anti-fRb) as well as the peptide antiserum immunoprecipitated a phosphoprotein of about 110 kD in normal cells, which was specifically absent in

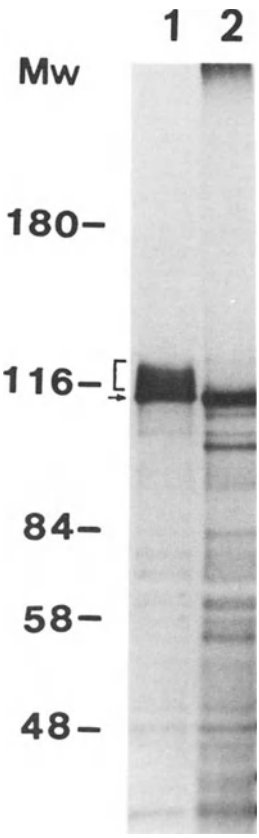

Figure 4. Modification of the Rb protein. Molt–4 cells (1×10^7) were labelled with ^{35}S–methionine for 3 hours. Cellular lysates were immunoprecipitated with anti-Rb IgG. After washing, one quarter of the immunoprecipitate was directly dissolved in SDS sample buffer (lane 1) and three quarters was treated with potato acid alkaline phosphatase at 37°C for 1 hour prior to loading (lane 2). Proteins were analyzed by 7.5% SDS–polyacrylamide gels. Bracket indicates the phosphorylated Rb and arrow points to the unphosphorylated Rb.

cultured cells from five of five retinoblastomas [43]. Further characterization showed that the Rb protein migrated in SDS–PAGE as a diffuse band at Mr 110,000–114,000 (figure 4, lane 1). After potato acid phosphatase treatment, the complex banding pattern reduced to the single fastest moving band (figure 4, lane 2). The slower migrating forms were strongly labelled with ^{32}P, suggesting that the apparent size heterogeneity was due to variable phosphorylation [44, 45]. Rb protein is primarily located in the cell nucleus as defined by cellular fractionation and immunocytochemical studies (figure 5).

Identification of the Rb protein offers a sensitive method for detecting RB mutation in tumor cells. RB mRNA is ubiquitously expressed in various tissues; therefore, absence of Rb protein or presence of an aberrant Rb

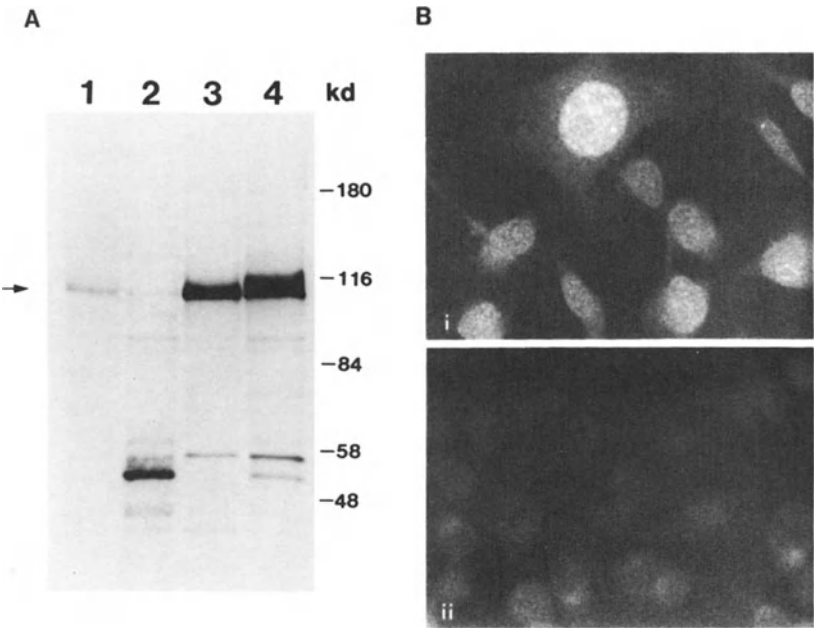

Figure 5. Localization of the Rb protein. A. ^{35}S–methionine labelled LAN–1 cells (lane 4) were fractionated into membrane (lane 1), cytoplasm (lane 2), and nucleus (lane 3). Rb protein was immunoprecipitated with anti-Rb IgG. The immunoprecipitates were then analyzed by SDS–PAGE. B. Immunofluorescence studies of Rb protein localization within osteosarcoma cell line U20S. Cell reacted with anti-Rb IgG (top) and preimmune rabbit IgG (bottom). Most fluorescence was found within nucleus. [From Lee et al., Nature 329:642–645, 1987.]

protein is indicative of RB mutation. Since the RB gene spans 200 kb, minor rearrangement of the gene frequently escapes the detection by genomic Southern hybridization. Only gross changes in mRNA can be readily identified by Northern blotting. Therefore, direct study of Rb protein expression is by far the most sensitive assay for possible RB mutation.

Mutational inactivation of the RB gene in human breast cancer

The ubiquitous expression of RB gene in every normal tissue suggests a common RB function and poses the question whether RB inactivation in cells other than retinoblasts will also lead to oncogenesis. As mentioned earlier, patients with hereditary retinoblastoma have a higher risk of developing second primary tumors later in life, suggesting the role of RB inactivation in other tumor types, e.g., osteosarcoma, soft-tissue sarcoma, etc. Studies of these tumor types indeed demonstrated mutation of the RB gene and suggested a crucial role of RB in tumors other than retinoblastoma [45–49]. Besides these second primary tumors, RB alteration at the DNA,

RNA, and protein level is found in more than 90% of the small-cell lung carcinoma cell lines and tumor specimens [50–52].

Expression of Rb protein in breast tumor cell lines

Breast cancer was not originally associated with retinoblastoma, though a higher risk of breast cancer has been noted in mothers of children with osteosarcomas or soft-tissue sarcomas [53]. Based on the RFLP analysis of DNA from tumor and somatic cells of the same patient, loss of heterozygosity in breast tumors was found for alleles on chromosome 11p and 13q [54, 55], suggesting that the loss or inactivation of several tumor suppressor genes might be involved in breast cancer. To test whether the RB gene, located at 13q14, is involved we have initiated the study of RB expression in breast tumors by surveying a panel of breast tumor cells for the expression of Rb protein [56]. Seven of nine cell lines contained normal-sized Rb protein (as detected by immunoprecipitation of lysates from cells metabolically labeled with ^{32}P, whereas Rb protein was undetectable in two cell lines,

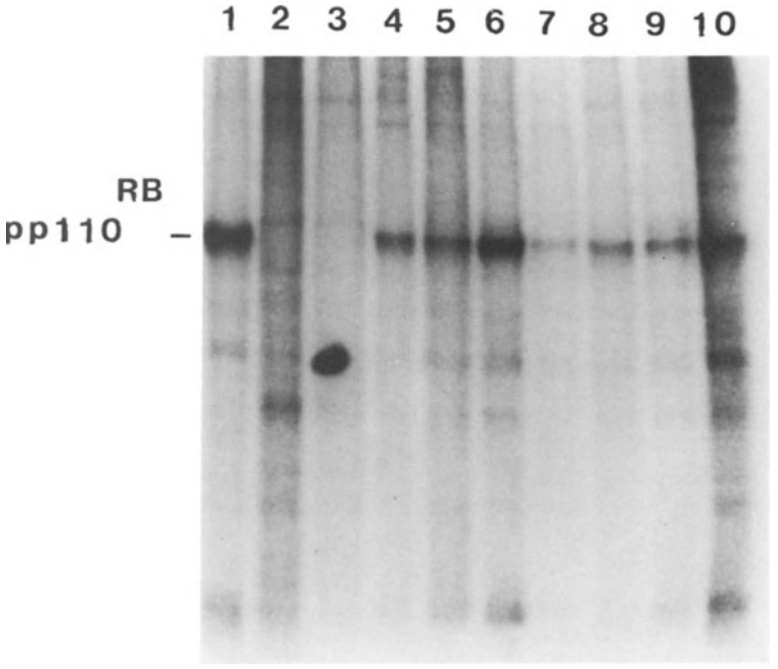

Figure 6. Immunoprecipitation of ^{32}P-labeled Rb protein in breast tumor cell lines. LAN–1 (lane 1) neuroblastoma and nine breast tumor cell lines (lane 2 to lane 10) were labeled with 150 uCi of ^{32}P–phosphoric acid/ml of phosphate free medium for 3 hours. Cell lysates were immunoprecipitated with an affinity-purified IgG specific against Rb protein. Immunoprecipitates were separated in 7.5% SDS–polyacrylamide gel and then autoradiographed overnight. [From Lee et al., Science 241:218–221, 1988.]

MDA–MB436 and MDA–MB468 (figure 6). Two other antisera-recognizing synthetic peptides based on the RB sequence were also unable to precipitate specific proteins from these two cell lines. Since Rb protein is found routinely in most cultured cells [43], its absence from these two cell lines suggested mutational inactivation of the RB gene.

Detection of RB gene mutation in breast tumor cells

To address the importance of RB inactivation in breast cancers, it was required not only to demonstrate RB mutation in primary tumors but also to correlate specific mutations of the RB gene with the lack of Rb protein. Northern blotting was performed using polyadenylated RNA from four breast tumor cell lines as well as a cell line (HBL 100) derived from nonneoplastic human mammary epithelium. Normal-sized RB transcripts of 4.7 kb were found in the mammary epithelial cell line and in two breast tumor cell lines, MDA–MB415 and MDA–MB435S, that expressed intact RB proteins. However, a slightly larger RB transcript (100–200 added nucleotides) was found in MDA–MB436, whereas no RB transcript was detectable in MDA–MB468. Expression of esterase D was demonstrated in all cell lines as a control for mRNA quality and to suggest that chromosome region 13q14 was generally intact. Lack of Rb protein in these two breast tumor cell lines therefore reflected characteristic alterations in RB gene expression (absence of RB mRNA or changes in its size) similar to those seen in retinoblastomas.

While many types of mutations might lead to aberrant gene expression, gross genomic rearrangements may be detected by Southern blotting analysis. Genomic DNA was extracted from these two cell lines, digested with restriction endonucleases *Bam*HI, *Hin*dIII, and *Msp*I, and analyzed with probes derived from RB cDNA (figure 7). In MDA–MB436 DNA digested with *Hin*dIII, an extra 5 kb fragment was present in addition to five expected bands with probe RB0.8 (figure 7, lane 2). Furthermore, the largest *Msp*I fragment in this cell line was displaced from the normal 7.5 kb to 12.5 kb. The rest of the RB gene appeared grossly normal, using RB3.8 as probe (figure 7). Comparison with the normal RB genomic map suggested duplication of exons 5 and 6 in both RB alleles, generating an extra *Hin*dIII site and creating a larger *Msp*I fragment (figure 8). Duplication of exons 5 and 6 would add 107 nucleotides to the mRNA transcript [42], which is consistent with RNA blotting analysis. Moreover, the reading frame of this transcript would be shifted with premature termination at nucleotides 659–661, consistent with the lack of intact RB protein. In cell line MDA–MB468, all three restriction digestions demonstrated homozygous deletion of most of the RB gene (figure 7). The 12 kb *Bam*H I fragment containing exon 2 was shortened to 8 kb, while a 19 kb *Hind* III fragment containing exons 1 and 2 was intact; therefore, the deletion junction was located within intron 2, as shown (figure 8). No DNA fragments were hybridized using

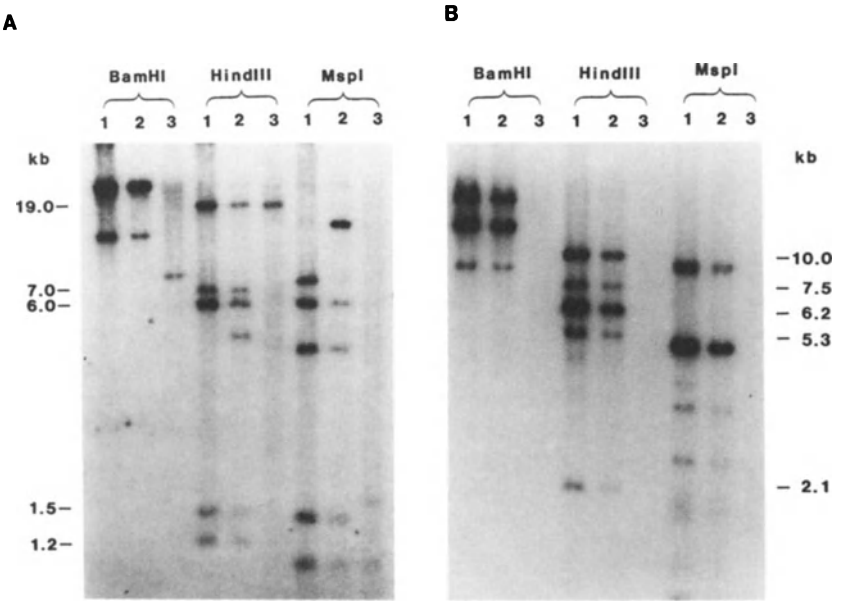

Figure 7. DNA blotting analysis of the RB gene in breast tumor cell lines MDA–MB436 and MB468 and normal DNA. DNA (3 μg per lane) from normal lymphocytes (lane 1), MDA–MB436 (lane 2), and MDA–MB468 (lane 3) were digested with restriction endonucleases *Bam*HI, *Hind*III, and *Msp*I and analyzed by Southern blotting with probes RB0.8 (A) and RB3.8 (B). [From Lee et al., Science 241:218–221, 1988.]

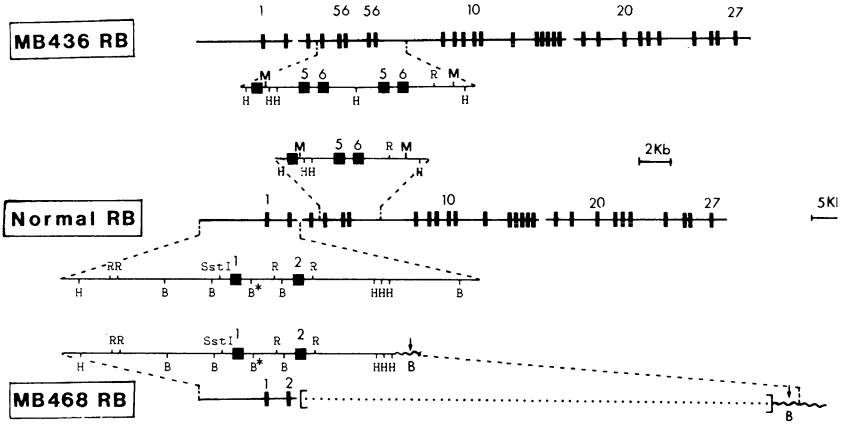

Figure 8. Inferred map of the RB gene in MDA–MB436 and MDA–MB468. The normal RB gene map [Bookstein et al., 1988; Hong et al. 1989] is shown with selected *Msp*I sites added. Using data from figure 7, the structure of mutant RB genes was inferred. In MDA–MB436, the RB gene contains two extra exons 5 and 6, resulting from duplication of a 5 kb region. In MDA–MB468, a large deletion (dotted line) extends from intron 2 to the 3′ end of the RB gene. The deletion junction was mapped between *Hind*III and *Bam*HI sites in intron 2. Exons are represented as solid vertical bars. R = *Eco*RI, H = *Hind*III, B = *Bam*HI, M = *Msp*I. * Indicates polymorphic site. [From Lee et al., Science 241:218–221, 1989.]

RB3.8 as probe (figure 7, lane 3), indicating that the deletion in MDA–MB468 must extend beyond the 3′ end of the RB gene. Further analysis of the deletion junction confirmed that the 3′ deletion end point was 9 kb 3′ to the last exon of the RB gene [57]. So far, divergent mutational events including point mutation, the deletion of variable sizes of DNA fragments, and duplication have been found within the RB gene; in some instances aberrant proteins with different mobility and biochemical properties were identified [45–52, 58, 59].

RB mutation in primary breast tumors

T'Ang et al. reported additional cell lines, including BT549 and Du4475, etc., and three primary breast tumors with mutated RB gene. Using RB cDNA as probe in Southern hybridization, deletion of various regions of RB gene was detected [60]. Using a survey of 77 primary breast carcinomas, Varley et al. concluded structural abnormality in 19% of tumors, although only one DNA rearrangement was shown [61]. Using antibody generated against a synthetic peptide, they further examined the expression of Rb protein in tumors by immunohistochemical methods. Of the 56 tumor samples examined, 29% had no Rb expression in a proportion of the cells [61]. However, several points remain to be clarified. The nuclear staining of Rb protein was clearly shown in certain cell types, including U20S (figure 5), but unequivocally positive or negative staining was difficult to judge in certain types of cells. Therefore, the sensitivity and background staining of their peptide antisera needs to be fully illustrated. Second, it is unclear why tumors with RB gene rearrangement contained different percentages of Rb expressing cells, since the mutation in each tumor will either change or have no effect on the epitope recognized by the antiserum. If indeed a mixed population of Rb expressing and nonexpressing cells were present in the tumor, DNA analysis should have also reflected the heterogeneity. However, this seems not to be the case.

In summary, regardless of these experimental difficulties, RB mutations have been found in breast tumor cell lines and primary carcinomas. Various types of lesions that lead to RB inactivation were identified. Although no clear genetic link of breast tumor and retinoblastoma is reported, in light of the common cellular function of the RB gene in several tumor types (see below), the finding of RB mutation in breast tumor suggests that it may have a role in the genesis of this malignancy.

Tumor suppression function of the RB gene

Mutations of the RB gene were observed in a significant percentage of tumors other than retinoblastoma. These included osteosarcoma [45–49], small-cell lung carcinoma [50–52], breast carcinoma [56, 60, 61] and a

continually growing list, as more tumors are surveyed. However, only a subset of cases of each type have demonstrable RB mutations. Since these cancers have no well-defined pattern of inheritance, the significance of RB mutation is not clear. To test the role of RB inactivation in these tumor types, we have proposed to study the effect of replacing the Rb protein on the neoplastic behavior of these tumor cells.

Vectors for expressing Rb protein in tumor cells

In the initial experiment, replacement of Rb protein was performed in retinoblastoma and osteosarcoma tumor cells [62]. Two retrovirus constructs were used in the study. One, Rb, consisted of the long terminal repeat sequences of Moloney leukemia virus (MuLV LTRs) coupled to a modified RB cDNA and the neomycin-resistance (*neo*) gene under Rous sarcoma virus (RSV) promoter control. The *neo* gene encodes Tn5 neomycin phosphotransferase, which confers resistance to the neomycin analogue G418 used to select against noninfected cells. The other, Lux, was identically constructed, except that RB was replaced by the luciferase gene. This gene served not only as a control for specific effects of the RB gene but also as a means to examine expression efficiency of the viral construct in different cell types. Questions concerning the level of expression and regulated expression of Rb protein require design of different vectors.

Tumor suppression function of the RB gene in retinoblastoma and osteosarcoma cells

Expression of exogenous RB protein initially had complex but characteristic effects on each cell type. In brief, the osteosarcoma cell Saos–2 become enlarged by threefold to tenfold in average diameter, and growth of bulk cultures was profoundly inhibited. Retinoblastoma cell line WERI–Rb27 cells, which grew in suspension, became mildly enlarged, and growth of bulk cultures was moderately inhibited. In contrast, both cell types were unchanged after Lux infection. Tumorigenicity tests were initially conducted only on bulk-infected cultures of WERI–Rb 27 cells because sufficient numbers of Rb-infected Saos–2 could not be accumulated in culture. Seven nude mice were injected with 2×10^7 Rb- or Lux-infected WERI–27 cells in opposite flanks and were observed for two months. Complete suppression of tumorigenicity was observed in RB flanks even as Lux flanks progressed to large subcutaneous tumors.

Suppression of the tumor phenotype of breast tumor cells by the replacement of Rb protein

Similar experiments were conducted using breast tumor cell lines with mutated endogenous RB gene. Morphological changes in the Rb-infected

cells included enlarged cells in some cases and highly granuated cytoplasm in others. Since many Rb-infected, neomycin-resistant cells did not continuously express Rb protein, we have subsequently isolated single cell clones from the bulk-infected population. Cell lines continuously expressing Rb protein were established. Preliminary results indicated that Rb expressing breast tumor cells have diminished soft agar colony-forming ability compared to that of cells not expressing Rb. Taking together the similar changes in breast tumor cells and osteosarcoma cells, we have proposed the potential suppression role of the RB gene in tumors other than retinoblastoma. How the replacement of Rb protein could lead to phenotypic changes in many cell types is very intriguing, and studies of the cellular function of RB gene may reveal some clues.

Regulation of RB gene function

Posttranscriptional regulation of the RB gene

The ubiquitous expression of the RB gene in all tissues and the similarity of its promoter to that of the house keeping genes suggest that transcription of the RB gene is not a key modulation step. Instead, regulation at post-transcriptional levels are more likely. First, an AU rich region is found in the 3′ untranslated region of RB mRNA. This sequence is similar to an unstable signal for the *fos* mRNA [63] and might be related to the stability of RB mRNA. Second, the 5′ untranslated region contributes to a low translatability in an in vitro translation system. Switching of this 5′ untranslated region with that of b–globin and AMV RNA4 increases the translation efficiency fivefold to tenfold [64]. Whether this feature is significant in the quantitative control of Rb protein is intriguing specifically in light of the increasing evidence that points to the role of 5′ untranslated region of mRNA in regulating protein translation. Third, Rb is a phosphorylated nuclear protein. It is known that phosphorylation plays an important role in regulating the activity of a wide spectrum of proteins [for review see 65]. We and others have shown that there are multiple phosphorylated forms of Rb [44, 45]. The significance of phosphorylation on the functional regulation of Rb is discussed in two aspects: cell cycle and DNA-binding activity.

Oscillation of Rb phosphorylation during the cell cycle

It was noticed that Rb protein in resting cells is less phophorylated than that in rapidly dividing cells. To further investigate the possible modulation of Rb during the cell cycle, we studied primary human peripheral lymphocytes before and after induction of cell division with phytohemagglutinin [66]. Only the unphosphorylated form of Rb was found in resting cells;

phosphorylated forms of Rb appeared when cells entered S phase. Further studies with synchronized cells in culture confirmed that phosphorylation of Rb varies at different stages of the cell cycle. Phosphorylation of Rb proteins was observed at the G1/S; dephosphorylation followed when cells left S phase; and maximum phosphorylation occurred again at M phase [66]. Therefore, the phosphorylation status of the Rb protein oscillates during the cell cycle. However, Rb protein is not absolutely required for cell cycle progression, since Rb negative cells continue to divide. On the other hand, we have shown that cells induced to differentiation by TPA or retinoic acid express only unphosphorylated forms of Rb [66]. Observations of the retarded cell growth in bulk populations of retinoblastoma and osteosarcoma cells after the replacement of Rb protein and of the oscillation of Rb phosphorylation during the cell cycle indicate that the Rb protein is involved in cell growth. Furthermore, phosphorylation may act as a switch for the on/off regulation of cell cycle progression. Whether this process is related to differentiation remains unknown. The notion that phosphorylation can modify regulatory function has gained support from studies of transcription activators [for review see 67]. Based on the complexity of growth control, it is proposed that sets of genes are regulated by the RB gene. Whether certain oncogenes are among the target genes is highly speculative [25], but to date there is no direct evidence for the negative regulation of oncogene expression by the Rb protein.

DNA-binding activity of Rb protein

Consistent with the hypothetical regulatory role of the Rb protein, we have shown that the protein is associated with DNA-binding activity in a DNA-cellulose-binding assay [43]. This activity is intrinsic to the protein, since purified Trp E–RB fusion protein expressed in *E. coli* retains the property. To identify the DNA-binding domain whinin Rb, we have expressed fusion proteins encompassing various regions of the RB gene and assayed their DNA-binding activity. The C-terminus of the Rb protein, containing exon 23–27 but not exon 19–23 nor exon 9–16, has the strongest DNA-binding activity [68].

Rb-associated proteins

Extensive studies of gene regulation have demonstrated the importance of interactions between multiple *cis*-regulatory elements and multiple transcription factors [67]. If indeed Rb protein regulates the expression of a set of genes, it is likely this regulation also requires the interaction of Rb with other cellular proteins. Although no specific cellular protein has been identified, it is interesting that the Rb protein forms a complex with many oncogenic products of the DNA viruses, namely EIA protein of Adenovirus, large T antigen of SV40, and E7 protein of the papilloma virus [69–71]. Association

with Rb protein and modulation of Rb function may provide the mechanism for the oncogenic properties of these viruses [69–71]. Based on this finding, the possible functions of other T- or EIA-associated cellular proteins were reexamined. One of these proteins, p53, was initially classified as oncogene, since it has the oncogenic potential in transfection assay [72, 73]. Further studies have led to the discovery that the original isolated p53 is a mutated form and may not possess the properties of the wild-type p53. No transformants were observed when primary rat embryo fibroblast were co-transfected with a wild-type p53 plus an activated *ras* gene [74, 75]. Recent work has shown that p53 is frequently mutated in cell lines and primary tumors [20, 75]. It is speculated that the wild-type p53 is a tumor suppressor gene, in contrast to the earlier claim of it being an oncogene [20, 75]. Indirect evidence supporting this notion comes from transfection of primary rat embryo fibroblasts with EIA, *ras*, and p53. Transformation is enhanced by mutant p53 but inhibited by the wild-type p53. Furthermore, colonies obtained by co-transfection with wild type p53 either do not express p53 or express the mutant p53 protein [76]. P53 is a nuclear phosphoprotein [77]. However, little is known about its function. Taking together the mutation of RB and p53 in a variety of cell types, it is likely both proteins play essential roles in growth regulation. Whether these two proteins have parallel functions or interactions is intriguing.

Perspective

Molecular genetic studies of retinoblastoma have led to an important breakthrough in the study of human oncogenesis. Our view of cancer has widened from the tumor-promoting genes that facilitate tumor formation to the tumor-suppressing genes that inhibit this process. Both types of genes appear to be ancient, since their presence can be traced back to *Drosophila* [78, 79]. The balanced function of these two types of genes appears important for regulating cell growth.

Complicated factors are involved in adult tumors, and multiple steps for tumor development have been proposed. The progression of human colorectal tumors is one of the best-illustrated examples [19]. In contrast, genetic studies of the childhood tumors suggest the involvement of one crucial gene. For example, in retinoblastoma, RB plays a central role. This conclusion is reinforced by molecular analysis of retinoblastoma and replacement of the Rb protein back into the tumor cells. While our studies indicate an important functional role for RB in many adult tumors, including breast carcinoma, it is plausible to suggest that mutations of other genes are also involved.

Breast tumor is one of the most common malignancies in Western societies. Based on the specific loss of heterozygosity, recessive genes on chromosome 13 and 11 are suggested to be involved. Whether mutations on

both genes are required has not been addressed directly. On the other hand, evidence of the suppressing role of RB in multiple types of tumors has been discussed. However, it is important to study a large size of samples before an estimate of the frequency of RB inactivation in breast tumors can be made. Alternatively, establishment of chimeric mice models will facilitate the study of RB inactivation and tumor formation. Recent advances in the use of homologous recombination to inactivate one allele of recessive genes in the embryonic stem cells and microinjection of the cells into blastocysts following implantation of the embryos into pseudo-pregnant mice have lead to the creation of mouse models for studying specific genes [for review see 80]. With this approach it will be possible to generate mice with a RB mutation in one allele and therefore to mimic the heritable retinoblastoma in humans. Studies of the factors and genetic alterations that cause breast tumors in this mouse model will facilitate our understanding of the role of tumor suppressor genes in this malignancy. Furthermore, based on the observation of tumor suppression by Rb replacement, this chimeric mice model will also offer a system for addressing the feasibility of gene therapy in cancer.

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II.

Growth Factors and Their Receptors

3. Relationship of growth factors and differentiation in normal and neoplastic development of the mammary gland

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Relationship of cell types present within the normal and neoplastic mammary gland in vivo

The mature normal mammary gland

The mammary gland of nonpregnant mammals is composed of an epithelium embedded in a fatty stroma. The epithelium consists of a branching ductal tree terminating in alveolar buds (ABs) in rats or in terminal ductal-lobuloalveolar units (TDLUs) in humans [1, 2]. The boundary of the epithelium is formed by a basement membrane, on the inner surface of which is a more or less continuous layer of elongated myoepithelial cells possessing smooth muscle-like myofilaments and pinocytotic vesicles [3–6]. One or more layers of cuboidal epithelial cells constitute the core of the ducts, with the inner layer bordering a lumen that is continuous throughout the ductal tree [7]. The luminal, cuboidal epithelial cells have apical microvilli and specialized junctional complexes with associated desmosomes. In the terminal ABs and TDLUs that form distended lobules, the luminal layer is composed of secretory or alveolar cells that synthesize and secrete milk products during lactation [2, 8]. More recently a battery of immunocytochemical probes has been used to define, on a more molecular basis, the cuboidal epithelial cell of the ducts, the epithelial cells of the ABs/TDLUs, the myoepithelial cells, and potential transitional cells [9–11]. These probes have been important in understanding the developmental relationship between the different cell types found in the mature mammary gland.

Development of the normal mammary gland

At birth in rodents and humans, the mammary ductal tree is a very rudimentary structure and, at least in the rat, myoepithelial cells are absent until about seven days after birth [3, 12]. Most of the development of the mammary gland occurs between birth and puberty [7, 13, 14]. Growth of the mammary gland occurs by the extension of the rudimentary ductal tree

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to the limits of the mammary fat pad by the elongation of primitive ducts, the dichotomous branching of the growing tip, and the monopodial branching of collateral buds [15–17]. Until puberty the ducts terminate in globular structures called terminal end buds (TEBs) in rats, and these structures contain the majority of the mitotic parenchymal cells [14, 18–20]. The number of globular structures increases until puberty, at which stage their number decreases rapidly, a consequence of not only their transition to terminal ducts but also their differentiation with each estrous cycle to small lobulated structures termed ABs in rodents or TDLUs in humans. This decrease is much more variable in humans than in rodents [14, 18, 19]. The ABs and TDLUs are the direct precursors of secretory alveoli.

Immunocytochemical and pulse-chase studies have shown that the TEBs, lateral buds, and to a lesser extent the ABs are composed not only of well-differentiated epithelial and myoepithelial cells but also of irregular, loosely-adherent cap cells located mainly at the periphery. The cap cells exhibit a gradation both to the fully differentiated epithelial cells within the end bud and to the myoepithelial cells of the subtending duct [21–23]. Similar, albeit weaker, evidence exists for such gradations in human TDLUs [11, 24, 25].

Development of rodent carcinogen-induced mammary tumors

The susceptibility of the rodent mammary gland to chemical carcinogens such as 7,12–dimethylbenz[a]anthracene (DMBA) and N–nitrosomethylurea (NMU) decreases markedly after fifty days (puberty) and correlates with the presence of TEBs and terminal ducts [26–29]. Detailed analysis has revealed that these tumors are largely benign and immunogenic [30], consisting of cuboidal epithelial and elongated, myoepithelial-like cells in ductlike arrangements surrounded by a basement membrane that is often thicker than normal [31–34]. The myoepithelial-like cells are relatively undifferentiated in appearance compared with the myoepithelial cells of mature ducts [33]. Hormonal stimulation of the host leads to the production of alveolar-like cells and casein. However, both the amount of casein produced and the number of alveolar-like cells are only 1% to 5% of the normal values; this may reflect the neoplastic origins of these cells [35–37].

In contrast, chemical induction of carcinogenesis in partially immunodeficient rats followed by non-specific immunostimulation yields non-immunogenic tumors of much higher metastatic capacity. These tumors disseminate by hematogenous and/or lymphatic routes, giving rise to widespread metastases [38]. Unlike their benign counterparts, these metastatic tumors do not contain myoepithelial cells nor do they produce alveolar-like cells and casein under the appropriate hormonal conditions. The majority also lack a basement membrane (see 'Differentiation and tumorigenicity,' below) [9, 33, 39].

Development of the human neoplastic mammary gland

With the exception of the radiation-induced breast cancers in the victims of the atomic bomb explosions at Hiroshima and Nagasaki [40], the primary carcinogens in humans are unknown. Prepubertal/adolescent humans appear to be most susceptible to radiation-induced carcinogenesis, although this finding is not as clear-cut as the finding in chemically-induced carcinogenesis in rats [40]. This may be due to humans exhibiting a more variable degree of differentiation of terminal ductal structures at a given age than do rats [14, 18, 19].

An increased risk of neoplastic disease correlates with the presence of atypical epithelial-cell proliferations in terminal ductal structures [41]. These proliferations are thought to represent a spectrum of changes from benign lesions to carcinoma in situ, the direct precursor of mammary carcinoma [42]; however, there are contrary views [43]. Ultrastructural [43, 44–46] and immunocytochemical [45, 47–52] analyses have demonstrated that some myoepithelial-like cells and basement membranes are always present in the major forms of benign breast disease (epitheliosis, adenosis, and fibroadenoma). In contrast, the myoepithelial cells are virtually absent from infiltrating ductal carcinomas, and fragmented basement membranes are observed in only a small number of them, usually of the Grade I category [50]. The epithelial cells of benign tumors can differentiate in pregnant/lactating women to produce casein, a marker of alveolar cells, whereas the cells of carcinomas appear to be unable to do so [53, 54].

Thus the pattern of differentiation and the generation of the different cell types and structures of the mature normal mammary gland is similar in rats and humans. Moreover, the characteristics of differentiation of both benign and malignant tumors also exhibit marked similarities in rats and in humans, with a common central theme of an increasing lack of the myoepithelial and alveolar cell phenotypes with increasing metastatic potential [9].

Differentiation of epithelial stem cells in culture

Primary cultures and cell lines

Partial collagenase digestion of the normal rat mammary gland or the benign, carcinogen-induced rat mammary tumors separates most of the fatty stroma from the epithelial elements, generating organoids of glandular elements surrounded by a basement membrane; fragments of blood vessels are also produced [55, 56]. Within 2 hours of plating, the greater part of the stromal cells adheres to the substratum, while the organoids adhere only after 12 to 24 hours. Epithelial cells spread out from these organoids some time

Table 1. Origins of the mammary cell lines discussed.

Mammary tissue	Identity	Cell line	Reference
Normal Furth–Wistar rat (inbred)	Rama 704	Epithelial	[60]
	Rama 704E	Myoepithelial-like	
	Rama 401	Myoepithelial-like	[90]
Sprague–Dawley rat (outbred), benign DMBA rat tumor	Rama 25	Epithelial	[62]
	Rama 259	Epithelial, truncated alveolar-like pathway	[73]
	Rama 25–I2	Cells intermediate between epithelial Rama 25 and myoepithelial-like Rama 29	[86]
	Rama 25–I1		
	Rama 25I–4		
	Rama 29	Myoepithelial-like	[62]
Furth–Wistar rat (inbred), benign DMBA syngeneic rat tumor	Rama 37CL–A3	Epithelial	[61]
	Rama 37–E8	Cells intermediate between Rama 37CL–A3 and Rama 37–E5	
	Rama 37–E5	Myoepithelial-like	
Weakly-metastasizing rat tumor, TR2CL	Rama 600	Epithelial	[39]
Moderately-metastasizing rat tumor, TMT–081	Rama 800	Anaplastic epithelial	[100]
Strongly-metastasizing rat tumor, SMT–2A	Rama 900	Anaplastic epithelial	[98]
Normal human transformed by SV40	SVE3	Epithelial	[70]
	Huma 7	Epithelial	
	SVE3-floaters	Myoepithelial-like	
	Huma 25	Myoepithelial-like	
	Huma 50	Myoepithelial-like	
	Huma 62	Myoepithelial-like	
Human ductal carcinoma	Ca2–83	Epithelial	[108]

later [55]. Essentially, all the different cell types that are observed in vivo, using both ultrastructural and immunocytochemical techniques are also seen in these cultures [57]. When reintroduced into the cauterized fat pads of syngeneic rats, the fast-sticking stromal cells give rise to a fatty outgrowth, while the slower-sticking epithelial elements give rise to the entire mammary ductal tree [58]. Finally, confluent cultures of epithelial cells yield hemispherical domes [59], and under the influence of the mammatrophic hormones, they produce small amounts of casein [56]. Most of the cells in these cultures die out after a few passages.

Spontaneously transformed, immortalized epithelial cells occasionally grow out of primary cultures, and single cells can be removed from such outgrowths and grown up into bulk culture. Thus single-cell-cloned mammary epithelial cell lines have been obtained from 7-day-old inbred Furth–Wistar rats [60], from DMBA-induced benign tumors in inbred Furth–Wistar [61] and outbred Sprague–Dawley rats (table 1) [62], and from an NMU-induced rat mammary tumor [63].

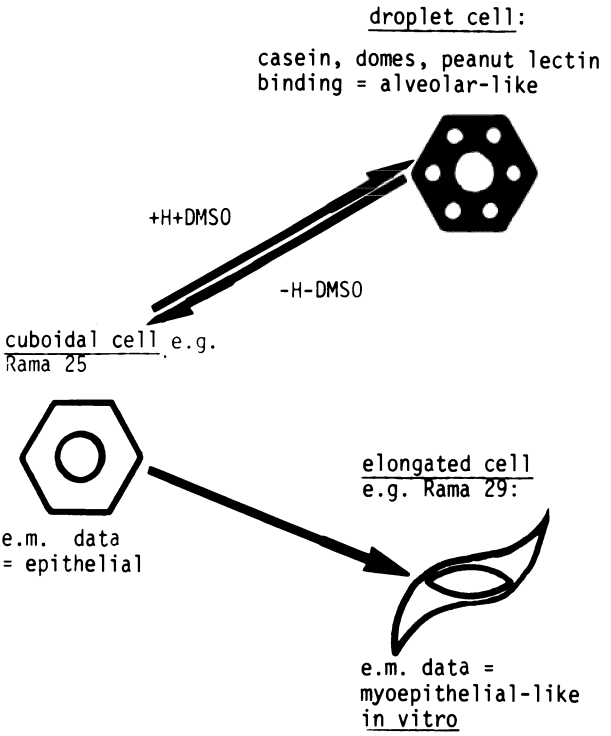

Figure 1. Differentiation pathways of Rama 25 epithelial cells. Rama 25 cuboidal epithelial cells derived from a benign rat mammary tumor can convert to droplet cell/doming, alveolar-like cells with mammatrophic hormones (H) (prolactin, estradiol, hydrocortisone, insulin), and dimethylsulfoxide (DMSO), prostaglandin E_1, or retinoic acid. They can also convert to elongated, myoepithelial-like cells (e.g., the cell line Rama 29). e.m. = electron microscopy.

Primary cultures of reduction mammoplasty specimens from otherwise normal human breasts have been obtained in a similar manner [64–67]. As in the rat, a variety of ultrastructural and immunocytochemical techniques shows that these cultures have cellular morphologies and structures similar to those seen in the human mammary gland in vivo [68]. The human cells, unlike cells of the rat, fail to transform spontaneously, and invariably the primary cultures keratinize and senesce after a few passages [67, 68]. Immortalization of such cultures with Simian Virus 40 (SV40) has been used to circumvent this problem [69], and clonal cell lines similar to those of the rat have been obtained (table 1) [70].

The properties of the different rat and human cell lines in terms of their ability to differentiate are very similar; so one epithelial cell line, Rat mammary (Rama) 25, derived from a benign DMBA-induced rat tumor (table 1) [62] will be discussed in some detail. This cell line exhibits two distinct differentiation pathways (figure 1).

Differentiation pathway to alveolar-like cells

Rama 25 is a cuboidal epithelial cell line that was originally isolated from a benign DMBA-induced tumor in an out-bred Sprague–Dawley rat (table 1) [62]. When Rama 25 cells are confluent and become densely packed, they form small, dark polygonal cells containing peripheral vacuoles or droplets, termed droplet cells [62]. The droplet cells form domes as a result of the action of the ouabain-dependent Na^+/K^+ ATPase [71]. Agents that stimulate the differentiation of Friend erythroleukemic cells [72] can accelerate the differentiation of an originally homogeneous cell population along this pathway (figure 1). These agents include dimethylsulfoxide [62], prostaglandin E_1 (PGE_1) [73], or retinoic acid [74] in the presence of the mammatrophic hormones prolactin, estradiol, hydrocortisone, and insulin. Under these conditions the cultures produce rat β–casein, although the amount of casein synthesized is only 1% to 2% of that observed in lactating glands. This may reflect the neoplastic origins of the cells [75]. Intermediate morphological forms of cells along this pathway [76] produce a small number of discrete stage-specific polypeptides [77]. The immunocytochemical staining characteristics together with the level of casein produced suggest that the cells that have differentiated in culture along the alveolar-like pathway resemble the cells of the ABs rather than the extensive casein-producing cells of the alveoli. Since many aspects of this differentiation pathway are observed in primary cultures and in other mammary epithelial cell lines [9] (table1), it is likely to represent a reasonable model for events that occur in vivo.

Differentiation pathway to myoepithelial-like cells

Although the Rama 25 cell line has been single-cell cloned three times, it consistently gives rise at a frequency of 1% to 3% to colonies of elongated

cells that can be grown up as cell lines. In culture both ridges of elongated cells and elongated cells floating freely in the medium are observed. Such elongated cells and clonal cell lines are related to myoepithelial cells on the basis of ultrastructural and immunocytochemical analysis [63, 78–85]. However, the staining for certain myoepithelial markers, e.g., actin, myosin, and human keratin, is variable, and some cell lines stain poorly, e.g., Rama 29 [86]. In general, cells that have recently converted express the most myoepithelial markers [60], and subcloning can lead to a loss of some of these characteristics, e.g., the microfilamental systems [87]. For these reasons the elongated cells derived from cuboidal epithelial cells in culture (table 1) are termed myoepithelial-like rather than mature myoepithelial cells [70, 79]. Furthermore, mature myoepithelial cells in both human and rat mammary glands do not divide [88, 89]. In the rat the most myoepithelial-like cell lines are derived from normal mammary glands, e.g., Rama 401 [90] and Rama 704E [60], and not from benign tumors, e.g., Rama 29 [62].

A series of clonal cell lines intermediate in character between the cuboidal epithelial and the elongated myoepithelial-like cells have been isolated separately from Rama 25 cells. They form a graded series between cuboidal epithelial and elongated myoepithelial-like cells in the order Rama 25, Rama 25–I2, Rama 25–I1, Rama 25–I4, Rama 29 (figure 2). Morphological, immunocytochemical, and molecular analyses have revealed that there is a progressive decrease of epithelial characteristics and a concomitant increase in myoepithelial characteristics across the series [86, 91, 92]. That the intermediate cell lines represent true intermediate stages along the myoepithelial differentiation pathway in vitro is suggested by kinetic studies of the differentiation of Rama 25 cells to elongated myoepithelial-like cells [85, 93]. Such cellular intermediates with analogous morphological and immunocytochemical properties have also been observed in primary cultures of normal rat and human mammary glands [9, 57, 68, 94, 95] and normal rat [60] and human [70] cell lines. Thus the generation of such intermediate cells is not unique to a single cell line.

Moreover, using the same criteria, Rama 25–I2, Rama 25–I1, and Rama 25–I4 also resemble cells in the terminal ductal structures in vivo [86]. Somewhat less detailed comparisons have been made for phenotypic inter-

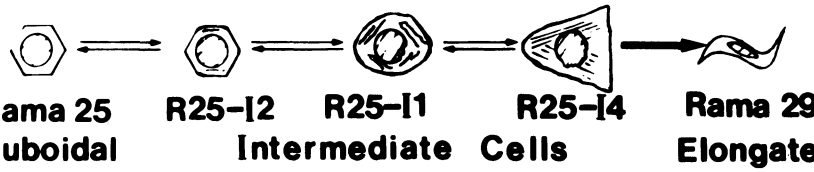

Figure 2. Differentiation of Rama 25 cells along a myoepithelial-like pathway. The cell lines intermediate in both morphology and characteristic markers between Rama 25 cuboidal epithelial and elongated, myoepithelial-like cells (e.g., Rama 29) are designated by R25–I2, etc. [86]. They are also thought to resemble similar cells in the direct conversion of Rama 25 cuboidal cells to elongated, myoepithelial-like cells. Only the last stage is irreversible.

mediates of the epithelial cell lines Rama 37 and Rama 704 [60, 61]. Thus the intermediate cell lines probably mimic stages of differentiation that are occurring in vivo [9].

Reduced differentiation of malignant epithelial cells

Neoplastic cells from rat mammary carcinomas have been isolated by collagenase digestion as described for normal and benign tumors above. However, this technique has been essentially limited to the weakly metastasizing tumors, e.g., TR2CL (table 1) [39, 96]. For tumors with a higher metastatic potential, other approaches have been employed, such as growing cells from ascitic versions of the transplantable tumor, e.g., TMT–081 and SMT–2A [97, 98], or by selection in vitro of cultured metastases [99] with other cell types present as feeders. All the clonal cell lines give the same histological appearance and patterns of metastasis in the syngeneic rats; thus no major changes have been brought about by experimental manipulation [97, 98, 100, 101]. The metastatic potential of the cell lines increases in the order Rama 600, Rama 800, Rama 900 (table 1) in parallel with their ability to grow as loosely adherent colonies, their dependence on feeder cells, and their anaplastic and heterogeneous cellular appearance. In contrast, cellular growth rates decrease with increasing metastatic potential in this series of metastatic cell lines [39, 98, 100].

The most weakly metastasizing cell line is Rama 600, and this cell line produces some undifferentiated elongated cells that have similar ultrastructural and immunocytochemical characteristics [39] to the recloned, dedifferentiated myoepithelial-like cell lines derived from Rama 401 and Rama 704E [87]. Although such cells retain some basement membrane components, they have lost most of their microfilamental systems (mentioned above). The elongated cells do not metastasize on their own, but they may serve to increase the growth rate of the parental Rama 600 cells [39]. Tumors of the Rama 600 cell line also contain some elongated cells; but if this represents differentiation along the myoepithelial pathway (figure 2), it is a very incomplete process [39] and may reflect only a vestige of the complete pathway seen with cell lines from normal rat mammary gland and its benign tumors (see above). However, even this incomplete process of differentiation appears to be sufficient to generate the fragmented basement membrane observed in some Rama 600 tumors in vivo. Similarly, the alveolar-like differentiation pathway (figure 1) is severely truncated in Rama 600 cells [39]. In contrast, cloned epithelial cell lines from the more metastatic tumors (TMT–081 and SMT–2A, table 1) fail to yield elongated myoepithelial-like cells in culture or to differentiate to any recognizable extent along the alveolar-like pathway (figure 1) [39, 93, 98, 100].

The culture of human mammary carcinomas has been extremely difficult [64, 65, 102], although a few epithelial cell lines have been established [103–106]. Collagenase digestion of over a hundred primary infiltrating ductal

carcinomas has yielded loosely adhering (>72 hours), malignant-looking cell clusters, and relatively fast-adhering (<48 hours), less malignant-looking epithelium on collagen gels [107, 108]. Metastases in lymph nodes and pleural effusions yield only the former type of cell, whereas normal mammary glands and fibroadenomas yield only the latter [107, 108]. Thus, as in the rat, the most metastasizing cell populations are represented by the slow-growing, loosely adherent cells [103, 106, 109]. The fast-sticking population, whether from benign fibroadenomas or primary carcinomas, yields epithelial cells, myoepithelial cells, and droplet/doming presumptive alveolar-like cells, exactly like those from normal breasts (see 'primary cultures and cell lines', above). The loosely adherent clusters yield only epithelial cells [108, 110] even under conditions known to promote formation of the other two differentiated cell types [PS Rudland, unpublished]. The loosely adherent cells usually die out after transfer in vitro [104, 111, 112]. The rare occasions when epithelial cells have emerged as permanently growing cell strains have usually involved their passage through a period of crisis characterized by a switch to more rapidly growing adherent cell sheets [106, 113, 114]. However, continued passage of one preparation of loosely adherent cells has yielded a continuously growing cell strain, Ca2–83, which has not yet undergone a period of crisis and still has a doubling time of 10 to 14 days (table 1) [108]. The properties of this cell strain are consistent with the properties of the original primary tumor in the patient [108], with those of the more metastatic rat mammary cells [64, 108], and with what is known of human carcinoma cells prior to their period of crisis in vitro [115, 116]. Like their rat counterparts, the malignant cell line Ca2–83 [108] as well as the similar line PMC–42 [109, 117] does not yield elongated myoepithelial-like cells or synthesize casein and differentiate to alveolar cells in culture under suitable hormonal conditions [9].

The studies described above are consistent with the pathology of benign and malignant human breast lesions and with the equivalent rat tumors outlined above. The observation of abnormal organoidal structures of epithelial and myoepithelial-like cells in some primary ductal carcinomas and their absence in metastatic tumors [108] probably reflects a progression towards a more malignant phase (see above). These findings may be more consistent with a mutational event occurring in an epithelial stem cell (with the gradual truncation of its differentiation pathways during the progressive phase of the disease [9]) than with simultaneous mutational events occurring in the epithelial stem cell and an adjacent nondifferentiating epithelial cell, which ultimately gives rise to the malignancy [118].

Differentiation and tumorigenicity

The epithelial cell line derived from normal rat mammary glands, Rama 704, fails to produce tumors in syngeneic rats [60], although SV40-transformed human epithelial cell lines SVE3 and Human mammary (Huma) 7 (table 1)

yield benign tumor nodules in nude mice [70]. However, even in the case of the SV40-transformed human system, the tumor nodules regress with time probably due to processes of terminal keratinization of the epithelial cells in the animals [119]. As anticipated, the epithelial cell lines isolated from the benign tumors, Rama 25 and Rama 37, produce progressively growing but relatively benign nonmetastasizing tumors in nude mice [120] and in syngeneic rats [61], respectively. Most of the recognizable mammary growth patterns are reproduced by these clonal cell lines in such tumors. These results suggest that the different histological forms of mammary tumor can be generated from a single epithelial or closely related mammary cell (see above) [70, 120].

Most of the myoepithelial-like cell lines derived from Rama 25, including Rama 29, are unable to induce tumors in nude mice. However, a spontaneous transformant of Rama 29 cells, Rama 521, exhibits a greatly increased tumorigenic potential, producing benign spindle-cell tumors pathologically different from those of Rama 25 in nude mice [120]. Similarly, conversion of Rama 25 to alveolar-like cells (figure 1) induced by the mammatrophic hormones is accompanied by a marked reduction both in rates of DNA synthesis and in tumorigenic potential. However, a variant of Rama 25, Rama 259, which has a truncated alveolar-like differentiation pathway, fails to reduce its tumorigenicity and rates of DNA synthesis under the same hormonal conditions [73, 74, 76]. Thus terminal metaplastic differentiation to keratinized structures, differentiation to more slowly growing alveolar-like cells, or differentiation to growing myoepithelial-like cells mitigates against the formation of tumors in animals. Therefore, it would seem likely that agents that promote the above differentiation processes in the mammary epithelial stem cells in vitro may also reduce their tumor-forming abilities in vivo. This is largely borne out in practice [121–125]. Subsequent transformation events may then be required to yield a cell that has regained its former neoplastic potential in vivo; but the resultant tumors will then be of a different pathological type.

Requirements for growth of tissue culture cells

Growth and differentiation of the mammary gland in rats and humans is controlled partially by systemic agents released from the pituitary, ovary, and adrenal as well as from the pancreas and thyroid glands [126–128] and partially by locally produced agents released by the fatty stroma [129, 130]. The growth of carcinogen-induced tumors in the rat is also controlled in a similar manner [131, 132]. However, when primary and secondary epithelial/myoepithelial cultures of normal rat mammary glands, of benign carcinogen-induced tumors, or of cell lines developed from them have their growth rates reduced by depletion of serum in the culture medium and when different agents are added back to the cultures, the results are more complex (table

Table 2. Agents tested that stimulate cell growth in mammary cells.

Agent	Alone	Type
Pituitary Factors: PMGF	+[a]	Polypeptide growth factors
Pituitary Factors: FGF	+[b]	Polypeptide growth factors
EGF-related molecules	+	Polypeptide growth factors
PGE_2	+	Arachidonic acid metabolites
$PGF_{2\alpha}$	+[c]	Arachidonic acid metabolites
Insulin	−	Polypeptide hormone
Estradiol	−[d]	Steroid hormones
Progesterone	−	Steroid hormones
Hydrocortisone	−	Steroid hormones
Serum	+	Systemic agent(s)
Myoepithelial cell medium	+	Local agents
Fibroblastic cell medium	+	Local agents

[a] Epithelial cells.
[b] Myoepithelial and stromal cells.
[c] Greater effect on stromal cells.
[d] In sparse cultures.

2) [55, 56, 133–36]. The agents that stimulate DNA synthesis in such cultures can be classified broadly into three sometimes overlapping types. The type I agents can stimulate DNA synthesis by themselves. The type II agents stimulate DNA synthesis only in the presence of the type I agents. The type III agents are very impure substances released by the cultured cells themselves or are found in serum [58, 137]. The type I agents correspond largely to the growth factors, and the type II agents largely to conventional hormones (table 2). This classification is analogous to the one for cultured fibroblasts [138, 139]. Additional agents, such as transferrin, are sometimes required to enable the cells to pass through the rest of the cell cycle [140].

The type I agents consist of polypeptides, e.g., Epidermal growth factor (EGF) (56, 141–143], insulin-like growth factors [144], and pituitary-derived factors [58, 136, 137, 145], as well as the prostaglandins (PGs), e.g., PGE_2 and $PGF_{2\alpha}$ (table 2) [58, 137]. The pituitary activity is separable into several components, one of which is phosphoethanolamine [146], but none of these components corresponds to prolactin or growth hormone [147–149]. The activity for promoting the growth of the fast-sticking stromal cells is separable from the activity for promoting the growth of the slower-sticking parenchymal cell fractions [58, 137]. The growth-promoting activity associated with supra-physiological concentrations of growth hormone, luteinizing hormone, and follicle stimulating hormone is due to a contaminant growth factor in these preparations [150].

The type II agents are usually conventional hormones (table 2). Some, like insulin, synergize with all the growth factors tested, while others, like the glucocorticoids and progesterone, synergize with only the pituitary growth factors. Some, like thyroid hormone, have differential effects de-

pending on the identity of the growth factor [58, 137]. In sparse cultures of primary rat mammary epithelium designed to limit the accumulation of potential growth factors released from the cells, estrogens alone have little or no growth-promoting effects (table 2) [56, 58, 137]. However, at higher cell densities, estrogens are believed to exert their growth-promoting effects, at least in certain human breast cancer cell lines, by stimulating the release of self-acting growth factors into the culture medium. This model is discussed at length elsewhere in this volume.

The very impure type III agents are found in serum and are also released into the medium from primary cultures and cell lines of the myoepithelial and stromal cells [58, 137]. These activities probably arise from different substances, since at saturating concentrations their individual effects on cell proliferation are additive [PS Rudland, unpublished]. The observations that primary cultures of rat and human epithelial cells and the early stages of the development of the cuboidal epithelial cell lines show an absolute requirement for the presence of myoepithelial-like cells [60–62, 70], whereas transplanted normal and hyperplastic rodent mammary tissues have an absolute requirement for fat pads for growth in vivo [129, 130], suggests that the myoepithelial and stromal cells of the mammary gland may also produce stimulatory factors in vivo. Moreover, the growth-promoting activity in serum is probably related to that in the pituitary, since serum from hypophysectomized rats loses much of its growth-promoting activity, which cannot be restored by the addition of type II agents [58, 137]. Thus growth-promoting agents produced by the pituitary gland, by the myoepithelial cells, and by the fatty stromal cells may have a physiological role in the control of mammary gland growth in vivo.

Local growth factors

Isolation of PGE_2 from cultured preadipocytes

Conditioned medium from primary cultures of stromal cells or rat fibroblastic cell lines, e.g., Rama 27, is mitogenic for normal and benign neoplastic rat epithelial cells and cell lines [58, 137]. Radioimmunoassay and thin layer chromatography of the culture medium has suggested that this activity is due to PGE_2 [151]. This result has been confirmed by the use of drugs that inhibit the synthesis of prostaglandins (indomethacin and flurbiprofen) and essentially abolish the production of growth-stimulating activity by the rat stromal cells. Similarly, prostaglandin receptor antagonists abrogate the growth-promoting effects of the conditioned medium from the stromal cells. Both classes of inhibitors scarcely affect the mitogenic activity of conditioned medium from the rat myoepithelial-like cell line Rama 29, suggesting that this activity is not due to PGE_2 or a related molecule [151].

The amount of PGE_2 secreted by rat stromal cells correlates with their

ability to differentiate to lipocytes. Thus fast-sticking primary fibroblastic cells (see above) that have been cultured for a relatively long period of time or similar cells from benign rat mammary tumors, both of which have lost their ability to differentiate to lipocytes, produce considerably less growth-stimulatory activity and PGE_2. Upon differentiation of the primary rat fibroblastic cells to lipocytes, the mitogenic activity and amount of PGE_2 secreted into the medium rise markedly, and these processes are accelerated by growth hormone [151]. The mitogenic effects of PGE_2 are enhanced with insulin, EGF, transferrin, and hydrocortisone. PGE_2 will stimulate the growth of epithelial cells from normal (Rama 704), benign (Rama 25), or weakly malignant (Rama 600) mammary glands. It has virtually no effect on most stromal or myoepithelial-like rat cell lines, with the notable exception of Rama 27 and Rama 29 cells, where in combination with insulin and hydrocortisone it can stimulate the cells to grow [151]. PGE_2 can also stimulate the growth of a mouse mammary epithelial cell line, and once again its mitogenic effect is potentiated by EGF, insulin, and also by eicosatetranoic acids [152].

Isolation of TGF–α from cultured myoepithelial-like cells

Although the growth-promoting activity for the rat epithelial cells and cell lines released by the myoepithelial-like cell lines is additive with that in serum and with that in the culture medium of stromal cells (see above), its effect is completely masked by saturating concentrations of EGF [PS Rudland, unpublished]. This result suggests that the trophic activity is EGF or a related molecule. Chromatography of this growth-promoting activity on reversed-phase HPLC columns shows that it comigrates roughly with mouse and human EGF [153]. However, purification or rat submaxillary gland EGF to homogeneity [154] shows that the rat EGF migrates ahead of this growth-promoting activity due to the truncation of the C terminus of the rat molecule and the consequent loss of the strongly hydrophobic tryptophan residues (figure 3) [155]. The growth-promoting activity of the conditioned medium, however, migrates exactly with rat Transforming growth factor–α (TGF–α) (figure 4) [153]. TGF–α is a member of the EGF

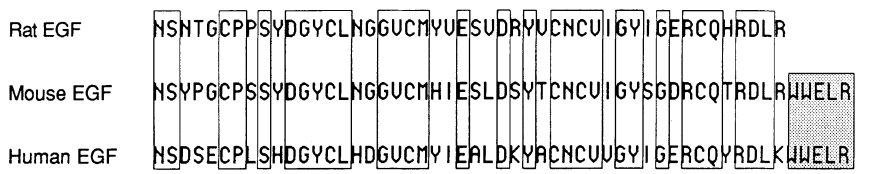

Figure 3. Amino acid sequence of rat EGF, mouse EGF, and human EGF. The alignment shows the truncation of the rat submaxillary gland EGF C terminus relative to that of the mouse and the human EGF (grey box). Amino acid residues are shown by the single letter code and conserved residues are boxed.

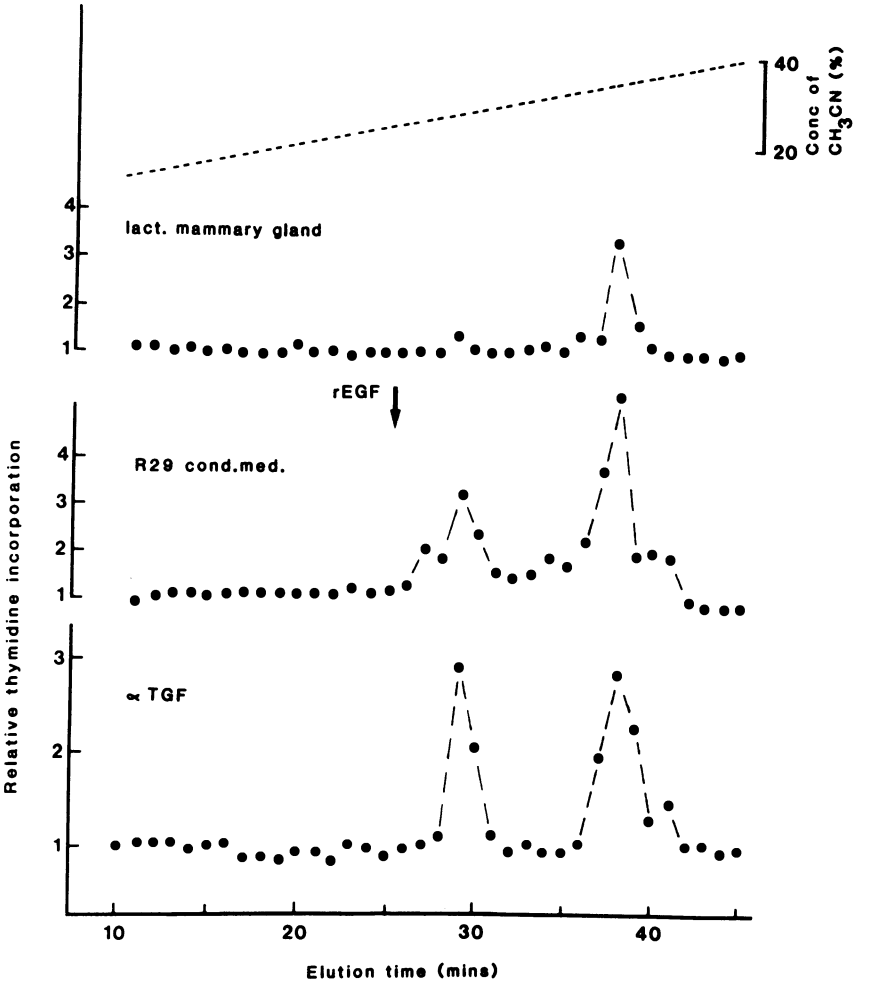

Figure 4. Chromatographic comparison of growth-promoting activity from medium conditioned by Rama 29 cells and αTGF. Samples of authentic rat αTGF (lower trace) and of growth-promoting activity from medium conditioned by Rama 29 myoepithelial-like cells (R29 cond. med) (middle trace) or from extract of lactating rat mammary gland (upper trace) were applied to a Spherisorb ODS 2 HPLC column and eluted using a gradient of acetonitrile as indicated by the dashed line. The amounts loaded were 1 μg αTGF, and the collected activity of 500 ml of conditioned medium or 6 g tissue; 2 μl, 20 μl, and 20 μl samples, respectively, were used for assay. The assay used was the stimulation of incorporation of [^{3}H]–thymidine into DNA of Rama 27 fibroblasts. The position at which rat EGF (rEGF) elutes is shown by the arrow.

family of growth factors and oncogenes [156]. It binds to the EGF receptor with a similar affinity to that of EGF and appears to exert its biological action through this receptor [157, 158]. Further confirmation of the presence of TGF–α is the finding of its mRNA in the rat myoepithelial-like cell lines and the ability of the chromatographed material to compete with EGF for

binding to the same receptor in cultured cells [153]. The TGF–α is secreted by myoepithelial-like cell lines from both benign mammary tumors (Rama 29) and normal (Rama 401) rat mammary glands. Epithelial cell lines, such as Rama 25, however, produce much smaller amounts of TGF–α [153].

The rat TGF–α [153], like EGF [150], is not specific for a particular cell type; it stimulates the growth of fibroblastic, myoepithelial-like and epithelial cell lines from normal mammary glands and from benign tumors. The amount of TGF–α secreted by the myoepithelial-like cell lines is sufficient to stimulate their own growth in culture. Thus differentiation of the epithelial cells along the myoepithelial-like pathway in vitro increases TGF–α to sufficient concentrations that it can act both in an autocrine manner in stimulating the growth of the myoepithelial-like cells and in a paracrine manner in stimulating the growth of the parental epithelial cells. Whether it performs either or both functions in vivo is uncertain. However, TGF–α has been identified in mammary glands from virgin rats [159], and its concentration increases sixfold in fully differentiated mammary glands from lactating animals [153]. Indeed, studies with slow-release implants in the mammary gland suggest that although EGF can stimulate lobuloalveolar development in the mammary gland of subadult animals, TGF–α is five times more potent than EGF and is effective even in the absence of added steroids [160, 161]. These observations taken together suggest that TGF–α has a role in the normal growth and development of the mammary gland, and that its secretion by myoepithelial-like cell lines is not a simple artefact of tissue culture.

Identification of receptors for EGF-like molecules in cultured cells

All our rat mammary cell lines possess high-affinity receptors for EGF with K_ds ranging from 0.4nM to 1.3 nM [153, 162]. Furthermore, epithelial and myoepithelial-like cell lines possess about 22,000 high-affinity receptors per cell (figure 5) with identical K_ds [162]. Thus there is no alteration in the EGF receptor when cells differentiate along the myoepithelial-like pathway in vitro (figure 2), further suggesting that locally produced TGF–α may stimulate the growth of both epithelial and myoepithelial cell types, including their cellular intermediates. These results also suggest that all mammary gland cell types in vivo possess cell-surface high-affinity receptors for EGF, in accordance with direct-binding studies in mice [163]. In contrast, the SV40-transformed human mammary epithelial and myoepithelial-like cell lines (table 1) express much greater numbers (10^5 to 10^6 per cell) of high-affinity receptors for EGF [DG Fernig, unpublished] than do either their rat counterparts above or human mammary cell lines not transformed by SV40. A fourfold increase in cell-surface EGF receptor upon SV40 transformation has also been observed in a separate human mammary system [164]. These results suggest that one of the mechanisms of transformation by SV40 may involve increasing the number of cell-surface receptors for EGF such that terminal differentiation (keratinization) to squamous forma-

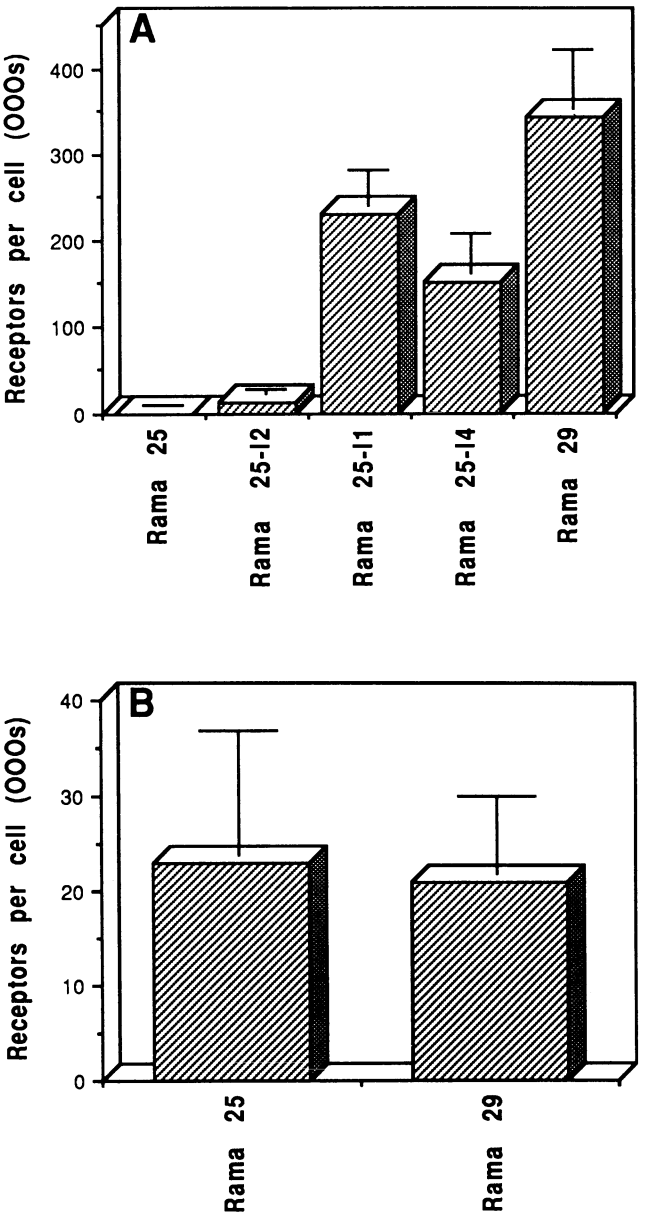

Figure 5. Quantitation of bFGF and EGF receptors on rat mammary cells. A. Numbers of bFGF receptors on epithelial Rama 25, on myoepithelial-like Rama 29 cells, and on intermediate cell lines that represent stable stages in the differentiation along the myoepithelial-like pathway in vitro (figure 2). B. Numbers of EGF receptors on epithelial Rama 25 and on myoepithelial-like Rama 29 cells.

tions in primary cultures of human mammary epithelium, the probable cause of their senescence [68], is largely suppressed [119].

Pituitary growth factors

Isolation and activity of FGFs in vitro

Purification over 10^7-fold to homogeneity of bovine pituitary extracts that stimulate the growth of the rat fibroblastic cell lines, e.g., Rama 27, has identified the active agent as fibroblast growth factor (FGF) [165, 166, 167]. This agent is also found in brain, and early studies of its effects were hampered by the impurity of many preparations (figure 6) [165]. The bovine brain and pituitary contain both the so-called acidic FGF (aFGF) and basic FGF (bFGF) molecules [168, 169] in the ratio 15 to 1 and 1 to 2, respectivity [JA Smith, unpublished]. Preliminary amino acid sequence analysis of both bovine pituitary FGFs isolated under nondegrading conditions suggests that, unlike the molecules originally sequenced [170], they possess extra residues at the amino-termini, which are also both acetylated [JA Smith, unpublished], in agreement with recent reports on brain FGFs [171]. The presence of extra residues is further confirmed from gene sequences [172]. The variety of FGF molecules isolated in these tissues is such that the acidic and basic components are not easily separable on ion exchange or heparin-affinity chromatography.

The most active species, bFGF, stimulates the growth of not only the rat fibroblasts [56] and their cell lines but also of the rat myoepithelial-like cell lines [150]. The bFGF fails to stimulate the growth of the parental rat epithelial cells [56] and cell lines [150]. These results are consistent for cell lines isolated from normal rat mammary glands and also from benign rat mammary tumors [150]. The aFGF also stimulates the growth of the myoepithelial-like and fibroblastic cell lines, but about ten-times more material than bFGF is required for maximal stimulation; the epithelial cell lines fail to be stimulated [JA Smith, unpublished]. In the rat cell lines intermediate between the epithelial and myoepithelial-like cells (table 1), the maximum stimulation of growth by bFGF is also intermediate, increasing in the same order as their stage in differentiation to myoepithelial-like cells in vitro [162]. Thus differentiation along the myoepithelial-like pathway (see above and figure 2) is associated with the acquisition of responsiveness to the growth-promoting effects of pituitary FGF.

Differentiation to myoepithelial-like cells in vitro is accompanied by the appearance of receptors for bFGF-like molecules

Although the physiological role of FGF in the development of the mammary gland is unknown, the rat mammary fibroblastic (Rama 27) and myoepithelial-

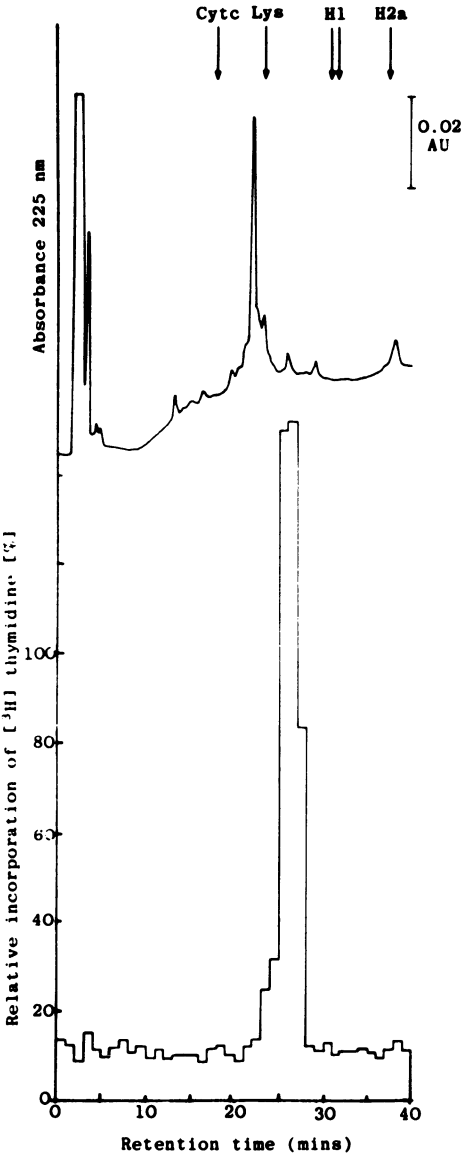

Figure 6. Ion exchange HPLC of a commercial preparation of pituitary FGF. A 10 μg sample was loaded onto an Altex spherogel TSK IEX 535 CM column and chromatographed at a flow rate of 1 ml/min with a gradient of 25 mM/min NaCI in 0.1 M sodium phosphate pH 6.0. 1 ml fractions were collected and 10 μl were used for assay as in figure 4. The growth-promoting activity elutes after the bulk of the protein in the sample. Standard proteins used were cytochrome C, lysoszyme, rat thymus histone H1 (two peaks), and rat thymus histone H2a.

like cell lines (Rama 29 and Rama 401) (table 1) possess high-affinity cell-surface receptors for bFGF, with K_ds ranging from 30 pM to 280 pM. In contrast, the parental cuboidal rat epithelial cell lines (Rama 25, Rama 37CL–A3, and Rama 704 [table 1]) do not express cell surface bFGF receptors [162, DG Fernig, unpublished]. Since this result is consistent for cell lines isolated from both normal rat mammary glands and benign rat mammary tumors, the observed pattern of binding may be applicable to rat mammary cells in general. Two complexes of Mr 180KDa and 160 KDa are observed when [^{125}I]–bFGF is specifically affinity cross-linked to its receptor on the fibroblastic or the myoepithelial-like cell lines [162]. The relationship between these two forms of the bFGF receptor is unknown, but similar forms have been identified in other cell systems [173]. Consistent with the binding experiments, [^{125}I]–bFGF fails to affinity cross-link specifically to receptors on the epithelial cell line Rama 25 [162]. Thus the inability of bFGF to stimulate the growth of the epithelial cell lines is probably due to the absence of its high-affinity receptors rather than to a defect at the level of intracellular signalling.

In the rat cell lines intermediate in character between the epithelial and myoepithelial-like cells (table 1, figure 2), it appears that bFGF receptors are first expressed at the cell surface at an early step in the differentiation pathway of Rama 25 to a myoepithelial-like cell, and this step precedes the stage represented by Rama 25–I2 cells (figure 5). Thereafter, the number of receptors for bFGF increases up to a maximum value for the myoepithelial-like cells (figure 5) [162]. The bFGF stimulates the growth of all cell lines that express a detectable number of bFGF receptors at their cell surface. However, bFGF is unable to stimulate growth maximally in those cell lines (Rama 25–I2, Rama 37–E8) that express less than 50,000 bFGF receptors per cell. In contrast, the cell lines expressing more than 100,000 receptors per cell are all maximally stimulated by bFGF. Maximal stimulation is observed at 0.3–1 ng/ml bFGF, a concentration that corresponds to the K_d of the high-affinity site [162]. In those cell lines that have a detectable high-affinity binding site for bFGF, there is also evidence for a low-affinity site, but its K_d of 100 nM precludes its direct involvement in the delivery of the growth response of bFGF [162]. Thus epithelial cells that progress along the myoepithelial-like differentiation pathway in vitro express higher levels of high-affinity bFGF receptors (figure 5). In contrast, the number of cell-surface EGF receptors is unaltered along this pathway (figure 5, see above). These observations suggest that the appearance of the high-affinity receptor for bFGF may be a characteristic of the differentiation pathway of myoepithelial cells in vivo. Thus it is possible that bFGF, or a related molecule, may play a significant role in selectively stimulating the growth of the myoepithelial component of the mammary parenchyma during development of the mammary gland in vivo. The source of the FGF-like molecule may be systemic from the pituitary, or it may be produced constitutively by cells within the mammary gland in an analogous fasion to the FGF-like molecule

int–2 [174], which induces relatively benign tumors in mice infected with Mouse Mammary Tumor Virus [175].

Identification and activity of PMGF in vitro

The activity for promoting the growth of the rat epithelial cells and cell lines can be separated from FGF [137] and has been purified about 300-fold from an initial extract from bovine pituitaries. At this stage it shows maximum stimulation at about 1 μg/ml, which suggests that a further 10^3-fold to 10^4-fold purification is still required to yield a homogeneous preparation. Even at this stage, it is separable from bovine prolactin, growth hormone, and the other pituitary hormones [150]. Although only partially purified, this material stimulates the growth of the rat epithelial cell lines (Rama 25, Rama 704) but not their derivative myoepithelial-like cell lines (Rama 29) or the rat fibroblastic cell line (Rama 27) [150]. Thus differentiation of the cuboidal epithelial cell lines to myoepithelial-like cells in vitro causes a loss in the ability to respond to the growth-promoting effects of Pituitary derived mammary growth factor (PMGF), and a similar loss probably also occurs when the same rat epithelial lines differentiate to alveolar-like cells in vitro [137].

Although FGF does not yet have a clearly identifiable physiological role in the development of the mammary gland, PMGF has been implicated in this process. Thus the activity of PMGF from pituitaries of early lactating or perphenazine-treated rats [134] when the mammary glands are growing rapidly is four-times to ten-times greater than the activity found in untreated virgin females (table 3) [150]. Pure rat or bovine prolactin fails to stimulate the growth of the rat epithelial cell lines (table 2) [150]. On the other hand, in combination with other hormones, bovine or rat prolactin stimulates the production of casein-secreting, alveolar-like cells in confluent cultures (see above), but PMGF is without such effect [PS Rudland and MJ Warburton, unpublished]. Thus although prolactin and the growth factor PMGF may be under similar hypothalamic control in the pituitary, prolactin acts on the epithelial cells of the mammary gland to induce processes of differentiation,

Table 3. Pituitary growth-promoting activity and physiological state.

Physiological state of rat	Relative growth-promoting activity on cell lines	
	Epithelial[a]	Fibroblastic[b]
Male	0.5	—
Female	1	1
Perphenazine-treated[c] female	4	0.25
Lactating female	10	—

[a] Activity separated from other pituitary hormones including prolactin, equivalent to PMGF.
[b] Largely due to FGF.
[c] Stimulates breast growth in vivo.

whereas other components, such as PMGF, may serve to promote growth of the glandular epithelial cells.

Effects of growth factors and hormones on malignant, metastasizing cells

Cell lines isolated from highly-malignant metastasizing rat (Rama 800, Rama 900) and human (Ca2–83) mammary carcinomas, on the whole, grow much slower than do epithelial cells from their benign counterparts (see above). They can be stimulated to grow in culture by those growth factors that are produced locally by cells isolated from the mammary gland, namely PGE_2 and TGF–α as well as by submaxillary EGF [98, 100, 108]. Indeed, such cells are very much more dependent for their isolation on cocultivation with feeder cells producing these growth factors. In the case of the moderately metastatic rat cell lines (Rama 800s) that spread mainly to the lungs and lymph nodes [100], their growth rate is also stimulated substantially when cocultivated with fragments of either of these tissues [176], suggesting that the growth environment of these tissues may, in some way, compensate for that found within the environs of the mammary gland [177, 178]. Moreover, the highly malignant metastasizing rat mammary cell line Rama 900 (table 1), which disseminates predominately via the lymphatics like the human disease, was isolated and can still grow as an ascites in the pleural cavity of syngeneic rats. This cell line requires cocultivation with a second non-neoplastic cell type found in the ascitic fluid, the mesothelial cell, both for growth in culture and for widespread dissemination from subcutaneous sites. The growth-promoting agents released by the mesothelial cells are probably different from PGE_2 or EGF-related molecules. Variants that no longer require the mesothelial feeder cells in culture for growth also no longer require them for widespread dissemination from subcutaneous sites in rats [98]. Thus failure of the malignant cells to differentiate to myoepithelial cells and their movement away from the fatty stromal cells has probably caused them to adapt to the growth effectors produced in their immediate vicinity. Therefore, carcinoma cells isolated at different stages in malignancy may require different growth factors to support their growth.

Unlike the growth factors produced by neighboring cells, PMGF fails to stimulate appreciably highly malignant metastatic carcinoma cells of rats (Rama 800, Rama 900) or of humans (Ca2–83). This is consistent with results in vivo, whereby hypophysectomy of rats or humans bearing such advanced tumors fails largely to cause their regression [38, 179]. In contrast, benign tumors of rats and humans largely regress after using antipituitary agents [180–182], and such epithelial cells are still responsive to the growth-promoting effects of PMGF in culture [150, PS Rudland, unpublished]. Thus in addition to its failure to differentiate, the highly malignant metastasizing mammary carcinoma cell is no longer under control of agents released from the pituitary gland. One of the simplest interpretations is that the malignant

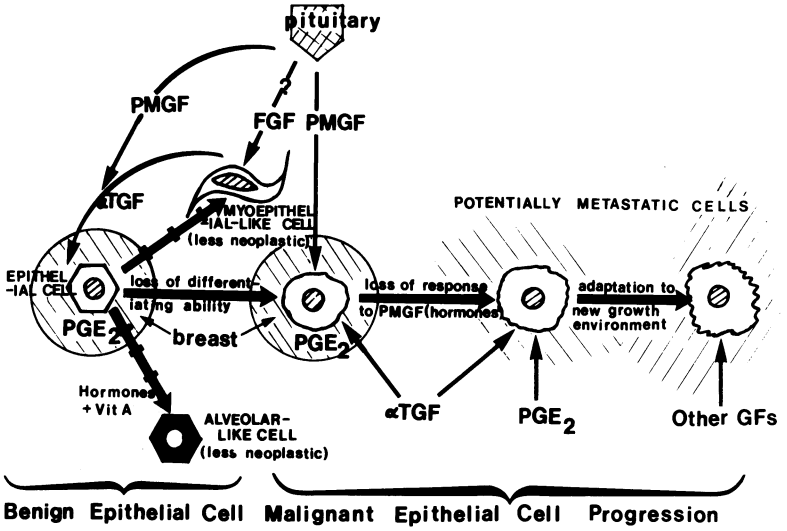

Figure 7. Schematic representation of some of the possible changes in malignant progression of breast carcinoma. The benign breast lesion contains neoplastic epithelial stem cells that can differentiate to myoepithelial-like or alveolar-like cells (barbed arrows). Growth of these epithelial cells is controlled by systemic and local growth factors (thin arrows). The former includes PMGF and the latter include PGE_2 and αTGF. The myoepithelial-like cells are stimulated to grow by FGF, possibly released from the pituitary. The emergence of a malignant cell occurs in stages (thick arrows) including the following: loss of its differentiating ability, loss of response to PMGF, and adaptation to a new growth environment. Each stage requires a corresponding change in the utilization of growth factors: a new source other than myoepithelial cells for αTGF, a new source other than fatty stromal cells for PGE_2, growth-independence from PMGF, and adaptation to utilize other growth factors (GFs). Hormones of pregnancy and vitamin A-related agents may only protect against the neoplastic process by intervening at an early, relatively benign stage before the epithelial cell has lost its capacity to differentiate.

cells are refractory to the growth-promoting effects of PMGF. However, the loss of differentiating ability and failure to respond to pituitary-related growth factor(s) are changes that may not occur simultaneously in the malignant cell, since weakly metastasizing rat cell lines are responsive to PMGF in culture [150], and about a quarter of all human breast cancers, when first diagnosed, are responsive to endocrine (including pituitary-ablative) therapy [179]. The human tumors that are responsive to endocrine therapy, however, invariably recur in a hormone-unresponsive form at a later stage of the disease [179]. Thus initiation of the loss of the differentiating ability of the neoplastic epithelial cell may occur at an earlier stage in the malignant process than does the loss of response to pituitary (possibly PMGF) control of cell growth.

If the above model (figure 7) has some validity, then those agents that induce differentiation of the epithelial cells to alveolar cells, e.g., mammatrophic hormones, pregnancy, retinoids [121–123], have to act at an early, premalignant stage of the disease to ensure that the neoplastic cell can be

switched to an alveolar cell with reduced tumorigenic potential. The same differentiation-inducing agents would be largely ineffective in causing regression of full-blown malignant breast cancer [124, 125]. Thus the interplay of both local and putative systemic growth and differentiation factors on a potentially neoplastic mammary epithelial cell with various possibilities of differentiation could lead to different outcomes, depending on the stage of the disease.

Summary

The different mammary cell lines described herein appear to be representative of the cell types found in both normal glands and benign tumors of rats and humans. The epithelial cell lines can differentiate to both alveolar-like and myoepithelial-like cells in culture. The epithelial cell lines and particularly those cell lines representing intermediate stages in the myoepithelial differentiation pathway are candidates for the epithelial stem cells found in rat and possibly in human terminal ductal structures. The systemic mammatrophic hormones that are thought to control the growth of the mammary gland in vivo have little or no stimulatory effect alone on the growth of normal and neoplastic rat mammary cells in culture. The pituitary growth factors (fibroblast growth factor [FGF] and pituitary-derived mammary growth factor [PMGF],) and the growth factors released from the different cell lines, (stromal prostaglandin E_2 [PGE_2] and myoepithelial transforming growth factor alpha [TGF–α]) are much more potent mitogenic agents for the mammary cell lines. The ability of FGF and epidermal growth factor (EGF) -related molecules to simulate growth of the different mammary cell types in culture correlates with the presence of their high-affinity receptors. Thus these growth factors are promising candidates for some of the primary effectors of mammary growth in vivo. Malignant mammary epithelial cells have a greatly reduced rate of growth compared to their normal and benign counterparts. They also fail to differentiate or to respond to PMGF but can still respond to PGE_2 and TGF–α. In addition, highly malignant variants appear capable of adapting to a new growth environment in vivo. This suggests that simple molecular explanations based solely on the autostimulation of cell growth may not be sufficient to explain some of the properties of the slowly growing, highly malignant cells.

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4. Local effects of growth factors

Charles W. Daniel, and Gary B. Silberstein

Background: The problem with growth factors

Regulation of mammary gland growth and morphogenesis provides a striking example of the interplay of systemic 'classical' hormones with more recently discovered growth factors. Both are probably required for mammary morphogenesis and functional differentiation; an understanding of both is critical to understanding the problem of mammary cancer.

The identification, characterization, and action of the systemic mammogens has been a subject of intense study by generations of endocrinologists. In making comparisons with growth factors, it is interesting to note that the starting point for endocrine studies has been the physiological effect of a particular hormone usually discovered either as a result of endocrine ablation surgery or as a consequence of pathological processes at the level of the endocrine gland or the target organ. Thus the physiological significance of the work was never in doubt, since it was based from the beginning upon in vivo phenomena. Endocrinologists could use the well-established technique of ablation surgery to recognize the source of secretion, then purify the hormone through bioassays based upon replacement therapy. Biochemical and molecular tools could be brought to bear on the question of hormone action, often using in vitro techniques as a means of simplifying and manipulating complex physiological processes.

Our knowledge of growth factors, gained mostly during the past thirty years, has been developed, as it were, by starting at the opposite end. Growth factors were discovered not in animals but in cultured cells or in cell products appearing in conditioned medium. Assays were based upon growth factor effects on other cultured cells. In many cases the cells used were established lines that had lost the differentiated phenotype of their tissue of origin, having been immortalized and transformed to varying degrees. The ability of these growth factors to influence proliferation and differentiation of cells in vitro brought them to the attention of investigators interested in cell regulation, especially the cancer research community. In contrast to studies of endocrine phenomena, however, growth factor research has been faced with the task of returning to the animal to determine the physiological role, if any, of these powerful and fascinating peptides.

M. Lippman and R. Dickson (eds.), REGULATORY MECHANISMS IN BREAST CANCER.

The search for physiological roles for growth factors has been complicated by the observation that in most cases their production appears to be widespread in the body, making the technique of surgical endocrine ablation unusable. Target tissues, defined by the presence of high-affinity receptors or by growth factor effects when placed in vitro were often widely distributed as well. This has led to the assumption that growth factors, with some exceptions [1], probably operate on an autocrine or paracrine basis. That is, their effects are local, involving cell- or tissue-level regulation. This exciting concept now profoundly influences our understanding of normal and neoplastic tissue processes. However, tools other than those used in traditional endocrinology must be developed to explore growth factor effects in situ, to establish physiological roles, and to probe mechanisms of action.

Slow-release implants

Injection of growth factors or other biological molecules inevitably leads to systemic effects that may mask their local, primary action. Results obtained by systemic administration become difficult to interpret and have been of limited value in growth factor research. In contrast, in vitro techniques provide a manageable system for directly exposing cells or tissues to materials of interest, and they permit direct analysis of cell products and secretions. Consequently, in vitro techniques have provided the backbone of growth factor research.

Conceptually, a method for the sustained, localized delivery of growth factors, or of other bioactive materials whose effects may bear on the action of growth factors, can provide a method for testing direct effects of such molecules in a physiological environment without the necessarily artificial conditions of tissue culture.

To take a single example, local delivery of epidermal growth factor (EGF) to the mitotically inactive mammary gland of ovariectomized mice results in restoration of growth and reappearance of the mitotically active end buds (figure 1) [2, 3]. The interpretation of this result is an indication of the power of the implant technique. Noting that the EGF effect is limited to the area of tissue immediately surrounding the implant, leaving unaffected other regions of the same gland as well as other glands within the same animal, leads to the conclusion that the local morphogenetic effect is a direct action of implanted EGF. This result does not indicate the precise cellular target, since the gland contains both epithelial and stromal elements. Nor is it possible to completely exclude systemic contributions, since some amount of EGF is undoubtedly taken into the circulation. Nevertheless, the conclusion that EGF is capable of directly stimulating mammary ductal morphogenesis appears indisputable.

Highly localized, sustained delivery can be provided by certain plastics that are capable of releasing bioactive materials without chemical alteration

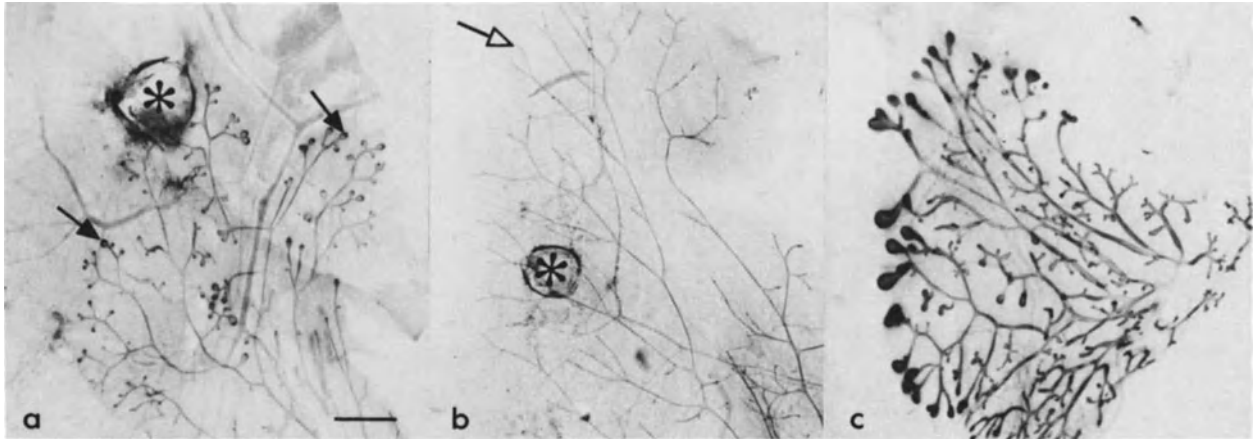

Figure 1. Local effects of epidermal growth factor on mammary ductal growth and morphogenesis in ovariectomized mice. (a) EVAc implant containing 2 μg of EGF in BSA carrier was placed in the right number 3 thoracic gland of an ovariectomized animal for 4 days. The treated gland displays end buds (arrows) and increased ductal diameter when compared to (b) gland from the contralateral side of the same animal, treated with an implant containing only carrier protein (BSA). (c) Number 3 mammary gland from a 5-week-old hormonally intact female. Note large end buds indicating vigorous growth. Bar = 2 mm; * indicates EVAc implant. [From Coleman et al., 2.]

or denaturation. The most thoroughly investigated slow-release material is EVAc (ethylene vinyl acetate copolymer; Dupont Chemical Co., Universal City, CA). The physical characteristics of EVAc and the kinetics of release of various molecular species by it have been described in some detail [4, 5]. In adapting the EVAc implant technique to the mammary gland [6], consideration was given to the following points.

Bioactivity of released substances

The release of a wide variety of both organic- and water-soluble molecules in an unaltered, bioactive form has been reported [5, 7]. In mammary studies, release of bioactive enzymes [6], steroid hormones [6, 8], peptide hormones and growth factors [9], cAMP-active agents (2), and antibodies (GB Silberstein, unpublished) have been documented.

Lack of inflammatory response

EVAc is remarkably biocompatible with mammary tissues and does not produce a foreign body response, as indicated by the lack of histologically observable encapsulation by fibrotic tissue [6, 10]. In connective tissues immediately surrounding mammary EVAc implants, no increases in levels of type I collagen mRNA or in the synthesis of sulfated glycosaminoglycan synthesis were observed [10].

Intrinsic bioactivity

EVAc alone, or when containing inert carrier proteins such as serum albumin, has not been reported to display mammotrophic activity, and there has been no indication of identifiable biological effects associated with the polymer. Other materials used for implant construction may be less inert, such as cholesterol [3], which may be expected to alter the physical properties of cell membranes in neighboring tissues, perhaps affecting membrane transport or receptor-mediated activities.

Release rate

Organic-soluble materials, such as steroid hormones, dissolve directly in the EVAc solvent and are gradually released from the copolymer in a nearly linear manner [8] (figure 2). Peptides and other water-soluble molecules are released in a burst during the first few hours followed by a longer period of more gradual release [2, 11, 12] (figure 2). In all cases the release rate is a function of both loading and of implant size, with larger implants providing more sustained release [7]. In the case of peptides, the amount of carrier protein incorporated into the implant also influences release kinetics by modifying the porosity of the EVAc matrix.

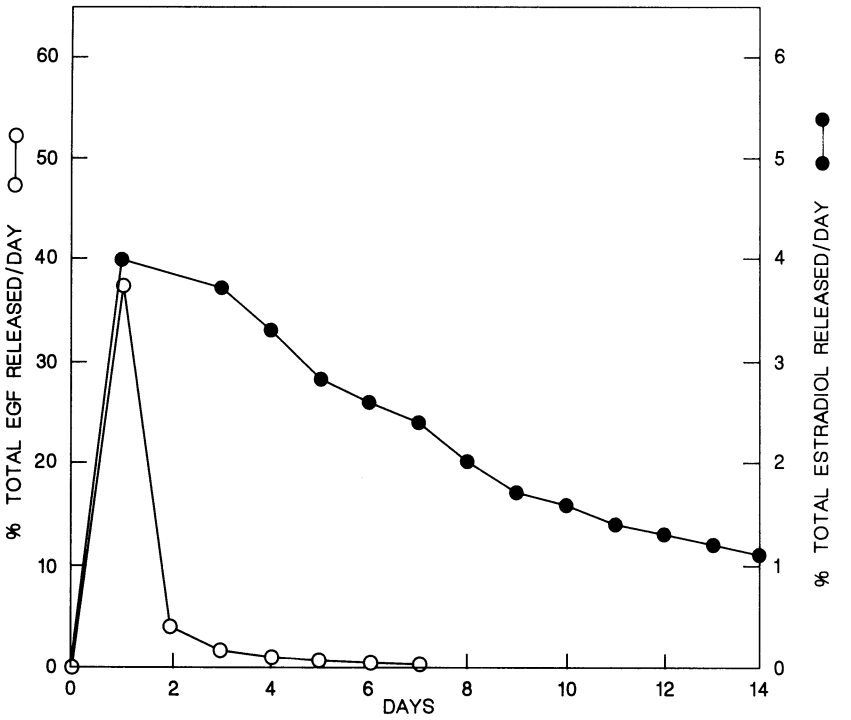

Figure 2. Comparison of the release kinetics of a peptide (EGF) and a steroid (estradiol) from EVAc. In each case radiolabeled ligand was incorporated into the plastic along with a standard dosage of the unlabeled molecule. Implant-sized pieces of EVAc (approximately 0.5–1 mg in weight) were cut, placed in nylon bags and incubated in saline for 24 hours, removed to fresh saline, and the previous volume was counted for released radioactivity. For estradiol, the percentage of the total counts released after 14 days was 33%; the percent of total EGF released after 7 days was 46.5% [2, 8].

The question of dosage

Several reports have demonstrated the usefulness of EVAc implants in carrying out dose-response studies [2, 8, 11, 12]. The effects of various dosages can be interpreted only in relative terms, however, since the levels of growth factors delivered to the tissues at any particular point in time are not known. Indeed, the level within the implanted tissue is not uniform, since the concentration of released material is highest close to the implant and decreases with diffusion distance, the diffusion pattern superficially resembling the dispersion of material in an agar Ochterlony dish (figure 3). The existence of this diffusion gradient may be useful in permitting visualization of graded levels of activity (figure 1).

A frequently overlooked aspect of local delivery systems is that materials are released directly into the interstitial tissue space. In this respect the delivery differs fundamentally from conventional injection where systemic

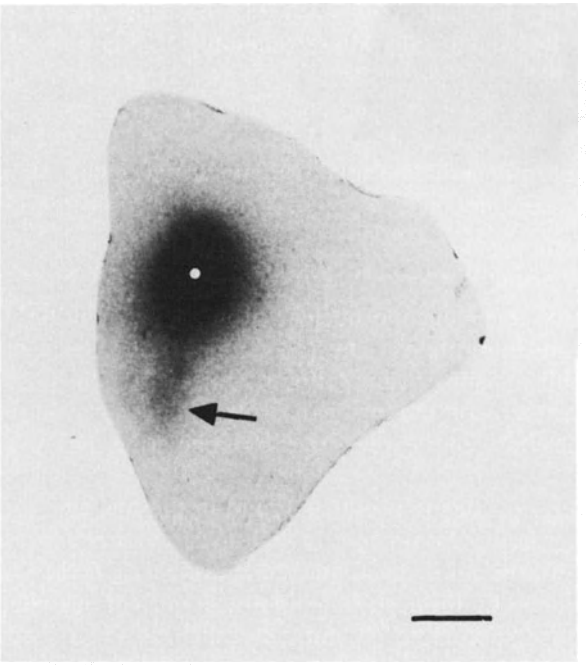

Figure 3. Zone of diffusion and retention of TGF–β1 released from EVAc implant. Implants containing 5×10^4 cpm of iodinated ligand were in place for 5 hours (approximately 30% of ligand released). After removing the pellet, the tissue was placed between sheets of X-ray film and exposed for 7 days. The white spot in the center indicates the implant, and the arrow points to the surgical channel (bar = 0.35 cm). [From Daniel et al., 11.]

distribution is carried out within the vascular compartment and where interstitial concentrations are mediated by capillary permeability. It must also be said that the traditional methods of measuring circulating levels of hormones or growth factors by immunoassay tells little about levels found in the microenvironment of tissue cells. This dilemma is compounded by recent observations that growth factors may bind with high affinity not only to specific membrane receptors but also to proteins of the extracellular matrix [13]. Immobilization mechanisms can raise the local concentrations of bound growth factors, independent of the type of delivery system.

In spite of these considerations, it is important to make whatever comparisons are possible between the minimum effective dosages of growth factors delivered by slow-release implants and the concentrations found effective in culture. Using EGF again as an example, several in vitro studies indicate minimum mitogenic levels to be in the 5–10 ng/ml range [14]. The dose response curve for EGF delivered by EVAc in vivo (figure 4) indicates that 2 μg implants provide a maximum response. Considering that implanted EGF is released by diffusion and that the effective distance from the

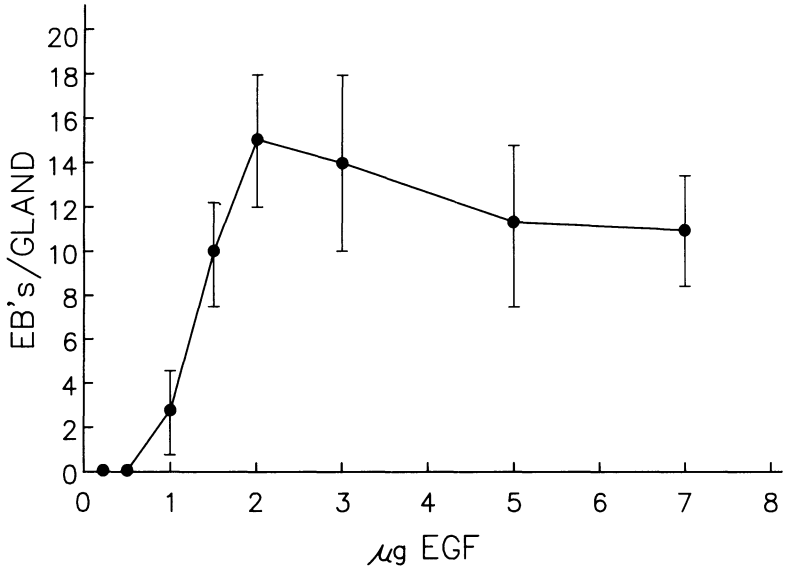

Figure 4. Dose response of mammary ducts to EGF. Glands in ovariectomized animals were treated with EVAc pellets containing 0.25 to 7.0 μg EGF, and the numbers of end buds were counted at 4 days. Bars indicate standard errors.

hormone-containing implant may be several millimeters, EGF is seen to be effective in areas of the gland where diffusion results in local concentrations that are only a small fraction of that observed around the implant. Assuming an effective volume of about 1 ml for the gland, local concentrations in the low ng range would appear to be quite reasonable. Thus the in vivo and in vitro concentrations required for stimulation are not likely to be substantially different.

Direct effects of growth factors and endocrine mammogens

Summary

Slow-release plastic implants have been used to investigate the local effects of a wide variety of growth factors and endocrine hormones on mouse mammary ductal and lobulo-alveolar growth. Focusing on ductal growth stimulation, a survey of these results (table 1) reveals that several different receptor systems, when stimulated, lead to end bud growth and ductal elongation. The mammogenic substances fall into three catagories: steroids (estrogen, DCA); protein factors (EGF, growth hormone, prolactin); and activators of adenyl cyclase (cholera toxin, prostaglandin E2). The existence of these several pathways is evidence that significant redundancy may normally underlie the regulation of ductal growth.

Table 1. Summary of growth effects of implanted hormones and growth factors.

Agent	Effect		Ref
	End buds[1]	Lobules[2]	
Classical mammogens			
17beta–estradiol	+[3]	0	[8]
17alpha–estradiol	0	0	[8]
Progesterone	0	0	
Deoxycorticosterone acetate	+	0	[6]
Growth hormone	+	0	[31]
Prolactin	+	0	[31]
Nontraditional mammogens			
Cyclic AMP agents	+	0	[12]
Epidermal growth factor	+	+	[2, 3]
Transforming growth factor alpha	0	+	[3]
Nonmammogenic agents			
Insulin	0	0	
Basic fibroblast growth factor	0	0	
Platelet-derived growth factor	0	0	
Transforming growth factor beta–1 + EGF in ovx	0	n/a	[28]
Transforming growth factor beta–1 − EGF in 5 weeks	−	0	[11, 28]
Glucocorticoids	−	n/a	
Testosterone	0	n/a	
Bovine serum albumin (control for implants with proteins)	0	0	[31]

[1]To test for stimulation, ovariectomized animals in which the ductal system had completely involuted were implanted on one side only, with the contralateral gland serving as a control. Implants were left in place for up to 7 days, after which glands were stained with hematoxylin and examined in whole-mount. The staining procedure is described elsewhere [12].

[2]To test for lobulo-alveolar differentiation, implants were placed in either 5-week-old or 3-month-old hormonally intact animals. Alveolar growth stimulation appears as "grape cluster" outgrowths on existing ducts [6] and was determined by inspection of stained whole-mount preparations.

[3]Key: + stimulation; −, inhibition; 0, no effect; n/a, not applicable. Note: results for agents not referenced in the table are from Silberstein et al., unpublished observations.

Local stimulation by the pituitary hormones was not unexpected, since Lyon, using cholesterol implants containing growth hormone, had already demonstrated direct stimulation of the rat gland [15], while local stimulation by EGF was predicted by its proliferative effects on mammary cells in vitro. The direct action of estrogen was not predicted and suggests that pathways may exist in which estrogen stimulates release of secondary peptide mammogens possibly of stromal origin [8].

A continuing puzzle concerns the dramatic stimulatory effects of cyclic AMP-active agents that apparently can replace all the hormones and factors required for local growth. At the least, this result strongly implicates the G-protein family of signal-transducing proteins as a nexus for pathways lead-

ing to ductal growth, and in addition, it may indicate the existence of undiscovered mammogens that act through receptor-mediated adenyl cyclase.

EGF and TGF–alpha

Although they represent quite different peptides, EGF and TGF–alpha appear to use a common receptor. Using cholesterol implants, Vonderhaar [3] demonstrated the ability of TGF–alpha to stimulate lobulo-alveolar development in intact, 5-week-old mice. EGF had a similar effect but required that the animals be pretreated with injected estrogen/progesterone. In none of these studies was the effect of growth factors on ductal elongation reported, and the implants appeared to be placed in regions of the gland away from the ductal tips where end buds would normally be found.

Coleman et al. studied the local effects of EGF on mouse mammary ductal elongation by inserting implants directly ahead of ductal tips in glands that had been rendered mitotically inactive by previous ovariectomy [2]. New end buds were formed in a time- and dose-dependent manner and were confined to the zone around the implant (figure 1). No lobulo-alveolar development or hyperplastic growth was observed in numerous experiments. In the same study, competitive binding assays and autoradiography were used to characterize and localize the EGF receptor. The ability of EGF to substitute for estrogen and other mammogenic mitogens, taken together with evidence for localized high and low affinity receptors, indicates that EGF must be considered a strong candidate for a naturally occurring mammary mitogen. The ability of TGF–alpha to reinitiate ductal morphogenesis has not been investigated, and although the distribution of its receptor is assumed to be identical with those for EGF, this has not been rigorously demonstrated and the existence of receptors specific for TGF–alpha cannot be ruled out.

In related but quite different studies, Coleman et al. [32] reported local effects of EGF on vigorously growing gland in intact animals, in contrast to earlier studies on growth-arrested gland. Implanted EGF was strongly growth inhibitory, resulting in loss of identifiable end buds and a decline in epithelial DNA synthetic activity. The effect was again dose and time dependent but with kinetics that suggested a separate mode of action — the inhibitory effects of EGF required sustained exposure for several days, and a single short-term exposure to hormone was ineffective. Receptor-binding studies using ^{125}I–EGF autoradiography indicated a loss of hormone-binding activity in treated gland, suggesting receptor down regulation as the mechanism of inhibition. Inhibitory effects of EGF are not without precedent; EGF has been reported to inhibit tooth development [16, 17] and to down regulate its receptors in several tissues [16, 18].

These specific, fully reversible mammogenic effects suggest an in vivo role for EGF and perhaps for TGF–alpha as well. Although circulating EGF appears to influence mammary development [1] and lactation [3],

an absolute requirement for either of these growth factors has not been demonstrated. Indeed, developmental dependence upon a growth factor has been shown only in the case of Nerve Growth Factor, where in vivo neutralization by specific antibody resulted in abnormal development of sympathetic nerves and ganglia [19]. Slow-release implants would appear to provide opportunity for similar experiments in the mammary gland, using EVAc to release specific antibody to local areas of the gland.

Assuming a role for EGF/TGF–alpha, there remain important questions concerning site and regulation of production. EGF is produced and secreted by the salivary gland [20], and sialoadenectomy has been reported to influence normal [1] and neoplastic [20] development, indicating an endocrine mode of action. However, EGF is also found in several fetal and adult tissues [20], lactating mammary gland [DiAugustine, personal communication], and virgin mammary gland [Coleman, unpublished], suggesting that local paracrine or autocrine action is also feasible. Especially interesting is the possibility that EGF/TGF–alpha action may be influenced by estrogen regulation of growth factor receptors [30]. It would appear that the use of EVAc implants, combined with appropriate studies using molecular probes and immunological reagents, could eventually unravel the hierarchy of hormone and growth-factor activities.

Transforming growth factors–beta

TGF–B1, the most thoroughly investigated of the TGF–B family of peptides, has been shown in vitro to influence, in association with other hormones and growth factors, both proliferation and differentiation of a variety of cell types [22]. Of particular interest are the studies of Knabbe et al. [23] that demonstrate that the hormone-dependent breast cancer cell line MCF–7 both secretes TGF–B1 and is growth-inhibited by it. These studies also show that antiestrogens increase the secretion of TGF–B1, suggesting estrogenic regulation of an autocrine regulatory loop.

In vivo TGF–B1 and its receptors are widely distributed in both embryonic [24] and adult tissues [13]. Although a physiological role for TGF–B1 has not been determined, studies on effects of exogenous hormone in vivo have demonstrated an acceleration of wound healing [25], inhibition of liver regeneration [26], and a fibrotic response when implanted subcutaneously [22, 27].

The mammary gland has a clearly defined set of responses to TGF–B1 when administered in EVAc implants. When implanted directly ahead of advancing end buds, growth was rapidly inhibited, as demonstrated by reduction in end bud numbers and a decline in DNA synthetic activity (figure 5) [28]. After four days of treatment, the inhibited ductal tips had differentiated into blunt ended, quiescent structures and were structurally indistinguishable from ducts that had terminated growth because of normal regulatory processes within the gland. Normality was further indicated by experiments demonstrating that the process was fully reversible.

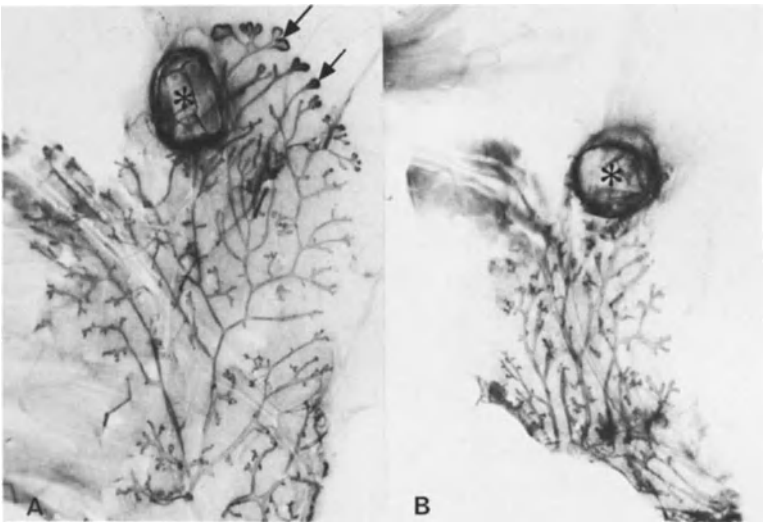

Figure 5. Local effects of TGF–β1 on ductal growth. Endocrine-intact mice were treated for 4 days with EVAc implants containing 150 ng of TGF–β1. (a) Control gland implanted with a BSA-containing implant, displaying prominent end buds and vigorous growth. (b) Treated gland from contralateral side of the same animal shows regression of end buds and inhibition of ductal growth.

In a subsequent series of experiments, Daniel et al. reported the specificity and characteristics of the TGF–B1 response [29]. TGF–B1 action was found to be highly specific for ductal epithelium. Doses of TGF–B1 that were capable of fully inhibiting ductal elongation had no effect on the proliferation of lobuloalveolar structures in pregnant or hormone-treated mice. Inhibition was epithelium-specific, resulting in cessation of DNA synthesis in end buds but not in surrounding stromal cells. A further level of specificity was demonstrated within epithelial tissues in which stem cells leading to bud formation were prevented from entering DNA synthesis, but 'maintenance' DNA synthesis associated with cell replacement was unaffected (figure 6). These observations indicate that TGF–B1 has the potential to suppress lateral branching of ducts and to maintain the open pattern of branching that is the signature of this stage of mammary development. The time course of local end bud inhibition was determined to be rapid, with nearly complete cessation of DNA synthesis occurring by 12 hours [11]. Thus TGF–B1 has the ability to inhibit lateral buds rapidly enough to prevent crossing of ducts, which is rarely observed.

Slow-release implants have also been used to investigate possible mechanisms of tissue regulation by TGF–B1. Using a collagen I antisense riboprobe for in situ hybridization, Silberstein et al. [10] showed that in the presence of TGF–B1, stromal fibroblasts were induced to synthesize collagen mRNA. Collagen transcription was enhanced in the immediate vicinity of affected end buds but not in the stroma between the end buds and the

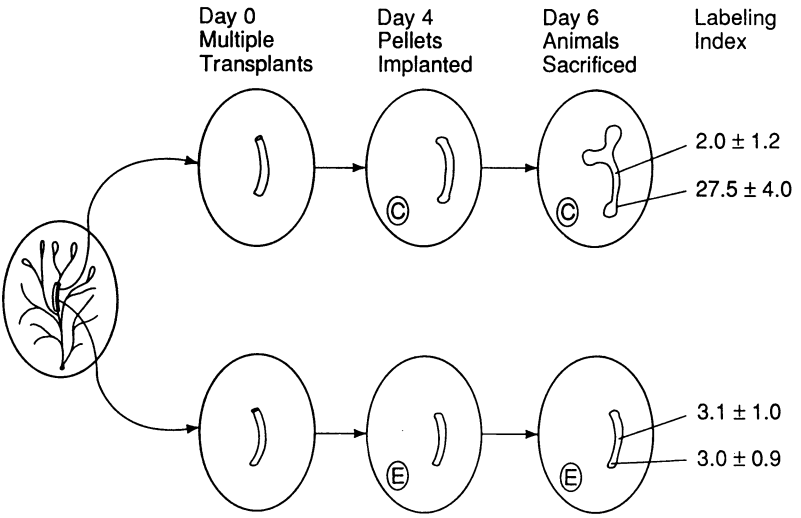

Figure 6. Effects of TGF–β1 on budding in fragments of mature ducts. Duct segments were transplanted into parenchyma-free mammary fat pads at day 0, allowed to establish for 4 days, then treated with 150 ng of TGF–β1 for 2 days. At day 6 animals were sacrificed and DNA synthesis was determined by thymidine autoradiography. Data are presented as mean ± standard error. Counts made at the center of duct segments measure maintenance DNA synthesis. C, control implant; E, TGF β1 implant. [From Daniel et al., 11].

TGF–B1 implants or even in the stroma immediately surrounding the implants. These results indicate that in the mammary gland, TGF–B1 can modulate an epithelium–stroma inductive interaction. This strict dependence on mammary epithelium also suggests that a second epithelial factor is probably released. The synthesis of another matrix component, sulfated glycosaminoglycans, was also found to be stimulated by TGF–B1, and again, synthesis demonstrated a strict epithelium dependence. Because the production of these extracellular matrix components has been associated with cell differentiation and tissue stabilization in other developmental systems, TGF–B1 stimulation of matrix synthesis appears to be a fruitful area for further investigation of mammary developmental regulation.

Estrogen

Although estrogen is considered a classical hormone rather than a growth factor, recent in vitro evidence indicates that estrogen may regulate TGF–B1 secretion [23] and EGF receptor levels [30]. The question of whether estrogen acts exclusively through mediation of pituitary mammogens or whether in addition it exerts direct effects on the gland becomes critically important in sorting out the role of locally active growth factors. To settle this long-standing question, Daniel et al. used EVAc implants to treat static gland in previously ovariectomized mice [8]. Estrogen (17B–estradiol) was extremely effective in restoring end buds and normal morphogenesis in a highly

localized and specific manner (the 17–alpha isomer was inactive). In addition, steroid autoradiography revealed high-affinity estrogen receptors in the lumenal end bud epithelium in the ductal lumenal cells, and in stromal cells adjacent to ducts.

Conclusions

In contrast to traditional mammogenic hormones that have well-established physiological roles, the importance of peptide growth factors in mammary development and functional differentiation is largely unknown. The use of slow-release plastic polymers provides an essential technology for studying local direct effects of growth factors in situ on the mammary gland. Epidermal Growth Factor has been shown to be locally active in stimulating ductal growth in the static gland of ovariectomized mice and is growth-inhibitory when administered to rapidly elongating mammary ducts of endocrine-intact mice. EGF receptors are present in certain epithelial and stromal mammary cells. Transforming Growth Factor Beta–1 is a potent, fully reversible inhibitor of normal ductal growth. It simultaneously inhibits epithelial cell proliferation and stimulates stromal production of extracellular matrix; thus TGF–B1 displays many of the biological activities associated with a predicted mammary regulator. Using implant techniques, estrogen has also been shown to have direct, local, mammogenic activities and to bind to high-affinity mammary receptors. Becasue of its suspected role in modulating TGF–B1 and EGF, estrogen is tentatively considered to reside at the top of a complex regulatory hierarchy involving an orchestration of traditional endocrine mammogens and growth factors.

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5. The insulin-like growth factors, their receptors, and their binding proteins in human breast cancer

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Introduction

The ability to grow breast cancer cells in vitro has lead to the identification of several polypeptide hormones that may regulate their growth. The insulin-like growth factors (IGFs) have been identified as potent mitogens for breast cancer cells in vitro and increasing evidence has suggested that the IGFs may have an important function in the proliferation of mammary epithelia. This chapter will examine the data that support a role for the IGFs in regulating the growth of breast cancer cells.

The family of insulin-like growth factors includes IGF–I, IGF–II, and relaxin. Like insulin, IGF–I and IGF–II are first synthesized as prehormones and then posttranslationally modified to yield 70 and 67 amino acid polypeptides respectively [for review see 1]. IGF–I and IGF–II have a high degree of amino acid and nucleic acid sequence homology to insulin and to each other [2, 3]. Relaxin is more distantly related to insulin than to either IGF–I or IGF–II [4] and it functions to relax the pelvis during parturition. IGF–I (also known as somatomedin–C) is important in the linear growth of the skeleton and mediates the effects of growth hormone. The liver is thought to be the principal site of protein synthesis, although IGF–I mRNA and protein have been found ubiquitously in human tissues, [5, 6]. Because of the widespread distribution of IGF–I mRNA expression, autocrine, paracrine, and endocrine effects have been proposed for IGF–I in normal growth and development [1].

IGF–II levels are elevated during fetal life, and the highest levels of IGF–II mRNA are found in fetal tissues. In the rat, IGF–II levels drop precipitously after birth and little circulating IGF–II can be detected in the adult [7, 8]. This has lead to the hypothesis that IGF–II is primarily a fetal somatomedin. In contrast, humans have high circulating levels of IGF–II throughout life, and the function of IGF–II in the adult is unknown [9, 10].

The cognate receptors for IGF–I and IGF–II have been identified and, unlike the ligands, are not homologous [11, 12, 13]. The type I IGF receptor (IGF–I receptor) is homologous to the insulin receptor. It is translated as

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a single gene product, then posttranslationally modified to form a heterotetramer composed of two 130 kD alpha and two 95 kD beta chains. The alpha chains are entirely extracellular and possess the ligand-binding domains. The beta chains are covalently linked to the alpha chains and have a small extracellular domain, a transmembrane domain, and an intracellular domain that possesses tyrosine kinase activity. Although often referred to as the IGF–I receptor, this receptor also has high affinity of IGF–II and is more properly referred to as the type I IGF receptor. Insulin can also bind to this receptor but with reduced affinity.

The type II IGF receptor (IGF–II receptor) is a single chain 250 kD protein. Molecular cloning of this receptor has demonstrated that it has a large extracellular domain, a transmembrane portion, and a small intracellular domain [12, 13]. Interestingly, the type II IGF receptor is identical to the cation-independent mannose–6–phosphate receptor that functions in targeting enzymes for lysosomal storage. Although some functions of the type II IGF receptor have been identified, its physiologic function is unclear. It has been suggested that the mitogenic effects of the IGFs in fibroblasts are mediated through the type I IGF receptor [14].

In addition to a family of ligands and receptors, there is also a family of IGF-binding proteins [15]. These proteins have high affinity for the IGFs and are found in blood and extracellular fluid. By binding the IGFs, they alter the ability of the ligands to interact with their receptor. In the circulation, IGF–I is bound to a protein of 150 kD. This complex is composed of an IGF-binding protein and an acid-stable subunit that does not directly bind IGF. The IGF-binding portion, named BP–53, has been cloned, and when expressed in mammalian cells, its molecular weight (as determined by affinity cross-linking studies) is 53 kD [16]. The molecular weight predicted by the cDNA sequence is only 28.7 kD, suggesting that the protein undergoes substantial posttranslational modification. Like IGF–I, the synthesis of this protein is growth hormone dependent.

Several smaller-molecular-weight IGF-binding proteins are also produced. One such protein is found in amniotic fluid and has a molecular weight of 28 kD. Cloning of the gene encoding this protein has demonstrated that its predicted molecular weight is 25 kD; it has been referred to as BP–25. Like BP–53, BP–25 undergoes posttranslational modification [17, 18]. Comparison of BP–25 and BP–53 primary sequences shows a highly conserved area of cysteines, suggesting that this area is important in IGF binding. The function of these binding proteins is currently being investigated. Brewer et al. have shown that BP–25 contains an Arg–Gly–Asp (RGD) sequence, which suggests it is associated with the cell membrane [18]. It has been proposed that alternate posttranslational modifications of BP–25 determine whether it will enhance or inhibit the effects of IGF–I [19]. Additionally, it has been shown that BP–53 can also either inhibit or augment the effects of IGF–I, depending on the experimental conditions [20]. In addition to these

well-characterized binding proteins, several other low-molecular-weight forms have been identified [15]. Although it is clear that binding proteins modulate the effects of the IGFs, further investigation is necessary before their functions are entirely understood.

Therefore, the IGF system is composed of a family of ligands, receptors, and binding proteins. The interactions of all these components determine the effects of IGFs in both normal growth and development and possibly in malignancy.

IGF receptors in breast cancer

We and others have previously shown that both IGF–I and IGF–II support the short-term growth of some breast cancer cells in serum-free media [21, 22, 23]. In the estrogen-dependent cell line MCF–7, 5 nM IGF–I or 10 nM IGF–II were as potent as 1 nM estradiol in stimulating monolayer growth. IGF–I also stimulated anchorage-independent growth in several estrogen-dependent breast cancer cell lines, and in some cases estrogen-stimulated colony formation was increased in the presence of IGF–I [24]. These observations suggest that specific receptors for these growth factors are expressed by breast cancer cells. Furlanetto and DiCarlo demonstrated the presence of the type I IGF receptor on several breast cancer cell lines by competitive-binding studies and affinity cross-linking. Additionally, DeLeon et al. have used affinity cross-linking to demonstrate the presence of both the type I and type II IGF receptors in some breast cancer cell lines [25].

Therefore, binding data and growth studies imply that breast cancer cell lines have receptors for IGFs. However, if breast cancer cells express insulin receptor, type I and type II IGF receptor, and IGF-binding proteins, then ligand binding studies may be difficult to interpret. Additionally, the presence of multiple receptors that can bind IGFs and potentially trigger a physiologic response make it difficult to discern which receptor mediates the mitogenic effects of the IGFs. To better characterize the receptors expressed by breast cancer cells and tissues, we examined IGF receptor mRNA expression and IGF binding in these cells [26].

Using the ribonuclease (RNase) protection assay, we have shown that virtually all breast cancer cells expressed insulin receptor, and type I and II IGF receptor mRNA [26]. Binding studies demonstrated high-affinity binding for both IGF–I and IGF–II. Interestingly, low concentrations of unlabelled IGF–I or IGF–II increased the affinity of MCF–7 cells for labelled IGF–I before competition for binding was seen. This biphasic response to unlabelled ligand suggested that positive cooperativity of receptor binding occurs in MCF–7. The mechanism for this cooperation in binding is not clear; however, it is intriguing to speculate that it could be due to interactions of the type I IGF receptor with either the type II receptor or the binding proteins.

The monoclonal antibody αIR3, which blocks the binding of IGFs to the type I IGF receptor [27], inhibited 80% of the IGF–I binding to these cells. In contrast, unlabelled IGF–II displaced labelled IGF–II binding, while αIR3 could not. Although there are several interpretations of this result, it appears that the labelled IGF–II preparation we used did not bind well to the type I IGF receptor [J Perdue, personal communication]. Scatchard analysis revealed that MCF–7 cell had 8,000 type I receptors per cell with a kD of 2 nM. The kD of the type II receptor is approximately the same, but there appeared to be greater than tenfold more type II IGF receptors. The increased numbers of type II receptors may partially explain why blockade of the type I IGF receptor did not appear to inhibit the binding of IGF–II to the cell; most IGF–II was bound to the type II IGF receptor. Therefore, MCF–7 cells expressed insulin receptor and type I and type II IGF receptors.

Since each of these receptors could potentially mediate the mitogenic effects of insulin and the IGFs, we also examined the effects of αIR3 on cell response to the growth factors. αIR3 has been reported to block IGF binding to the type I receptor without eliciting a physiologic response [27]. In MCF–7, 12 μg/ml of αIR3 inhibited the mitogenic effects of both IGF–I and IGF–II. Although this antibody did not detectably inhibit IGF–II binding to cells, blockade of the type I IGF receptor abolished the mitogenic effects of IGF–II [26]. These data indicate that the type I IGF receptor mediated the mitogenic effects of the IGFs and that the type II receptor is not sufficient, and perhaps not necessary, for IGF–II-induced stimulation. In contrast, αIR3 did not affect the mitogenic action of insulin. Previous observations have suggested that insulin exerts its mitogenic effects through the type I IGF receptor [27]; however, we find that insulin stimulates a mitogenic response through its own receptor in these cells.

Several groups have investigated the ability of αIR3 to inhibit breast cancer cell growth. Rohlik et al. demonstrated the ability of this antibody to inhibit the growth of the estrogen receptor-positive cell line MCF–7 in the presence of serum [28]. Since serum contains IGFs, it is possible that the growth inhibitory effects of αIR3 in this system were due to blocking the IGFs derived from serum. When the cells were grown in estrogen-depleted serum, αIR3 could blunt the cell proliferation induced by the addition of estradiol, although the degree of αIR3-mediated inhibition was greater in the absence of estradiol. The authors interpreted this finding as showing that an IGF species may participate as an estrogen-regulated autocrine growth stimulator of breast cancer cells, although the exact nature of the IGF was not determined (see below). However, since the experiment was performed in the presence of serum, another interpretation is that estradiol could partially rescue the cells (perhaps by inducing other non-IGF growth factors) from blockade of serum-derived IGF–I and not from estrogen-stimulated autocrine production of IGF–I.

Arteaga et al. have used αIR3 to examine anchorage-independent growth

and tumor formation in athymic mice [29]. They found that αIR3, in the presence of, serum inhibited anchorage-independent growth, suggesting that IGFs are important in this measure of transformation. In this study, it is not clear if the IGFs were derived primarily from the serum or if they were produced by the breast cancer cells. Additionally, they found that in athymic mice, αIR3 inhibited tumor formation by the estrogen-independent cell line MDA–MB 231 but not by the estrogen-dependent cell line MCF–7. Although these studies do not prove autocrine growth stimulation of breast cancer cells by IGFs, they do support the hypothesis that the IGFs are important in tumor growth.

As in breast cancer cell lines, detection of type I and type II IGF receptor transcripts in breast cancer tissue RNAs was also common [26]. All breast cancer tissues contained type II IGF receptor mRNA and most demonstrated type I IGF receptor mRNA. Insulin receptor mRNA was also found in most breast cancer specimens. RNA derived from tissue sources contains RNAs derived from a variety of different cell types, and it has been suggested that malignant epithelial cells contribute proportionally more RNA to the total quantity of RNA than do the stromal cells [31]. Although this argument does not exclude the possibility that nonepithelial cells are overexpressing the IGF receptor RNA, these data suggest that the IGF receptors may be expressed in vivo by malignant epithelial cells.

In support of this observation, several investigators have documented the presence of IGF receptors in both benign and malignant breast tissue specimens by using affinity cross-linking techniques. Pollack et al. have examined subcellular membrane fractions derived from tumor specimens to demonstrate type I and II IGF receptors [32]. Using a similar technique, Peyrat et al. have also shown that the type I receptor was present in malignant breast tissues [33] and in benign breast disease [34]. Therefore, several lines of evidence suggest that IGF receptors are expressed by breast cancer cells and that the IGFs could be important in regulating the growth of breast cancer.

Production of IGFs by breast cancer cell lines

Since breast cancer cells express receptors for the IGFs and since both IGF–I and IGF–II are mitogens for these cells, several investigators have sought to examine whether IGFs could stimulate breast cancer cell proliferation via an autocrine pathway. Huff et al. examined media conditioned by breast cancer cells and discovered a 13 kD protein could displace authentic IGF–I in a radioimmunoassay (RIA) [35]. This protein was associated with an IGF-binding protein, and acid conditions were required to separate the IGF–I-related activity from binding protein. Minuto et al. also examined conditioned media from breast cancer cell lines and found two peaks of IGF–I immunoreactive material [36]. The larger peak at 35–45 kD demonstrated

IGF-binding activity, while the smaller peak co-migrated with labelled IGF–I and did not bind IGF–I. Therefore, the larger peak likely represented interference of IGF-binding protein with the IGF–I RIA, while the smaller peak could have been an IGF–I-related protein.

Examination of breast cancer cell line mRNA expression with an IGF–I cDNA probe revealed several hybridizing RNA species [35]. However, these transcripts were of different size from those seen in normal human liver. This finding has been demonstrated by other investigators [37] and suggests that either the IGF–I gene is alternately spliced in breast cancer or that the RNA species detected in breast cancer cell lines were mRNAs with nucleotide sequence homologous to, but not identical with, authentic IGF–I. In order to examine the nature of these transcripts, we examined cell line RNA for IGF–I mRNA expression by RNase protection assay using an anticomplementary IGF–I RNA probe transcribed from the IGF–IA cDNA. This is a sensitive and specific technique for the detection of IGF–I transcripts; only RNA transcribed from the IGF–I gene will protect the probe from RNase digestion. Using this technique, we found that none of the cell lines produced IGF–I mRNA [38]. Additionally, anticomplementary RNA probes transcribed from genomic IGF–I exon probes also failed to demonstrate IGF–I mRNA [D Yee, unpublished data]. These data suggest that the transcripts detected by Northern blot analysis are not transcribed from the IGF–I gene but represent related RNA species.

Since IGF–II is closely related to IGF–I in both protein and nucleotide sequence, we also used RNase protection with an IGF–II probe to examine breast cancer cell line mRNA [21]. We found that only one breast cancer cell line, T–47D, consistently expressed IGF–II mRNA in vitro, although some subclones of MCF–7 demonstrated IGF–II mRNA expression. Of note is that the transcript sizes detected are similar to those produced in developing fetal tissues [21, 39], suggesting that promoters utilized in fetal tissues are also active in cancer.

The majority of breast cancer cell lines produce neither IGF–I nor IGF–II mRNA, suggesting that the insulin-like growth factor-related protein produced by many breast cancer cell lines is not IGF–I or IGF–II. Therefore, it is possible that breast cancer cell lines secrete an as of yet unidentified IGF- related activity.

T–47D did express IGF–II mRNA and is an estrogen receptor-positive cell line. Since it has been suggested that estrogen may mediate its growth stimulatory effects by inducing autocrine growth factors [40], we studied the response of IGF–II mRNA expression to estrogen stimulation to determine whether IGF–II could fulfill this role. We found that IGF–II mRNA steady-state levels were increased by exposure to 1 nM 17β–estradiol, and the maximal increase in IGF–II mRNA occurred eight hours after exposure [21]. Therefore, IGF–II mRNA expression was responsive to estrogen and could potentially mediate some of its growth stimulatory effects.

In order to investigate the effect of estrogen on tumors in vivo, we grew

several breast cancer cell lines as xenotransplants in athymic mice [41]. In this system RNase protection was specific and anticomplementary RNAs transcribed from the human IGF–II cDNA did not detect mouse IGF transcripts. The growth response to estradiol was directly correlated with the change in steady-state levels of IGF–II mRNA. That is, when exposed to hormone, xenotransplants that were growth-stimulated by estradiol (MCF–7) increased their levels of IGF–II mRNA, while xenotransplants that were growth-inhibited by estradiol (T61) had decreased levels of IGF–II mRNA after estradiol exposure. In tumors derived from MDA–MB–231, which is neither inhibited nor stimulated by estrogen, IGF–II mRNA levels did not change after estrogen exposure. Of note, the parent MDA–MB–231 and MCF–7 cells used in this study expressed IGF–II mRNA in vitro. Other passages of these cell lines used for previous experiments [21] did not express IGF–II mRNA, suggesting that either clonal variation or cell culture conditions are important in determining IGF–II mRNA expression in breast cancer cell lines. However, the observation that the change in IGF–II mRNA levels directly correlated with the growth response of cell lines to estrogen suggests that IGF–II expression may be involved in regulating breast cancer tumor growth.

IGF mRNA expression in breast tissues

We found that, in contrast to breast cancer cell lines, IGF–I and IGF–II mRNA were commonly expressed in RNA extracted from breast cancer tissue samples [21, 38]. However, both nonmalignant breast hyperplasia and non-pathologic breast tissue adjacent to breast cancers also had high levels of both IGF–I and IGF–II mRNA. Travers et al. demonstrated similar results for IGF–II using dot-blot hybridization to detect IGF–II mRNA. All RNAs derived from nonmalignant breast disease contained IGF–II mRNA, yet only half the breast carcinomas had detectable IGF–II [42]. Unlike RNA obtained from breast cancer cell lines, breast tissue RNA is composed of RNAs derived from a heterogeneous mixture of cells, including malignant epithelial cells, benign epithelial cells, cells of stromal origin, cells of the vascular system, and infiltrating hematopoietic cells. Therefore, when examining total cellular RNA derived from tissue specimens, the identity of the IGF-producing cell will not be clear. Examination of human fetal tissues has suggested that IGF–I and IGF–II mRNA originate primarily from stromal tissues [43].

In order to identify the site of IGF–I mRNA production in breast cancer tissues, we have used anticomplementary RNA probes for in situ hybridization. IGF–I is homologous to both IGF–II and insulin; RNase digestion after hybridization increases the specificity of detection. We found that IGF–I mRNA expression in human tissues was confined to areas of normal breast ductules, and microscopical examination demonstrated that the

strongest signals were over normal stromal cells [38]. In the specimens we studied, no IGF–I mRNA could be detected in the malignant epithelial cells. These studies suggest that the IGF–I mRNA we detected in human breast cancer specimens originated from normal stromal elements that were included in the sample and did not originate from the malignant epithelial cells. Therefore, we found that the pattern of authentic IGF–I mRNA expression supports its role as a potential paracrine stimulator of growth for breast cancer cells.

We had found that, in contrast to IGF–I, some cell lines and breast cancer xenotransplants expressed IGF–II. When breast cancer samples were examined by RNase protection assay, we found that virtually all samples contained some IGF–II mRNA and that the level of expression varied approximately twentyfold among samples [21]. Preliminary in situ hybridization studies demonstrated that IGF–II mRNA expression can be seen in stromal cells, suggesting that, like IGF–I, IGF–II may stimulate breast cancer cells via a paracrine pathway. Additionally, samples can be found in which it appears that IGF–II mRNA originated from the malignant epithelial cells [S Paik, unpublished observation]. Therefore, in situ hybridization and in vitro studies support the concept that IGF–II may be an autocrine growth factor for breast cancer cells.

In addition to autocrine and paracrine regulatory roles for IGF–I and IGF–II, it is also possible that they function as endocrine modulators of breast cancer growth. Estrogen increases IGF–I mRNA levels in the rat uterus [30]. In humans it has been demonstrated that estrogen can increase the level of serum IGF–I [44]. Additionally, the antiestrogen tamoxifen decreased detectable circulating levels of IGF–I [45]. These observations suggest that estrogens could exert their growth-promoting effects on breast cancer cells by increasing serum IGF levels and could stimulate breast cancer growth by endocrine pathways.

Expression of IGF-binding proteins by breast cancer cells

Recent work has demonstrated that several species of IGF-binding proteins (IGF–BPs) are produced by breast cancer cell lines. The determination of the molecular-weight-species of IGF–BPs varies according to the assay conditions. Affinity cross-linking, immunoprecipitation, immunoblotting, and Western ligand blotting will all yield slightly different molecular weights for the same purified IGF–BP. Additionally, reducing conditions generally retard the mobility of IGF–BPs, since they contain multiple cysteine bonds. These technical aspects need to be considered when studies using different assay conditions are compared.

DeLeon et al. used affinity cross-linking studies of conditioned media to demonstrate that breast cancer cell lines produced a heterogeneous pattern of IGF–BPs. The predominant molecular-weight-species of breast cancer

IGF–BPs varied between cell lines. They identified 27 kD IGF–BPs produced by T–47D and MCF–7 under nonreducing conditions, while MDA–MB–231 and Hs578T produced a 24 kD IGF–BP. Hs578T produced additional bands of 28 and 35 kD. Although both Hs578T and MDA–MB–231 expressed BP–25 mRNA, the authors did not identify this IGF–BP protein species in either of these cell lines [46].

In contrast, we used Northern blot analysis and RNase protection to demonstrate BP–25 mRNA production by the breast cancer cell lines MDA–MB–231 and Hs578T but not by other breast cancer cell lines. BP–25 protein was demonstrated in the media conditioned by MDA–MB–231 and Hs578T by both radioimmunoasay and immunoprecipitation with a specific polyclonal antibody [47]. These results differ from those of DeLeon et al., who were unable to identify BP–25 protein by cross-linking and Western immunoblot. This difference probably relates to the sensitivity of the methods; we examined concentrated conditioned media by immunoprecipitation and radioimmunoassay, while DeLeon et al. used unconcentrated media in cross-linking. However, it seems likely that et least one of the IGF–BPs detected by DeLeon et al. in Hs578T was BP–25. This species of IGF–BP has been shown to enhance the effects of IGF–I [19, 48]. It has been suggested that BP–25 can associate with the cell membrane, and alternate posttranslational modification of the IGF–BP will influence its ability to interact with the membrane and enhance the biological effects of IGF–I [18, 19]. Since it has been previously shown that the IGFs are potent mitogens, it becomes clear that expression of this IGF–BP by breast cancer cells could be important in regulating their growth.

We have also assayed concentrated, conditioned media for the presence of IGF–BPs by Western ligand blotting [49]. In this technique conditioned media was fractionated by polyacrylamide gel electrophoresis under nonreducing conditions and transferred to nitrocellulose; labelled IGF–I was allowed to bind to the blot [50]. Preliminary results have demonstrated that multiple species of IGF–BP are produced by breast cancer cell lines and that the pattern of expression is more complicated than that suggested be DeLeon et al. [46]. All breast cancer cell lines expressed a 24 kD binding protein. However, other species were selectively produced; MCF–7 expressed a 34 kD protein, and MDA–MB–231 expressed both BP–25 and a higher molecular-weight-species of approximately 41 kD. Examination of RNA produced by breast cancer cell lines demonstrated that the IGF–BP found predominantly in serum, BP–53, was also produced by many cell lines. It seems likely that the 41 kD IGF–BP identified in MDA–MB–231 was BP–53, since this was the most prominent band detected in serum by Western ligand blotting and BP–53 is the major component of serum IGF-binding activity [50]. The similarity of molecular weight sizes between the IGF–BP found in the spinal fluid [51] and the 34 kD IGF–BP produced by MCF–7 is suggestive evidence that they may be related.

Therefore, multiple species of IGF–BPs are expressed by breast cancer

cell lines, although the protein species expressed by the cells vary. An IGF–BP with a molecular weight of 24 kD on Western ligand blot appears to be commonly produced by breast cancer cell lines. Additionally, some cell lines produced the well-characterized BP–25 and BP–53. Western ligand blotting demonstrates that there are also other intermediate forms of IGF–BP produced by breast cancer cells, and at this time these forms are incompletely characterized. It is not clear if they represent other IGF–BP species or alternate posttranslational modification of the cloned IGF–BPs. The role of these BPs in breast cancer growth regulation will be better understood when the functions of each IGF–BP are known.

Summary

Various investigators have shown that the IGFs are mitogens for breast cancer cells. The expression of the IGF receptors is seen in most breast cancer cell lines and tissues, suggesting that most breast cancers have the ability to respond to the IGFs. Although authentic IGF–I is not expressed by breast cancer cell lines, it is possible that an IGF-related peptide that can be detected immunologically is expressed. Furthermore, in estrogen responsive xenotransplants, changes in the level of IGF–II mRNA correlate directly with estrogen-mediated changes in tumor growth. These observations suggest that IGF–II may be important in tumorigenesis and may serve as an autocrine growth stimulator of breast cancer cells.

When human breast cancer tissues are studied, IGF–I and IGF–II mRNA expression are commonly seen. However, in situ hybridization studies suggest that IGF–I mRNA is expressed mainly by the stromal elements, while IGF–II mRNA can be found both in stroma and malignant epithelial cells. These observations support the studies done with breast cancer cell lines; IGF–I may stimulate cells via a paracrine pathway, while IGF–II may act as both an autocrine and paracrine growth factor. In addition, IGF–BPs are commonly expressed by breast cancer cells in culture, and it is possible that expression of the IGF–BPs act to modulate the effects of either IGF–I or IGF–II.

We propose that the IGFs are important stimulators of breast cancer cells and that their growth promoting effects may be mediated by autocrine, paracrine, or endocrine mechanisms. Furthermore, interactions between the stroma and malignant epithelial cells may be important in regulating the growth of breast cancer. The biological importance of a fibroblast–epithelial cell interaction has been demonstrated in a normal mouse mammary cell line; morphological and functional changes in epithelial cells were induced when the cells were in direct contact with fibroblasts [52]. Similar mechanisms may be important in malignant breast epithelial cells. For example, many breast cancer cells produce platelet-derived growth factor (PDGF) yet have no PDGF receptor [53, 54]. PDGF has been demonstrated to increase IGF–I

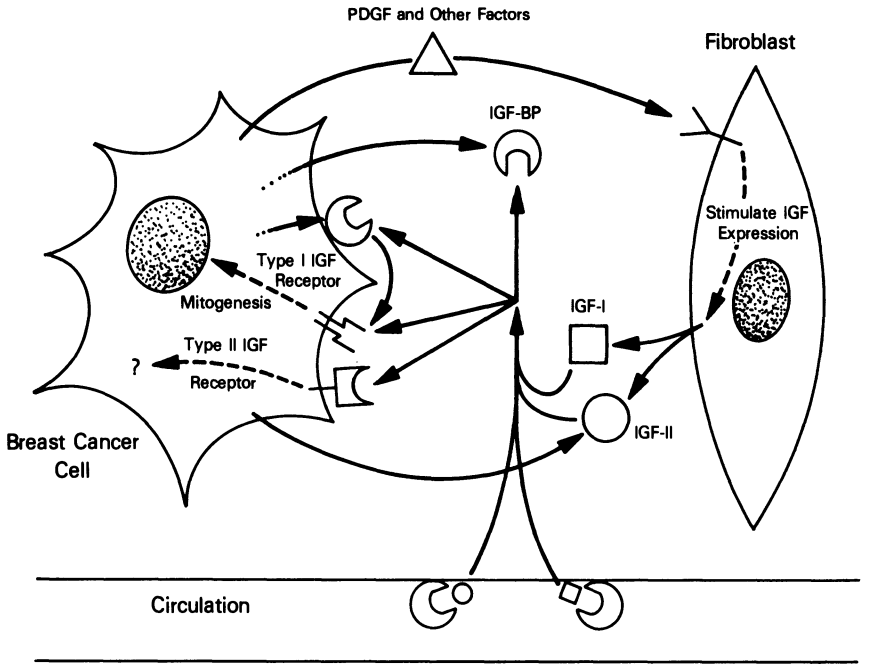

Figure 1. Schematic representation of the interactions between components of the IGF system, the fibroblast, and the breast cancer cell. Breast cancer cells produce only IGF–II, while both IGF–I and IGF–II may be produced by the fibroblast. Breast cancer cells express both type I and II IGF receptors and IGF–BPs produced by the breast cancer cell may influence the interaction between ligand and receptor. A dual paracrine pathway in which growth factors produced by the breast cancer cell act to stimulate IGF expression in the fibroblast may be important in the growth regulation of breast cancer cells. Finally, endocrine-mediated stimulation of breast cancer cells may originate from serum-derived IGFs.

production by fibroblasts [55], and a dual paracrine pathway involving PDGF and IGF–I expression by epithelial cells and stromal cells could be envisioned. The pathways through which the IGF system may function in human breast cancer are schematically represented in figure 1. Further work in our laboratory is directed at clarifying the role for the IGFs in breast cancer growth.

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6. The role of *ras* gene expression and transforming growth factor α production in the etiology and progression of rodent and human breast cancer

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Introduction

Cancer represents a spectrum of different diseases that generally arise through a series of multiple yet discrete steps that include initiation, promotion, transformation, progression, and metastasis [1]. Genetic alterations to specific host cellular genes or sets of genes are associated with some or all of these stages [1–3]. Damage to DNA can result in somatic changes due to the action of chemical carcinogens, mutagens, viruses, or radiation. A large body of evidence suggests that certain endogenous regulatory genes are likely targets for insult from exogenous environmental agents. These cellular regulatory genes or proto-oncogenes have been implicated in the control of cellular proliferation and/or differentation [3–7]. Activation of these genes through processes such as point mutation, amplification, rearrangement, insertional mutagenesis, chromosomal translocation, or overexpression can lead to neoplastic transformation in vitro and tumorigenicity in vivo [3, 5, 7]. Approximately 35–40 different proto-oncogenes have been identified to date and can be stratified in some cases into families based on their structure and function [3, 7]. These gene families were originally detected as the dominantly transforming genes that were associated with the genome of different acutely transforming retroviruses and that were transduced or captured as cellular proto-oncogenes [3, 4, 7]. DNA transfection assays have also demonstrated the presence of other additional groups of activated cellular proto-oncogenes that are associated with the DNA obtained from a number of rodent tumors and from a small fraction of primary human tumors and that are capable of transforming immortalized rodent fibroblasts, such as NIH–3T3 cells, in vitro [8–15]. In addition to this group of genes, there exists a second major group of genes, the tumor suppressor or antioncogenes, which are genes that give rise to tumors after allelic deletion or inactivation [16–18]. These recessive genes, such as the Rb–1 (retinoblastoma) gene, can code for nuclear phosphoproteins that can negatively regulate cell growth and that have the potential to interact with and to be subsequently inactivated by other

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specific nuclear oncogene proteins, such as the SV40T antigen protein and the Adenovirus E1A protein [19–22].

One phenotypic property that is almost universally exhibited by mesenchymal and epithelial cells that have been spontaneously transformed or that have been transformed by retroviruses, DNA tumor viruses, or chemical carcinogens is their ability to proliferate in an unrestrained fashion. This property in vitro is generally reflected by a decreased serum requirement for the growth of these cells, which is due in part to the partial or complete relaxation of the growth factor conditions that are necessary for maintaining the proliferation of these cells [23, 24]. Eventually, tumor cells may progress to a state in which there is a total loss in the requirement for specific in vivo host-derived or in vitro serum-derived growth factors or sets of growth factors. This autonomous situation may be partially due to the ability of these transformed cells to synthesize and secrete their own endogenous growth factors and by their ability to respond to these activities in an autocrine or paracrine manner [25–32]. Exacerbation of this situation can also be due to the ability of transformed cells to escape from the effects of normal growth inhibitory factors, such as transforming growth factor β (TGF–β) [22, 25, 27, 32]. In addition, transformed cells can also constitutively overexpress cell surface receptors for specific growth factors. An example of the latter situation is illustrated by the epidermal growth factor (EGF) receptor (EGFR), which has been demonstrated to be rearranged, amplified, and/or overexpressed in several different types of human malignancies [3, 7, 29, 30, 33]. Such an event could lead to a ligand-independent activation of the receptor if it were also structurally altered in the external binding domain for the growth factor. In addition, overexpression of the normal receptor protein could hypersensitize tumor cells to low concentrations of exogenous or endogenous growth factors to which nontransformed cells would not normally be responsive [34–36]. All of these possibilities can probably occur and are particularly relevant to the activation of specific proto-oncogenes, because some of these genes have the capacity to code for proteins that are growth factors or potential growth factors (e.g., c–*sis*/B chain of platelet-derived growth factor [PDGF] and *int*–2/fibroblast growth factor [FGF]-related peptide) or proteins that are receptors or putative receptors for growth factors (e.g., c–*erb*B–1/EGFR; c–*fms*/colony-stimulating factor–1 [CSF–1] receptor, and c–*erb*B–2 [*neu*]) [3, 5, 7, 10, 11, 37–39]. In addition, these genes may encode proteins that can function as intracellular coupling factors or protein kinase effectors for growth factor receptors (e.g., c–*ras*, c–*mos*, and c–*raf*) or proteins that may be involved in the nuclear signal transduction pathway for growth factors, such as DNA-binding transcriptional activating factors (e.g., c–*myc*, c–*fos*, and c–*jun*) [40–43]. Alternatively, cellular proto-oncogenes may indirectly regulate the expression of or response to a spectrum of different endogenous cellular growth factors that may be involved in mediating some of the biological effects that are produced in transformed cells after activation of these proto-oncogenes [28, 30, 44–46].

Oncogenes and growth factors in breast cancer

Breast cancer is one of the most prevalent types of cancer observed in women. Approximately 1 of 12 women will develop breast cancer during the course of her life [47–49]. One intriguing aspect about breast cancer is that specific mammotrophic hormones, such as estrogens and possibly prolactin, perform an important role in the origin and/or the progression of this disease in rodents and humans and may therefore be functioning as classical tumor promoters [1, 50]. Nearly 50% of human breast cancers are estrogen dependent and possess functional estrogen receptors (ER), which is one reason that antiestrogens have been used extensively as adjunct therapeutic agents [49, 50]. However, the mechanism(s) by which these mammotrophic hormones, such as estrogens and their pharmacological antagonists the antiestrogens, can regulate the growth and differentiation of normal and neoplastic mammary epithelial cells is still unclear. It is conceivable that estrogens may indirectly function as mitogens for mammary epithelial or surrounding stromal cells by their ability to induce either locally or systemically the synthesis and secretion of different endogenous autocrine or paracrine peptide growth factors, and that this responses(s) may be modified by antiestrogens [50–55]. In addition to this hormonal component, a number of different activated cellular proto-oncogenes, such as c–Ha–*ras*, c–*myc*, c–*erb*B, c–*erb*B–2, or *int*–2, and recessive antioncogenes, such as the Rb–1 gene, have been implicated in the pathogenesis of breast cancer (table 1) [10, 11, 15, 33, 37–39, 56–59]. The inappropriate activation of these proto-oncogenes or deletions or inactivation of antioncogenes may indirectly lead to the constitutive overproduction of growth factors and/or their receptors, to a loss of receptors for growth inhibitory factors, or to a change in the response of different populations of cells within the mammary gland to various growth factors and growth inhibitors [22, 33, 50]. In fact, several distinct growth factors and growth inhibitors have been demonstrated to be synthesized by or associated with a number of malignant rodent and human breast cancer cell

Table 1. Proto-oncogenes and antioncogenes associated with primary rodent and human breast carcinomas.[a]

Proto-oncogene	c–*onc* protein	Changes observed
c–*Ha–ras* (11)	$p21^{ras}$	Overexpression/deletions/point mutations
c–*myc* (8)	$p62^{myc}$	Amplifications/rearrangements
c–*erb*B (7)	EGFR(p170)[b]	Overexpression
c–*erb*B–2 (17)	$p185^{erbB-2}$	Amplifications/overexpression
int–2 (11)	FGF-related peptide (p25)	Amplifications
Rb–1 (13)	$p105^{Rb}$	Deletions

[a] Numbers in parentheses represent human chromosomal locations. The $p105^{Rb}$ is a tumor suppressor protein that interacts with SV40T and adeno E1A proteins.
[b] Associated tyrosine kinase activity.

Table 2. Growth factors or growth inhibitors produced by rodent and human breast carcinoma cells.[a]

Transforming growth factor–α (2)[b]
Transforming growth factor–β (19)[c,d]
Insulin-like growth factor–I (12)[b]
Insulin-like growth factor–II (11)[b]
Platelet-derived growth factor (A chain, 7; B chain [c–*sis*] 22)[b]
Basic fibroblast growth factor (4)
Mammary-derived growth factor–I
Mammary growth inhibitor
p52 protein (cathepsin D) (11)[b]

[a] Numbers in parentheses represent human chromosomal locations.
[b] Estrogen-induced.
[c] Antiestrogen-induced.
[d] Estrogen decreased.

lines or primary breast carcinomas (table 2). It has also been observed that normal rodent and human mammary epithelial cells or stromal cells are capable of synthesizing and secreting some of these activities in vitro and in vivo, suggesting that the presence of these growth regulatory agents is not unique to tumor cells but may be performing a more basic function in controlling the proliferation and/or differentiation of potential stem cell populations within the mammary gland [50, 51, 54, 60–63]. Furthermore, several of these growth factors may be functioning as paracrine activities that could regulate the proliferation of surrounding stromal cells and/or capillary endothelial cells within the mammary gland. These events could be important in the onset and development of desmoplasia and angiogenesis, which are observed in breast tumors.

The ras *gene family*

There is a substantial body of experimental and clinical evidence, which will be presented at a further point in this review, suggesting that genomic alterations in or changes in the level of expression of the c–Ha–*ras* gene can occur by overexpression, deletions, and, in some rare instances, point mutations and may be involved in the genesis and/or progression of a subset of mouse, rat, and human breast tumors. The *ras* genes were originally discovered as the transforming genes that were found in a series of acutely transforming rat sarcoma retroviruses [10, 11]. The *ras* genes are a highly conserved multigene family that has now been identified in organisms ranging from yeast to mammals. In mammals there are at least three major *ras* genes, designated Ha (Harvey), Ki (Kirsten), and N (neuroblastoma derived). The *ras* genes code for a group of homologous proteins of 21 KD ($p21^{ras}$), which in the case of the retroviral oncogene proteins are phosphorylated on a threonine residue. The *ras* gene family is most likely a member of a more extended supergene family consisting of proteins that exhibit varying degrees

of amino acid sequence homology to the p21^{ras} proteins. These include members such as *rho*, *ral*, *rev*–1, and *rab*; the α subunit of guanine nucleotide-binding proteins that are associated with the adenylate cylase system, phospholipase C, and cyclic GMP phosphodiesterase; and the translation elongation factors [10, 64–66]. Oncogenic activation of the *ras* genes can frequently occur by single point mutations in codons 12, 59, 61, and 63, which can lead to amino acid substitutions and constitutive biochemical activation of the protein. The presence of point-mutated *ras* genes in primary human tumors is a relatively rare event, occurring at a frequency of approximately 10%–20%, yet higher incidences have been found in several types of primary human tumors, most notably in colorectal and pancreatic carcinomas [10–13]. However, in several experimental animal tumors that arise in the skin, liver, lung, colon, and mammary gland after induction by chemical carcinogens (e.g., dimethylbenz(a)anthracene [DMBA] or nitrosomethylurea [NMU]), the presence of point-mutated *ras* oncogenes occurs at a frequency of approximately 85% [10]. Point mutations in *ras* genes can frequently be detected in the initial stages of tumor development for certain organs, because such changes can be found in premalignant lesions, including skin papillomas, colon polyps, and mammary hyperplastic alveolar nodules [10–13]. These results suggest that additional genetic changes or progression factors are necessary to complete the transformation process in vivo, because a majority of these benign growths generally regress and do not progress to frank carcinomas. This suggestion is in agreement with the in vitro observations that the transformation of primary rodent embryo fibroblasts requires the cooperative interaction between at least two activated proto-oncogenes (e.g., a *ras* gene and a second nuclear proto-oncogene, such as c–*myc*, E1A, SV40T, or p53) [5, 8, 67, 68]. More recently this cooperative thesis has been extended to two in vivo situations in which sequential and coordinate expression of the c–Ha–*ras* and c–*erb*B or c–*myc* genes have been found in the development of oral squamous cell and prostate carcinomas, respectively [69, 70]. Inappropriate overexpression of the c–Ha–*ras* proto-oncogene when ligated to a strong retroviral promoter can also lead to cellular transformation in vitro [71]. This may be important with respect to the observation that overexpression of the p21^{ras} protein and *ras* mRNA are more commonly found in human tumors [9–11, 57].

The p21^{ras} proteins are guanine nucleotide-binding proteins (G proteins) that possess intrinsic GTPase activity and are therefore related structurally and functionally to other members of this multigene family [7, 10, 65, 66]. They are located at the inner aspect of the plasma membrane, where they are attached to the membrane through a palmitic acid that is acylated to a cysteine residue in the COOH-terminal end of the molecule [10]. Point mutations within the molecule that can activate the oncogenic potential of this protein universally result in a severe reduction in or a total loss of the associated GTPase activity [72]. Under these circumstances, the p21^{ras} proteins are constitutively locked into the active form to which GTP is bound

[64]. Normally, the GDP/GTP exchange reaction is initiated by upstream activator signals, such as growth factors or other proliferative agents [65, 67]. Desensitization requires hydrolysis of the bound GTP to GDP by the GTPase, which is in turn activated by a 120 kD cytoplasmic GTPase-activating protein (GAP) that can become membrane associated [64, 73]. However, GAP has no effect on the low GTPase activity of the oncogenic, point-mutated $p21^{ras}$ proteins due to its failure to physically interact with the point-mutated $p21^{ras}$ molecule.

Interactions between ras *and growth factors*

Because $p21^{ras}$ proteins exhibit sequence homology to other mammalian and yeast G proteins, it is suspected that the $p21^{ras}$ proteins, acting through the downstream effector functions of GAP, may be functioning as potential signal transduction coupling proteins between certain growth factor receptors and effector molecules, such as phosphatidylinositol–4,5–bisphosphate (PIP_2)-specific phospholipase C [65, 67, 74–76]. Activation of phospholipase C is normally involved in initiating the breakdown of membrane-associated PIP_2 to 1,2–diacylglycerol (DAG) and inositol–1,4,5–triphosphate (IP_3), of which both may serve as intracellular second messengers for different hormones and growth factors, such as EGF, PDGF, bombesin, and bradykinin [75, 77–81]. DAG can function as an endogenous cellular activator for protein kinase C, whereas IP_3 is involved in the mobilization of intracellular calcium from internal stores and the subsequent activation of calmodulin-dependent portein kinases [75]. Interestingly, part of the biological activity that has been ascribed to antiestrogens, such as tamoxifen, may relate to their ability to inhibit protein kinase C activity and thereby interrupt the action of specific growth factors and activated proto-oncogenes, such as *ras* [82]. In this respect, PIP_2 turnover and DAG production are constitutively elevated in several *ras*-transformed rodent fibroblast cell lines [75, 83, 84]. In addition, the production of DAG and IP_3 in response to either bombesin or bradykinin is increased, whereas the enhanced turnover of PIP_2 in response to PDGF is muted in Ha– and N–*ras*-transformed NIH–3T3 and rat–1 cells [77, 85, 86]. Also, evidence indicates that the receptors for EGF/TGF–α, TGF–β, PDGF, and bombesin are associated or interact with potential G-binding proteins that may be functionally related to Gp (the putative G protein that mediates the activation of phospholipase C) and to the $p21^{ras}$ gene family [64–67, 74, 75, 77, 81, 82, 87, 88]. A direct link between certain growth factors that may function through specific G proteins and the phosphoinositide pathway probably exists, because EGF and PDGF can selectively increase the level of a PIP_2-specific phospholipase C or phosphatidylinositol kinase activity that can in turn serve as a substrate for or physically associate with the tyrosine kinase that is part of each of these respective growth factor receptors [81, 89–91].

These latter results collectively suggest that the expression of $p21^{ras}$ may be required for maintaining normal cell proliferation by mediating the intracellular effects of several growth factors that can function through receptors that

are tyrosine kinases. Experimental evidence to support this thesis comes from the observation that microinjection of recombinant, point-mutated $p21^{ras}$ proteins, but not their normal proto-oncogene counterparts, into quiescent rodent fibroblasts can induce a transient increase in cell proliferation that renders the cells refractory to the effects of specific growth factors, such as EGF and insulin [92, 93]. Conversely, after microinjection into NIH–3T3 cells, $p21^{ras}$-neutralizing monoclonal antibodies can block the proliferative responses induced by progression factors, such as EGF and insulin and other tyrosine kinase proto-oncogenes, such as *fes* and *fms* [94–96]. Mechanistically, this may relate to the ability of EGF or insulin, which act through functionally related tyrosine kinase receptors, to enhance the phosphorylation of and/or guanine nucleotide binding to the $p21^{ras}$ protein [97, 98].

The presence of a point-mutated *ras* proto-oncogene or high levels of expression of normal cellular *ras* proto-oncogenes that can be produced after ligation of the gene to a retrovirus long terminal repeat (LTR) can lead to the transformation of previously immortalized populations of rodent fibroblasts [8, 10, 71]. In contrast, activated *ras* proto-oncogenes alone are generally insufficient to transform primary cultures of rodent and human fibroblasts and epithelial cells and are ineffective in rescuing these cells from terminal senescence in vitro [6, 8, 10, 68]. Transformation of primary rodent embryonic fibroblasts requires the cooperative interaction between an activated c–*ras* and a second nuclear oncogene, such as c–*myc* [5, 8]. This is probably due in large part to the ability of these nuclear oncogenes, such as *myc* and E1A, to immortalize cells and thereby sensitize them to the biological effects of *ras* [3, 7, 8]. A similar cooperative interaction between *ras* and *myc* has recently been demonstrated to be operative and obligatory in vivo in the development of prostatic cancer [70]. Under these conditions, overexpression of *ras* alone produced dysplasias, whereas overexpression of *myc* produced hyperplasias. In contrast, *myc* and *ras* overexpression resulted in the development of carcinomas. In general, an enhanced level of c–*myc* expression occurs in rapidly proliferating cells and can be transiently induced by a number of different growth factors [43]. Reciprocally, cells that are overexpressing c–*myc* can exhibit a change in their behavior to exogenous growth factors. For example, rodent fibroblasts that have been transfected with and overexpress the c–*myc* gene generally become hypersensitive to the growth-promoting effects of exogenous or endogenously produced growth factors, such as EGF, basic FGF, TGF–β, TGF–α, and PDGF, depending on whether the cells are propagated either for anchorage-dependent growth in monolayer culture or for anchorage-independent growth in soft agar (table 3) [27, 28, 30, 99, 100].

One mechanism by which an activated *ras* proto-oncogene may contribute to the transformation of an already immortalized population of cells is by stimulating the production and secretion of endogenous cellular growth factors [7, 30, 31]. Todaro et al. [101] first observed that rodent fibroblast cell lines which had been transformed by a number of different retroviruses containing the *ras* (Ha or Ki), *fms*, *fes*, *mos*, and *abl* oncogenes lead to a

Table 3. Oncogene-induced growth factors or change in response to growth factors following oncogene transformation of immortalized rodent fibroblast cell lines.

Oncogene	Change in growth factors or receptors	Change in response to exogenous growth factors
v-/c-*ras* (Ha/Ki) v-*mos*, v-*fes*, v-*fms*, v-*abl*, and v-*src*	Increased production of TFG-α, TGF-β, IGF-I, and PDGF	Increased response to IGF-I; decreased response to EGF, bFGF, and PDGF; decrease in EGFR expression
v-/c-*myc*		Increased response to EGF, TGF-α, TGF-β, PDGF, and bFGF
v-*erb*B	Truncated EGFR	Decreased response to EGF
c-*erb*B	EGFR overexpression	Increased response to EGF and TGF-α

specific and chronic reduction in the number of cell surface receptors for EGF that were expressed on these cells. This effect was not limited to retroviral oncogene-transformed cells, because several carcinogen-transformed fibroblast cell lines also exhibited this phenotype. It was later discovered that the reduction in EGFRs was due in part to the ability of these transformed cells to synthesize and secrete a growth factor that was first described as sarcoma growth factor (SGF) and that could compete with EGF for binding to the EGFR [46, 102–104]. Subsequently, it was found that SGF was actually a crude mixture that consisted of two structurally and functionally distinct growth factors, TGF-α and TGF-β [28, 30, 105–107]. The presence in cells of other oncogenes or activated proto-oncogenes, such as *src*, *met*, and *trk*, can result in the increased expression and/or secretion of TGF-α and TGF-β and other growth factors, including basic FGF, insulin-like growth factor-I (IGF-I), and the A and B chains of PDGF (table 3) [108–111]. These changes in endogenous growth factor production may directly contribute to the unresponsiveness of *ras*-transformed rodent fibroblasts and epithelial cells to the stimulatory effects of exogenous growth factors, such as EGF, PDGF, and basic FGF, and may be responsible for the enhanced anchorage-dependent growth rate and possible anchorage-independent growth of these cells [44, 45, 112–115]. The enhanced production of one growth factor after cellular transformation may result in the increased synthesis of a second, heterologous growth-regulating activity(ies) through a cascade-like response. For example, TGF-β or EGF can induce the expression of either c-*sis* (PDGF B chain) or the PDGF A chain, respectively [116, 117], which may in turn enhance the expression of IGF-I [118]. Likewise, the same growth factor may amplify its own expression through an autoinduction process. This has recently been demonstrated for the expression of TGF-α, TGF-β, and the PDGF A chain [117, 119–121].

The enhanced expression of different cellular growth factor genes after transformation by an activated *ras* proto-oncogene may result from a change in the activity or level of expression of specific DNA-binding transcriptional

activating proteins, such as AP–1 (c–*jun*), which can also be modulated by phorbol ester tumor promoters, such as 12–0–tetradecanoylphorbol–13–acetate (TPA), and by serum [40–42, 122–125]. These *trans*-acting factors recognize and bind to discrete *cis*-acting consensus sequences or motifs in the promoter and enhancer regions of different groups of cellular genes [40, 42, 122]. In fact, it is well established that the activation of certain proto-oncogenes, such as *ras*, or that treatment of cells with TPA can indirectly regulate the expression of a series of cellular genes through these *ras* (TPA)-responsive, *cis*-acting regulatory elements [122–124, 126]. This may be germane to the recent finding that TPA or EGF can cause a threefold to sevenfold increase in the level of TGF–α mRNA and TGF–α protein within 2 to 5 hours after treatment of human keratinocytes and MDA–MB–468 human breast cancer cells [119, 121, 127]. Growth factors such as EGF and growth inhibitors such as TGF–β can also rapidly enhance the coordinate expression of c–*jun* and c–*fos* in a transient fashion [43, 128–130]. This is mechanistically significant, because the c–*fos* and c–*jun* (AP–1) proteins can interact and can form a functional complex that can bind to specific DNA consensus sequences in a cooperative manner [42, 131].

Transforming growth factor α (TGF–α)

TGF–α is of particular interest with respect to the phenotypic changes that occur after *ras* transformation, because fibroblasts and some epithelial cells that are transformed by this oncogene generally become refractory to the mitogenic effects of EGF, presumably due to the overproduction and secretion of TGF–α [6, 26, 30, 31, 44, 46, 103, 107, 113–115, 132]. TGF–α may be important with respect to the growth, development, and neoplastic transformation of the rodent and human mammary gland, because EGF is a potent mitogen for normal mammary epithelial cells and for some malignant rodent and human mammary carcinoma cells in vitro and in vivo [33, 51, 55, 62, 133–35]. In fact, the mitogenic effects of EGF or TGF–α on mammary epithelial cells may be due in large part to the ability of these two growth factors to differentially enhance the synthesis of various components that are associated with the basement membrane, such as type IV collagen and laminin [62, 136–138]. EGF is also important developmentally within the mammary gland because it is found in relatively high concentrations in human milk and because it can stimulate the lobuloalveolar development of the mouse mammary gland in explant cultures in vitro or after local administration to the mammary gland in vivo [139–143]. Finally, some evidence suggests that EGF may be involved in the in vivo initiation and/or progression of certain spontaneous or mouse mammary tumor virus (MMTV)-induced mammary tumors [55, 135].

Mouse, rat, and human TGF–α peptides are functionally related to EGF, although they exhibit only a 30% to 40% amino acid sequence homology to either mouse or human EGF [26]. In this respect, TGF–α belongs to the EGF

supergene family of proteins that includes several additional growth factors, such as vaccinia virus growth factor (VVGF), myxoma growth factor (MGF), Shope fibroma growth factor (SFGF), amphiregulin, cripto, and possibly the pS2 protein [124, 144–148]. Other proteins in this family that contain EGF-like repeat units at their amino terminus and cysteine residue homology but are not bona fide growth factors are tissue-plasminogen activator, the low-density lipoprotein receptor, various mammalian clotting factors, and the *Drosophila* notch gene protein [144, 148]. TGF–α binds to and interacts exclusively through the EGFR and is a potent mitogen for both mesenchymal and epithelial cells [26, 149]. TGF–α can cooperate with other growth factors, such as TGF–β, PDGF, IGF–II, or basic FGF, to reversibly induce the anchorage-independent growth of nontransformed cells in soft agar and can phenotypically produce a series of morphological changes in fibroblasts that are reminiscent of those changes that are observed after transformation with specific oncogenes, such as *ras* [27, 150–153]. These latter responses are probably due to the ability of TGF–α and of *ras* to alter either the synthesis and/or the subcellular distribution of a series of cytoskeletal proteins, such as specific isoforms of tropomyosin [154, 155]. In addition to these activities, TGF–α is alos a chemoattractant for some cells in vitro, which may ultimately relate to its ability to function as a strong angiogenic factor in vivo for capillary endothelial cells [156–158].

TGF–α is a low-molecular-weight, acid- and heat-stable, 50 amino acid peptide (M_r 5,600). TGF–α is initially synthesized as a high-molecular-weight, 159 (rat) or 160 (human) amino acid, transmembrane precursor of approximately 18–20 kD [26, 159]. The cell-associated precursor is biologically active [160, 161]. The precursor is usually *N*- or *O*-glycosylated in the extracellular NH_2-terminal domain, while palmitate is acylated to a cysteine residue(s) in the intracellular COOH-terminal domain. The 50 amino acid TGF–α peptide is found in the external NH_2-terminal region of the precursor and is flanked by a pair of alanine and valine residues from which it can be potentially cleaved by elastase-like proteases. A number of soluble high-molecular-weight forms of TGF–α are found in the conditioned medium (CM) of several rodent and human tumor cell lines. These are referred to as *meso* species and range in molecular weight from 25,000 to 45,000 [160]. They are apparently derived from the NH_2 terminus of the membrane-associated precursor by differential proteolytic processing and exhibit varying degrees of glycosylation [159, 160]. In some cell types, these secreted *meso* forms are biologically active and immunologically reactive [160].

The genes for mouse, rat, and human TGF–α have been cloned and sequenced from cDNA libraries [26, 28, 30]. Recently, 5′ upstream flanking regions of the human TGF–α gene have been sequenced from a genomic library [162]. Different enhancer-like motifs that have the potential of binding various DNA *trans*-acting factors, such as Ap–2 and SP–1, have been identified in a region ranging from −50 to −1150 base pairs (bp) upstream of the initiating methionine ATG codon. The TGF–α promoter lacks a classical

TATA box but does contain a sequence in or close to the transcription initiation site that might perform an analogous function. Using either nick-translated labeled TGF–α cDNA inserts or labeled TGF–α antisense RNA riboprobes, a major mRNA species of 4.5–4.8 kb and, in some instances, minor transcripts ranging from 1.6 to 2.2 kb have been detected in a number of rodent and human carcinoma and sarcoma cell lines and in several primary human carcinomas but not in lymphoid malignancies [107, 108, 115, 163–165]. TGF–α is not entirely restricted to malignant cells. For example, TFG–α mRNA and/or protein have been found in the developing rat and mouse embryo; in bovine and porcine pituitary cells; in rat Sertoli and peritubular cells; in regenerating and proliferating rat hepatocytes; in neurons from rat, bovine, and human brain; in human keratinocytes; in human mammary epithelial cells; and in activated human lung alveolar macrophages, suggesting that this growth factor may be performing some physiological function in a normal population of cells [53, 119, 166–173]. Nevertheless, overexpression of this growth factor may be sufficient for or contribute to the transformation of certain cells that possess a functional complement of EGFRs. In this regard, high levels of EGF or TGF–α expression after transfection or infection of appropriate expression vector plasmids into immortalized rodent fibroblasts, such as rat–1 cells or normal rat kidney (NRK) cells, or into epithelial cells, such as the NOG–8 mouse mammary epithelial cell line, can lead to the in vitro transformation and in vivo tumorigenicity of these cells [174–177]. TGF–α may also function as a local growth factor for benign lesions in vivo, because overexpression of this gene in mouse skin papillomas can lead to an increase in the size but not in the frequency of squamous cell carcinomas that ultimately develop from some but not all of these lesions [178].

The present review attempts to summarize the experimental and clinical information that is currently available regarding the involvement of *ras* gene expression and its potential relationship to the distribution or control of TGF–α production in the etiology and progression of rodent and human breast cancer. Such an exercise may not be entirely unreasonable, because it has recently been shown that gastric carcinomas express TGF–α mRNA and that coexpression of the p21 c–Ha–*ras* protein and TGF–α occurs in patients with invasive gastric carcinomas [179, 180]. In fact, patients with tumors that express both proteins have an extremely poor prognosis compared to patients with tumors in which only one of these proteins is expressed [179].

Expression of *ras* and TGF–α in rat and mouse mammary tumors and in transformed mouse mammary epithelial cell lines

Expression of ras *and TGF–α in rat mammary tumors*

Rodent mammary tumors represent appropriate animal model systems in which to study the potential interactions between carcinogens, retroviruses,

hormones, and growth factors, with respect to the initiation and progression of breast cancer. This is particularly true in Sprague–Dawley and Buffalo/N rats, in which well-differentiated ductal adenocarcinomas can be produced with a high frequency after the administration of chemical carcinogens, such as DMBA and NMU [10, 181–183]. The appearance of tumors occurs in a majority of these animals with a latency period of 60 to 90 days. In both cases, the effects of the carcinogen can be hormonally manipulated, because ovariectomy before carcinogen administration markedly decreases the incidence of tumor formation [181, 182, 184–186]. In addition, both sets of primary carcinogen-induced tumors are hormone dependent and responsive to antiestrogens in a fashion that is similar to that observed in human breast cancer. Ovariectomy and hypophysectomy result in tumor regression, suggesting that estrogen and other pituitary-derived mammotrophic hormones are important in regulating the growth of these tumors. This is the case because administration of estrogens, prolactin, and growth hormone to castrated rats can restore tumor growth in vivo [187]. In fact, both DMBA- and NMU-induced rat mammary tumors possess functional estrogen receptors and a low but detectable complement of EGFRs, suggesting that they have the capacity to respond to EGF and TGF–α [51]. After serial transplantation, a major fraction of both the DMBA-and NMU-induced rat mammary adenocarcinomas will develop into undifferentiated carcinomas that are hormone independent [51, 181, 183].

Barbacid et al. [184] first described the genetic and molecular changes that were associated with these primary carcinogen-induced rat mammary tumors. Approximately 85% of the NMU-induced tumors were found to contain an activated c–Ha–*ras* proto-oncogene early in the development of the tumor with a single point mutation in codon 12 (i.e., G to A mutation), resulting in a transforming $p21^{ras}$ protein with a glutamic acid in place of glycine. DNA extracted from these tumors could induce foci formation in NIH–3T3 cells after transfection, and such foci contained a point-mutated c–Ha–*ras* proto-oncogene at a position that was identical to that found in the original tumors. In the small percentage of primary NMU-induced carcinomas that were hormone independent, a similar frequency of tumors also contained a point-mutated *c–Ha–ras* proto-oncogene. Barbacid et al. [185, 186] further demonstrated that the primary DMBA-induced rat mammay tumors also contained a G to A mutation but in this case at codon 61 in the $p21^{ras}$ protein [185, 186]. Subsequently, it was observed that estrogen-dependent, DMBA-induced tumors also contained a fivefold to sevenfold higher level of $p21^{ras}$ protein than did the estrogen-independent tumors or normal virgin rat mammary glands [188].

Activation of a *ras* proto-oncogene may lead to a change in the response of mammary epithelial cells either to different growth factors or to an enhanced production of endogenous growth factors [46, 105, 107, 112, 114, 150]. This is apparently the case for these carcinogen-induced rat mammary tumor cells. Primary cultures of rat mammary tumor cells that were isolated from the

DMBA-induced rat mammary tumors were found to be generally less reponsive to the mitogenic effects of EGF than were mammary epithelial cells that had been isolated from normal virgin rat mammary glands [136, 189]. This difference could be partially accounted for by a reduction in the levels of EGFR expression that was observed on these tumor cells, suggesting that they may be elaborating an EGF-like factor [101, 102, 112]. Subsequently, it was found that an activity, mammary tumor factor (MTF), was elaborated by these tumor cells in vitro and in vivo [189]. MTF was partially purified from both the DMBA- and NMU-induced rat mammary tumors and was found to be an EGF/TGF–α-like growth factor. MTF existed as two species of 6 kD and 60 kD. Both forms could inhibit the binding of ^{125}I-labeled EGF to EGFRs in a radioreceptor assay (RRA), and both could stimulate the anchorage-independent growth of NRK fibroblasts in soft agar. Very little MTF activity could be detected in the transplantable DMBA–1 and NMU–II tumors that do not contain a point-mutated c–Ha–*ras* proto-oncogene [51, 189]. More recently, Liu et al. [190] have demonstrated that MTF actually represents TGF–α because authentic immunoreactive TGF–α protein and a specific 4.8 kb TGF–α mRNA transcript could be found in the primary DMBA- and NMU-induced tumors, and that they were present at a reduced or undetectable level in the transplantable DMBA–1 and NMU–II tumors. More importantly, it was demonstrated that estrogen could modulate TGF–α expression both in vitro and in vivo in the primary estrogen-dependent DMBA- and NMU-induced tumors. Ovariectomy resulted in a rapid decline of TGF–α mRNA levels in vivo within 6 hours, whereas primary cultures of DMBA- or NMU-induced tumor cells exhibited a fivefold increase in the levels of TGF–α mRNA and TGF–α protein after treatment with 17β–estradiol (10^{-8} M) for 4 days. Ethier et al. [191] have extended these studies and have shown that rat mammary tumor cells obtained from DMBA- or NMU-induced tumors exhibit a higher growth rate in a serum-free medium compared with the growth potential of normal rat mammary epithelial cells. In addition, they observed that a subset of either DMBA- or NMU-induced tumors eventually could give rise to a population of tumor cells in vitro that showed no growth response to either exogenous EGF or to IGF–I or insulin. The growth factor-dependent cell lines were able to develop into normal or benign hyperplastic outgrowths, whereas the growth factor-independent clones produced tumors when transplanted into syngeneic rats. These results suggest that the primary carcinogen-induced rat mammary tumors intrinsically contain a subpopulation of cells that no longer require exogenous growth factors for their growth and that these cells are highly tumorigenic. It is possible that these cells have now acquired the ability to synthesize their own endogenous growth factors. Moreover, different morphological populations of cells within these tumors may respond to or produce different sets of growth factors depending on their interaction with adjacent normal tissue [192, 193].

The development of mammary carcinomas in mice offers an even more diverse and complex picture than in the rat. For example, various carcinogens (such as DMBA and urethane), hormones (such as estrogens and prolactin), and viruses (such as different strains of MMTV) have been shown to be involved in the etiology and the progression of these tumors [194]. In addition, development of certain mouse mammary carcinomas, unlike the rat carcinomas, can be shown to arise from preexisting preneoplastic lesions, hyperplastic alveolar nodules (HANs) and ductal hyperplasias [194]. These lesions can spontaneously develop into well-differentiated adenocarcinomas after serial transplantation. However, the frequency of such conversions can be greatly enhanced by a number of agents, including DMBA and chronic hormonal stimulation. In contrast to normal mammary epithelial cells that exhibit a finite lifespan in vivo, HANs can be repeatedly transplanted in vivo as hyperplastic outgrowths (HPOs) and therefore represent a potential immortalized population of cells that are probably more sensitive to environmental insult and subsequent neoplastic transformation because 80% to 90% of DMBA-treated HANs give rise to carcinomas [195, 196]. HANs are generally hormone independent with respect to their growth in vivo, yet they are still hormonally responsive for the induction of milk proteins, such as casein and α–lactalbumin. The possible role c–Ha–*ras* proto-oncogene activation in the progression from HANs to mammary adenocarcinomas has been studied in serially transplanted HPOs treated with the chemical carcinogen DMBA. DMBA treatment dramatically increases the incidence of tumor progression of these preneoplastic outgrowths and activates the c–Ha–*ras* proto-oncogene, as demonstrated by the NIH–3T3 transfection assay, by causing a specific point mutation at codon 61 [195]. In contrast, several DMBA-induced premalignant mammary hyperplasias do not possess an activated c–Ha–*ras* proto-oncogene [196]. Collectively, these results suggest that the activation of the c–Ha–*ras* proto-oncogene may play an important role in the progression from hyperplasia to carcinoma induced by DMBA, but not in the initiation or maintenance of DMBA-induced hyperplasias in the mouse.

A novel approach to study the potential role of oncogenes during in vivo tumorigenesis has been to insert specific DNA sequences into the germline of transgenic animals. To ensure the expression of the transgene in the mammary gland, Andres et al. [197] have stably inserted the activated human c–Ha–*ras* proto-oncogene under the transcriptional control of the promoter region of the mouse whey acidic protein (Wap) gene into the germline of five transgenic mice. The Wap gene is normally expressed in mammary epithelial cells during late pregnancy and lactation in response to lactogenic hormones, such as prolactin, insulin, and hydrocortisone. A low incidence of mammary tumors was found in a female founder animal after a long latency period (325 days) and several pregnancies, suggesting that the activated human c–Ha–*ras*

proto-oncogene is not sufficient by itself and that other events or genetic changes are necessary for the subsequent transformation of mouse mammary cells in vivo. In addition, the same group has reported that transgenic mice carrying a Wap–c–*myc* fusion proto-oncogene develop hormone-independent, well-differentiated mammary tumors in pregnant females at a higher frequency (approximately 80%) and with a shorter latency period than do transgenic mice carrying the Wap–Ha–*ras* fusion proto-oncogene [198]. The different tumorigenic potential of the activated Wap–*myc* and the Wap–*ras* proto-oncogenes in the mammary epithelium may be attributed to the developmental stage in which the oncogene is activated, because the endogenous Wap promoter is hormonally stimulated during late pregnancy and during lactation in the mammary gland. Deregulation of *myc* proto-oncogene expression is apparently more efficient than deregulated Ha–*ras* gene expression with respect to the transformation of differentiated secretory mammary alveolar epithelial cells in vivo. In these *myc*-derived tumors, casein and α–lactalbumin production were found to be constitutive and independent of hormone stimulation. Collectively, these results suggest that the level of *myc* gene expression can be indirectly controlled by the differentiated state of the cells as well as by the type of recipient cell in which it is being expressed. Moreover, when driven by a tissue-specific and developmentally regulated promoter, c–*myc* can induce the transformation of mammary epithelial cells in both the early and the late stages of mammary gland development. In addition, Leder's group [199] has demonstrated that transgenic mice bearing either the c–*myc* proto-oncogene or the v–Ha–*ras* oncogene driven by glucocorticoid-inducible MMTV promoter/enhancer sequences develop sporadic focal mammary carcinomas with a long latency period, averaging 325 days for the *myc*-carrying animals and 168 days for the Ha–*ras*-carrying mice, respectively. Similar results with transgenic mice have been described by Tremblay et al. [200] after introduction of a comparable MMTV/v–Ha–*ras* fusion gene. When the MMTV/c–*myc* and the MMTV/v–Ha–*ras* founders are mated to yield hybrid mice, there is a synergistic effect of the two oncogenes on the frequency of tumor formation. Almost all of the animals develop tumors within 150 to 180 days, and the latency period is decreased to an average of 46 days [199]. However, because these tumors arise stochastically in the mammary gland and are of monoclonal origin, other genetic or somatic events in addition to the presence of an activated *myc* and *ras* proto-oncogene are most likely necessary for their malignant progression in vivo.

The role of c–Ha–*ras* proto-oncogene in the transformation process of mammary epithelial cells has been extensively studied in vitro. An activated c–Ha–*ras* oncogene is able to transform spontaneously immortalized mouse mammary epithelial cell lines. Hynes et al. [201] have shown that after transfection of human activated, point-mutated c–Ha–*ras* proto-oncogene into NMuMg cells, a normal polyclonal mouse mammary epithelial cell line isolated from the mammary gland of Namru mice, the *ras*-transfected cells

acquire anchorage-independent growth in semisolid medium and form invasive undifferentiated carcinomas when injected into nude mice. Moreover, the degree of transformation of these cells is dependent on the level of c–Ha–*ras* gene expression. After infecting NOG–8 cells, an epithelial subclone of NMuMg cells, with a recombinant v–Ha–*ras* containing retrovirus, several individual clones of cells expressing low (SR3 cells), moderate (SR1 cells), or high (SR2 cells) levels of v–Ha–*ras*-specific mRNA and of $p21^{ras}$ protein were isolated [202]. SR3 cells have a 0.05% cloning efficiency in soft agar and form tumors in nude mice with a latency period of approximately 20 weeks. In contrast, SR2 cells grow with cloning efficiency of approximately 40% in semisolid medium and form palpable tumors in nude mice after only 1 to 2 weeks, whereas SR1 cells exhibit an intermediate behavior both in vitro and in vivo [202]. Furthermore, the introduction of the v–Ha–*ras* oncogene into primary cultures of mouse mammary epithelial cells prepared from mid-pregnant animals can lead to a fully transformed phenotype only in mammary epithelial cells that express at least eightfold more v–Ha–*ras*-spcific mRNA and $p21^{ras}$ protein than the levels found in the highly malignant mammary epithelial SR2 cell line.

An activated *ras* proto-oncogene may affect either the response of mammary epithelial cells to specific growth factors or the production of endogenous growth factors. Therefore, the growth under anchorage-dependent and anchorage-independent culture conditions in response to exogenous EGF or TGF–α, and the levels of production of endogenous TGF–α were examined in NMuMG cells that had been transformed with a transfected plasmid containing the human point-mutated c–Ha–*ras* proto-oncogene (NMuMG/*ras* cells). Exogenous EGF or TGF–α was unable to stimulate the growth of these *ras*-transformed NMuMG cells both in monolayer culture and in soft agar. One mechanism by which fibroblasts transformed with various oncogenes (such as *ras*, *mos*, *fms*, *fes*, *abl*, *met*, and *trk*) may lose their growth responsiveness to exogenous EGF is through a reduction in or a total absence of available cell surface EGFRs on these cells [44, 46, 104, 105, 107, 108]. The reduction in EGF binding to these cells may be due in part to an increase in the secretion of endogenous TGF–α [105–108]. In fact, NMuMG/*ras* cells exhibited a three to five fold increase in the expression of a TGF–α 4.8 kb mRNA species and a comparable increase in the secretion of immunoreactive and biologically active TGF–α protein into their conditioned medium (CM). In addition, there was a 60% reduction in the total number of cell surface receptors for EGF that could be detected on these cells as compared to the nontransformed NMuMG cells [115]. To further examine whether TGF–α production is coordinately and functionally linked to *ras* transformation in mouse mammary epithelial cells, NOG–8 cells have been cotransfected with the pSV2*neo* plasmid and a plasmid containing the human activated c–Ha–*ras* proto-oncogene that has been placed under the transcriptional control of the glucocorticoid-inducible MMTV LTR [203]. The resulting cell line that was obtained following G418 selection and cloning was designated NOG–8

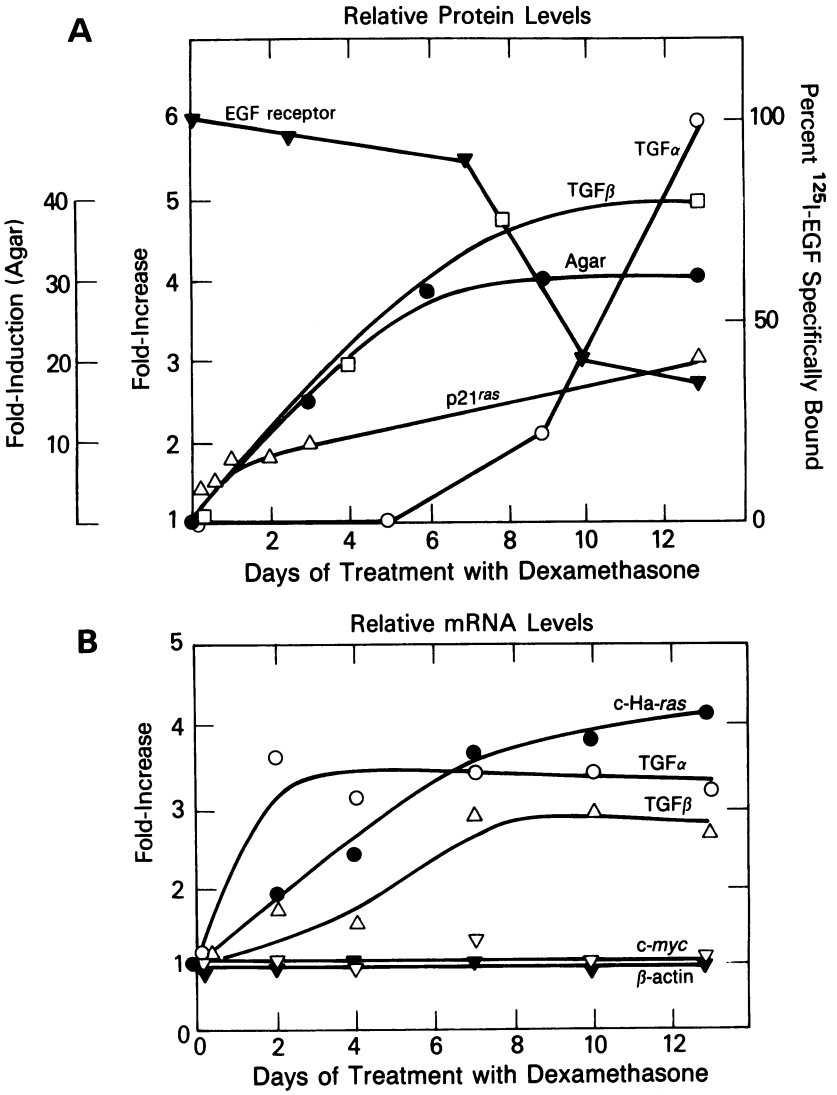

Figure 1. Temporal summary of the biological effects following induction of point-mutated c-Ha-*ras* in NOG-8 *ras* cells. Cells were treated with 10^{-6} M dexamethasone for the indicated time periods and subsequently analyzed for the indicated parameters. A. Phenotypic changes observed in NOG-8 *ras* cells: Δ-Δ $p21^{ras}$ protein levels as detected by Western blotting; ●-● soft agar growth; ○-○ TGF-α protein secreted into the CM as determined by RIA and RRA; □-□ TGF-β protein secreted into the CM as measured by RRA; ▼-▼ ^{125}I-EGF-specific binding. B. Densitometric scan of Northern blot analysis of poly(A)$^+$ RNA isolated from NOG-8 *ras* cells and hybridized to the appropriate ^{32}P-labeled cDNA probes, which detected the following mRNA transcripts: ●-● c-Ha-*ras*; ○-○ TGF-α; Δ-Δ TGF-β; ∇-∇ c-*myc*; ▼-▼ β-actin.

ras. Figure 1 summarizes the biological properties of NOG–8 *ras* cells treated for different periods of time with dexamethasone. In the absence of the glucocorticoid, NOG–8 *ras* cells appear flat and cuboidal, exhibit contact inhibition of growth, are unable to grow in anchorage-independent conditions in soft agar, and secrete low levels (5–10 ng/10^8 cells/48 hours) of TGF–α protein into their CM. After addition of dexamethasone, there is a rapid induction of $p21^{ras}$ protein within 1 to 3 hours that occurs concomitantly with a parallel induction of a 1.5 kb c–Ha–*ras*-specific mRNA. After 3 to 6 days of steroid treatment, NOG–8 *ras* are able to grow as colonies in soft agar. Analysis of TGF–α-specific mRNA levels demonstrates that there is an increased expression of TGF–α mRNA that occurs simultaneously with the elevation in c–Ha–*ras* mRNA and $p21^{ras}$ protein. The levels of biologically active and immunoreactive TGF–α in the CM of NOG–8 *ras* cells are increased five to six fold after 9 to 12 days of glucocorticoid treatment. During this interval, there is a concomitant 60% to 65% reduction in the number of EGFRs, as the cells assume a more transformed morphology (i.e., foci formation) and exhibit an enhanced growth rate in serum-free monolayer cultures. Exogenous TGF–α is able to stimulate the anchorage-dependent and anchorage-independent growth of nonsteroid-treated NOG–8 *ras* cells to a level that is comparable with that observed in these cells after dexamethasone treatment and after subsequent induction of the activated *ras* proto-oncogene. In addition to TGF–α, the secretion of other growth factors, such as IGF–I and TGF–β, is also increased in NOG–8 *ras* cells after *ras* induction [203]. The enhanced production and secretion of TGF–α, TGF–β, and IGF–I are specific for these growth factors, because no changes in the levels of c–*myc* and β–actin mRNA can be observed. Moreover, NOG–8 *ras* cells do not express specific mRNA transcripts for other endogenous growth factors, such as basic FGF and PDGF A or B chains. These results demonstrate that overproduction of TGF–α may be one important early event in the transformation process that is induced by an activated c–Ha–*ras* proto-oncogene in mouse mammary epithelial cells. Enhanced levels of TGF–α secretion may be necessary at some point in mediating the anchorage-independent growth, the morphological changes, and the enhanced growth rate of *ras*-transformed mammary epithelial cells, possibly in combination with other endogenously secreted growth factors, such as IGF–I and TGF–β. We have also recently found that the amount of TGF–α produced in Ha–*ras*-transformed mouse mammary epithelial cells is dependent on the levels of $p21^{ras}$ expression in these cells and correlates with the cloning efficiency of these cells in soft agar. For example, NOG–8 SR2 cells [202] express three to four fold more $p21^{ras}$ than do NOG–8 SR1 cells and exhibit twice the soft agar colony-forming ability as do NOG–8 SR1 cells. These differences are paralleled by a twofold to threefold higher level of TGF–α production in the NOG–8 SR2 cells compared with the NOG–8 SR1 cells.

The previous studies circumstantially suggest that overexpression of TGF–α in *ras*-transformed mouse mammary epithelial cells may play an

Table 4. Biological properties of transfected TGF–α-expressing NOG–8 mouse mammary epithelial clones.

Clones	Soft agar growth[a] (no. of colonies/dish)	TGF–α secreted[b] (ng/10^8 cells/ 48 hours)		EGF receptors[c] (total sites/cell)
		RIA	RRA	
NOG–8	0 (0/10)	7	7	95,000
TF C1 2 (pSV2*neo*)	4 (0/5)	14	7	91,000
TF C1 5	1048 (8/10)	312	296	12,000
TF C1 7	1297 (10/10)	610	595	16,000
TF C1 10	1054 (5/5)	339	177	44,000
TF C1 13	1103 (0/5)	120	36	41,000

[a]Soft agar growth represents the average of quadruplicate determinations. The Standard deviation was less than 10%. Values in parentheses represent the number of tumors per number of animals injected with 5×10^6 cells s.c.

[b]TGF–α protein from concentrated CM was evaluated in a TGF–α-specific radioimmunoassay (RIA) and in an EGF/TGF–α radioreceptor assay (RRA). Values represent the average of quadruplicate determinations. The standard deviation was less than 10%.

[c]EGF binding sites per cell were calculated by Scatchard analysis from the specific binding isotherms using different concentrations of mouse ^{125}I–EGF.

important role through an autocrine pathway as a proximal effector of *ras*-induced transformation. To ascertain whether the inappropriate, constitutive overexpression of TGF–α is sufficient to facilitate the transformation of an already immortalized population of mouse mammary cells in which there is a sufficient complement of functional EGFRs, NOG–8 cells were cotransfected with an SV40 promoter-human TGF–α expression vector plasmid and the pSV2*neo* plasmid [177]. After cotransfection, 9 of 180 G418-resistant NOG–8 colonies were cloned and expanded. All clones were subsequently analyzed for TGF–α mRNA expression by Northern blot analysis, TGF–α secretion, anchorage-dependent growth in serum-free medium, anchorage-independent growth in soft agar, and tumorigenicity in nude mice. The biological characteristics of representative TGF–α-transfected NOG–8 clones are summarized in table 4. Three TGF–α-transfected NOG–8 clones (TF CI 5, 7, and 10) secrete 10-fold to 80-fold higher levels of TGF–α protein into their CM (300–600 ng of immunoreactive TGF–α/10^8 cells/48 hours) than do the parental NOG–8 cells or the pSV2*neo*-transfected clones. The majority of the immunoreactive TGF–α that is secreted into the CM from these three clones is also biologically active, because it is able to compete with ^{125}I–EGF for binding to the EGFRs in an EGF/TGF–α RRA. In contrast, NOG–8 TF CI 13 cells produce intermediate levels of immunoreactive TGF–α of which only 30% is biologically active. The TGF–α-transfected NOG–8 clones that are producing elevated levels of TGF–α protein also express high levels of a 2.3 kb TGF–α mRNA species, which is consistent with the size of the mRNA originating from the expression vector plasmid cDNA insert. In addition,

Southern analysis of DNA isolated from these high TGF–α-expressing clones demonstrates the presence of multiple copies of the plasmid expression vector DNA. Whereas NOG–8 cells transfected only with the pSV2*neo* plasmid are not able to grow in soft agar, NOG–8 TF C1 5, 7, 10, and 13 cells are able to form colonies in soft agar at a cloning efficiency equivalent to that of NOG–8 cells transformed with a point-mutated c–Ha–*ras* proto-oncogene. In addition, high TGF–α-producing clones pile up and form foci-like structures at high cell density and exhibit a fourfold to sixfold increase in their anchorage-dependent growth rate in serum-free medium compared with the pSV2*neo*-transfected NOG–8 cells. More importantly, NOG–8 TF C1 5, 7, and 10 cells form undifferentiated, invasive carcinomas when injected into nude mice at a frequency of 80% to 100% within 2 to 4 weeks. In contrast, NOG–8 TF C1 13 cells, which secrete 5-fold to 16-fold less biologically active TGF–α than do NOG–8 TF C1 5, 7, and 10 cells and are able to grow as colonies in soft agar, are unable to form tumors in vivo. These results suggest that there may be a threshold level of TGF–α that has to be exceeded before tumorigenicity in vivo can be achieved, and this threshold is less than the level that is apparently necessary to induce anchorage-independent growth in soft agar. NOG–8 TF C1 5 and 7 cells, which secrete the highest levels of biologically active TGF–α, exhibit an 80% to 90% reduction in the specific binding of ^{125}I–EGF compared with parental NOG–8 cells or pSV2*neo*-transfected NOG–8 cells. NOG–8 TF C1 10 and 13 cells, which secrete intermediate levels of biologically active TGF–α, show a 50% to 60% reduction in ^{125}I–EGF-specific binding. These data suggest that the high levels of secreted TGF–α are inducing a chronic occupation and down regulation of the EGFRs on the cell membrane of these cells. A neutralizing anti-TGF–α mouse monoclonal antibody generated against the low-molecular-weight human TGF–α protein was able to inhibit in a dose-dependent fashion the colony formation in soft agar of TGF–α-transfected NOG–8 clones [177]. Collectively, the results of this study demonstrate that the enhanced constitutive overexpression of the TGF–α gene in an immortalized population of mouse mammary epithelial cells which is responsive to EGF may be sufficient at a critical threshold level to induce a transformed phenotype in vitro and tumorigenicity in vivo and that such events can occur mainly but not exclusively through an external autocrine-dependent loop in these cells.

Expression of *ras* and TGF–α in human breast carcinomas and in transformed human mammary epithelial cell lines

Several prognostic variables have been identified for human breast cancer. These include estrogen receptor (ER) and progesterone receptor (PgR) status, tumor size, histologic grade, nuclear grade, thymidine labeling index (TLI), and axillary lymph node involvement [48, 49]. Some of these, such as

axillary lymph node and steroid receptor status, offer a limited degree of reliability for monitoring tumor recurrence, patient relapse, and overall survival. Other novel markers are therefore needed to supplement these more classical indicators for prognostic purposes as well as for segregating patients into appropriate therapy protocols. The presence of activated proto-oncogenes or deleted antioncogenes in human breast tumors (table 1) may fulfill some of these criteria [33, 56, 58, 59]. Amplification, rearrangements, and deletions of specific proto-oncogenes may be particularly informative with respect to the onset and progression of human breast cancer. Such changes may in some cases be accompanied by alterations in the expression of distinct oncoproteins. For instance, c–*erb*B–2 appears to be an important independent indicator of prognosis in breast cancer [37, 59]. Amplification and/or overexpression of the c–*erb*B–2 proto-oncogene is found in approximately 20% to 30% of primary human breast tumors and is generally associated with poor prognosis. Likewise, the level of c–*erb*B (EGFR) expression is also important with respect to tumor recurrence and patient survival [37, 59]. This is particularly relevant to the potential role of TGF–α in breast cancer, because TGF–α interacts exclusively through the EGFR and because the presence of an activated *ras* proto-oncogene might modulate either TGF–α or EGFR expression. Therefore, it may be possible to phenotype breast carcinomas with respect to the presence and the amount of TGF–α protein and/or TGF–α mRNA and its association with other prognostic proto-oncogene markers.

Expression of ras *in normal and malignant human breast tissues*

The involvement of the *ras* gene in the pathogenesis of human breast cancer has received a good deal of attention because it was first assumed that a subset of these human tumors may contain a point-mutated *ras* gene, as had been demonstrated in rat mammary tumors [10, 184, 185]. However, no point mutations have been found in either the Ha–, Ki–, or N–*ras* proto-oncogenes in human breast tumors, suggesting that if these mutations exist, they are relatively rare. The levels and localization of *ras* expression in normal and malignant breast tissues have been examined and quantitated by analyzing breast tissue samples for the expression of *ras*-related mRNA transcripts by Northern blot analysis or by dot blotting and for $p21^{ras}$ protein by immunocytochemistry or by immunoblotting.

In the majority of cases that have been examined, c–Ha–*ras* mRNA is expressed in biopsies of both normal and malignant breast tissues. N–*ras* and Ki–*ras* mRNA transcripts are expressed in several human breast cancer cell lines as well as in normal human mammary epithelial cells (table 5). The initial reports on the expression of c–Ha–*ras* mRNA in human breast tissues indicated that the levels of c–Ha–*ras* mRNA were more prominent in tumor samples. For example, in breast tumors and adjacent normal tissue obtained from 12 patients, c–Ha–*ras* mRNA expression was significantly increased in

Table 5. Phenotypic properties of normal and transformed human mammary epithelial cell strains and cell lines.[a]

Cell line	Origin	ER	EGFR	EGF response	TGF-α production	*ras* expression	Tumorigenicity
MCF-7	Pleural effusion	1.5×10^5	1×10^4	Growth stimulated	+ E_2 inducible[a]	Amplified N-*ras*	+ E_2 dependent
MCF-7-*ras*	v-Ha-*ras* transfection of MCF-7	1.5×10^5	1×10^4	Growth stimulated	++	Overexpress v-Ha-*ras*	+++
MDA-MB-231	Pleural effusion	–	2×10^5	Not responsive	++	Point-mutated c-Ki-*ras*	+++
MDA-MB-468	Solid tumor	–	1.5×10^6	Inhibited	++	Not determined	+++
Hs578T	Solid tumor carcinosarcoma	–	9×10^4	Slight inhibition	–	Point-mutated c-Ha-*ras*	+++
184	Reduction mammoplasty	–	4×10^5	EGF dependent	++	c-Ki-*ras*	–
184A1N4	Benzo[a]pyrene-treated 184	–	3×10^5	EGF dependent	++	c-Ki-*ras*	–
184A1N4-*ras*	v-Ha-*ras*-transformed 184A1N4	–	3×10^5	Not responsive	++	Overexpress v-Ha-*ras*	+
184A1N4-SV40T-*ras*	SV40T and v-Ha-*ras*-transformed 184A1N4	–	2×10^5	Not responsive	++	Overexpress SV40T and v-Ha-*ras*	+++

[a] E_2, estrogen.

all the breast cancer samples [204], which is similar to observations made in another early report [9]. Subsequently, it was found that higher levels of c–Ha–*ras* mRNA expression could be detected in infiltrating ductal carcinomas from node-positive patients compared with tumors obtained from node-negative patients [205]. Theillet et al. [206] also observed that 16 of 22 (73%) breast tumors expressed a 1.4 kb c–Ha–*ras* mRNA, as detected by Northern blot analysis. No expression of N–*ras* or c–Ki–*ras* mRNA transcripts could be detected in this group of tumor samples.

The enhanced transcription of the c–Ha–*ras* gene that is observed in breast tumors suggests that this may result in an elevated level of $p21^{ras}$ protein expression. A number of immunocytochemical studies have demonstrated the presence of $p21^{ras}$ in breast biopsies using two different antibodies, Y13–259 and RAP–5. Y13–259 is a rat monoclonal IgG that was raised against the viral Ha–$p21^{ras}$ protein and recognizes Ha–, Ki–, and N–*ras* proteins in both frozen and paraffin-embedded tissues [207]. RAP–5 is a mouse monoclonal IgG_{2a} that was generated against a synthetic peptide corresponding to amino acids 10 to 17 of the c–Ha–$p21^{ras}$ protein and recognizes both the v– and c–*ras* forms of the three *ras* types [15]. Using the RAP–5 antibody, Horan–Hand et al. [15] found that 27 of 30 (90%) infiltrating ductal carcinomas were positive for $p21^{ras}$ staining, of which 19 cases (63%) exhibited staining of more than 20% of the tumor cells. Only 2 of 10 (20%) fibroadenomas and 0 of 11 fibrocystic disease samples showed comparably high amounts of staining for $p21^{ras}$, whereas 5 of 5 regional lymph node metastases and 4 of 4 distal metastases all demonstrated $p21^{ras}$ expression. In a subsequent study by the same group [208], 36 of 47 (77%) infiltrating ductal carcinoma samples showed $\geq$50% of the carcinoma cells expressing $p21^{ras}$. Gradually decreasing levels of $p21^{ras}$ staining were found in samples of hyperplasia with atypia (10 cases) and in samples of hyperplasia without atypia (16 cases). Little or no staining was detected in 20 benign fibrocystic lesions without hyperplasia. Eighteen hyperplastic lesions were also studied from patients for which 15-year follow-up information was available [208]. Four of 10 patients with atypia but only 1 of 8 patients without atypia subsequently developed ductal carcinomas. In all of the 18 samples, moderate $p21^{ras}$ staining was also observed in the normal mammary epithelial and myoepithelial cells adjacent to the hyperplastic areas. It was pointed out that there was marked heterogeneity of staining, especially in metastatic lesions. Further, in 45 breast cancer samples in the same study, no correlation was found between ER status and $p21^{ras}$ staining. These results were confirmed and extended by Querzoli et al. [209] using both the Y13–259 and RAP–5 antibodies. They found positive staining for $p21^{ras}$ in 97 of 142 (68%) infiltrating primary breast tumor specimens. A reasonable correlation was found between histological subtype and $p21^{ras}$ expression, because 79 of 92 (86%) ductal carcinomas were positive in contrast to only 12 of 42 (29%) lobular carcinomas. Furthermore, a greater intensity of $p21^{ras}$ staining was found in ER-positive tumors.

Expression of $p21^{ras}$ may not be limited to malignant breast tissues, because varying levels of $p21^{ras}$ have also been found in both benign and malignant breast epithelium, using the same RAP–5 antibody [210]. In this study, a total of 47 specimens were analyzed, including 2 normal breast samples, 7 fibroadenomas, 12 fibrocystic disease samples, and 22 malignant tissues, of which 7 were infiltrating ductal carcinomas. Normal, benign, and malignant breast epithelial cells all demonstrated similar $p21^{ras}$ staining patterns and intensities. In addition, stromal fibroblasts, smooth muscle, and sweat glands also stained with the $p21^{ras}$ antibody. The authors thus concluded that the protein detected by the RAP–5 antibody is the normal cellular $p21^{ras}$ protein, whose expression is not necessarily enhanced in all neoplastic lesions.

Immunocytochemical studies using the Y13–259 antibody have also yielded comparable results in that both normal and benign tissues were stained with this antibody. Expression of $p21^{ras}$ protein was seen in all types of mammary epithelium but predominately in the malignant lesions [211]. In this study, only 12 of 33 (36%) cystic lesions and 5 of 22 (23%) fibroadenomas were positive for $p21^{ras}$ expression, whereas 17 of 20 (85%) carcinomas stained positive. No difference in the intensity of staining was seen between ductal or lobular carcinomas. Three additional studies have yielded similar results [212–214]. In one study, 20 invasive carcinomas, 3 fibroadenomas, and 5 cystic disease samples all showed a similar staining pattern, with slightly higher staining intensity along the luminal borders and in normal epithelium [212]. In all of the samples staining of some stromal cells was also noted, although less intensely than in the mammary epithelium. In a more recent study, Walker and Wilkinson [214] examined 14 benign proliferative breast disease samples, one 20-week pregnant breast tissue sample, 5 fibroadenomas, and 45 carcinomas of which 32 were infiltrating ductal carcinomas for $p21^{ras}$ expression. All of the samples were found to express $p21^{ras}$ to varying degrees. Uniform staining was observed in the normal epithelium, with stronger staining confined to hyperplastic areas. However, endothelium, nerves, and smooth muscle, but not fibroblasts, were also stained. Heterogeneous staining was observed in the 45 carcinomas, with 22 samples staining weaker and 14 stronger than the adjacent normal epithelium. A significant correlation was found between the level of $p21^{ras}$ staining in the carcinomas and the degree of tumor cell proliferation as determined by the expression of a cell cycle-specific antigen, since increased $p21^{ras}$ expression was observed in 30% of the tumors associated with higher proliferation rates [215–217]. No significant association was observed between $p21^{ras}$ expression and the degree of histological differentiation or axillary lymph node involvement.

Studies utilizing immunoblot analysis or radioimmunoassay (RIA) have also been performed with the Y13–259 antibody to more fully characterize the $p21^{ras}$ protein and to more accurately quantitate the levels of $p21^{ras}$ expression in normal, benign, and malignant breast tissues, since immunocytochemical evaluation is only semiquantitative. DeBortoli et al. [218] found

approximately tenfold higher levels of $p21^{ras}$ in 15 of 22 (68%) ER-positive and PgR-positive human breast tumors than in normal breast tissue, which was obtained from reduction mammoplasties. In contrast, 13 of 21 (62%) ER-negative and PgR-negative tumors exhibited only a threefold increase in $p21^{c\text{-}Ha\text{-}ras}$ levels compared with normal breast tissues. Overall, 41 of 43 (95%) tumor samples contained at least twofold higher levels of $p21^{ras}$ protein than did any of the 10 normal tissues or fibroadenomas examined. In a second larger study, 204 of 314 (65%) primary breast carcinomas exhibited $p21^{ras}$ levels exceeding those found in 35 normal breast tissues [219]. All lymph node metastases, as well as 9 of 11 distant metastases showed very high levels of $p21^{ras}$ expression. In small tumors without lymph node involvement, a positive correlation was found between $p21^{ras}$ expression and the presence of ER. In contrast, in ER-negative tumors, high levels of $p21^{ras}$ correlated with lymph node involvement. In a third study by the same group, similar results were obtained, demonstrating significant correlation between more advanced tumor stage, lymph node involvement, and elevated levels of $p21^{ras}$ protein [220]. Thirty-seven of 54 (69%) carcinomas contained $p21^{ras}$ levels that were twofold to tenfold greater than in normal breast tissue. Higher levels of $p21^{ras}$ protein were generally detected in more advanced stage tumors and in patients who had positive axillary lymph nodes. Increased levels of $p21^{ras}$ in invasive ductal carcinomas have also been detected by a liquid-phase RIA, using the Y13-259 antibody [57]. Ten of 15 (66%) breast carcinomas had $p21^{ras}$ levels ranging from 35 to 50 pg/μg protein compared with 2 to 7 pg $p21^{ras}$/μg protein in normal, fibrocystic, and hyperplastic breast tissue samples.

Alterations in the c–Ha–*ras* gene may also contribute to the abnormal expression of c–Ha–*ras* mRNA that has been observed in breast tumors. Such alterations may involve either deletions or rearrangements of regulatory elements or amplification of the gene. The c–Ha–*ras*-1 gene is located on chromosome 11, where specific restriction fragment length (allele) polymorphisms have been identified [221]. No evidence for amplification or major rearrangements of the Ki–, N–, or Ha–*ras* genes have been observed in primary human breast tumors after Southern blot analysis [206, 222]. However, polymorphisms with an increased incidence of rare c–Ha–*ras* alleles have been detected in breast cancer patients [222]. The frequency of rare c–Ha–*ras*-1 alleles was found to be significantly increased in DNA obtained from peripheral blood lymphocytes in 208 samples from patients with breast cancer compared with 112 normal healthy individuals [222]. In addition, the frequency of more common alleles, such as the 6.5 and 8.0 kb alleles, was significantly diminished among the breast cancer patient population. Of these, one rare allele was significantly increased in the DNA from breast carcinomas. Two subsequent studies have also found an increased frequency of rare c–Ha–*ras*-1 alleles in 97 [223] and 92 breast cancer patients [224], respectively, compared with healthy controls, suggesting that women with rare c–Ha–*ras* genotypes may have an increased risk for developing breast

cancer. In addition, loss of heterozygosity at the c–Ha–*ras*-1 locus in DNA from 15 of 51 (27%) breast tumors obtained from patients who were heterozygous at this locus showed a significant correlation with histologic grade, lack of ER and/or PgR, and poor prognosis [206]. This loss of one c–Ha–*ras*-1 allele was more frequent in the metastatic lesions than in the primary tumors [225]. Allelic deletions of c–Ha–*ras* gene were also apparently more common in tumors of higher histologic grade and in tumors that recurred earlier [226]. However, this parameter did not correlate significantly with either tumor size, lymph node positivity, or tumor stage [226]. In contrast, in two recent studies, no significant differences were observed in the distribution of any c–Ha–*ras* alleles among 62 healthy individuals and 61 breast cancer patients [227] or between 101 controls and 80 breast cancer patients [228], respectively, suggesting that other environmental or genetic factors may contribute to the onset and progression of this disease [47].

TGF–α expression in normal and malignant human breast tissues

TGF–α mRNA is expressed in a number of different human tumors and human tumor cell lines, where a significant correlation was observed with concurrent EGFR mRNA expression [163]. Recent studies have demonstrated that between 40% and 70% of primary human breast carcinomas are positive for TGF–α mRNA expression [229–231]. In one study, total and poly $(A)^+$-selected mRNA from 40 primary human breast tumors were examined, and 28 of these tumors (70%) were found to express a 4.8 kb TGF–α mRNA transcript [231]. No significant correlation was observed between ER or PgR status and TGF–α mRNA expression. Travers et al. [229] have found that 4 of 18 (22%) nonmalignant breast samples and 17 of 44 (39%) breast carcinomas expressed TGF–α mRNA. Of the 17 tumors that were expressing TGF–α mRNA, 8 were ER positive and 9 ER negative. Ten of the 17 TGF–α-positive breast tumors (59%) also expressed EGFR mRNA, and 8 of these 10 (80%) were ER negative. In 6 of 6 lymph node metastases analyzed, the level of TGF–α mRNA expression was essentially the same as in the corresponding primary tumors. No significant correlations were found between either TGF–α mRNA or EGFR mRNA expression and histologic grade or lymph node status. These findings were confirmed by Ciardiello et al. [230], in that 9 of 18 (50%) primary infiltrating ductal breast carcinomas were found to express TGR–α mRNA, and no significant correlations were seen between TGF–α mRNA expression and ER or PgR status. In addition, no significant associations could be observed between TGF–α mRNA expression and axillary lymph node involvement or patient relapse. Further, the level of TGF–α mRNA expression did not correlate with c–Ha–*ras* mRNA expression in these 18 breast tumors. The same study also examined 79 primary human breast tumors for alterations in the TGF–α gene. No amplifications or gross rearrangements of the TGF–α gene were observed in DNA obtained from the tumors compared with DNA obtained from patient-matched peripheral blood lymphocyte preparations.

The levels of TGF–α protein in primary human breast carcinomas have also been analyzed in a limited number of normal, benign, and malignant breast tissues. Immunoreactive TGF–α protein, as detected by RIA and ranging from 1 to 7 ng/mg protein, was found in extracts prepared from 1 normal mammary gland, 2 fibroadenomas, 1 benign fibrocystic lesion, and 22 breast carcinomas [232]. Overall, TGF–α protein levels did not differ significantly between the nonmalignant and malignant breast tissue samples. However, if a level of 2.5 ng of TGF–α/mg protein was used as an arbitrary cut-off value, the value found in the normal breast tissue samples, then 11 of 22 (50%) tumor samples exceeded this value. All of these tissue samples were also analyzed for $p21^{ras}$ expression by immunoblotting using the Y13–259 antibody, and no significant correlation was seen between the protein levels of TGF–α and $p21^{ras}$ in any of the breast tissue samples. There was, however, a trend toward higher TGF–α levels in the ER-positive tumors than in the ER-negative tumors, but this was not statistically significant due to the small sample size. Macias et al. [233] analyzed the acid-ethanol extracts prepared from 54 primary invasive breast tumors for EGF-like activity in an EGF radioreceptor competition assay, with or without prior incubation of the tissue extracts with an antihuman EGF antiserum. Fourteen of 54 tumor extracts (26%) showed EGFR-competing activity that was not neutralized by the EGF antiserum and that showed an apparent M_r of 6,000 after gel filtration chromatography. It was therefore assumed that this material was TGF–α . Further, in 37 tumors analyzed for both TGF–α mRNA and EGFR mRNA expression, there was a trend for coexpression of both the EGFR and TGF–α. Gregory et al. [234] found that 15 of 15 breast tumors contained immunoreactive TGF–α. In characterizing this immunoreactive TGF–α in primary human breast tumors, they found that the majority of the activity corresponded to the previously reported low-molecular-weight species of TGF–α and that this material was biologically active in stimulating [^{3}H]thymidine uptake in serum-starved NIH–3T3 cells. In addition, tamoxifen treatment of the patients seems to result in a tenfold reduction in the levels of tumor-associated TGF–α. Collectively, these results demonstrate that approximately 50% to 60% of human breast cancers express TGF–α protein and TGF–α mRNA and that TGF–α mRNA expression is not significantly correlated with ER or PgR status or with $p21^{ras}$ expression, although its expression can be regulated by estrogens and antiestrogens. In addition, the presence of TGF–α is not limited to breast carcinomas because some nonmalignant breast tissues also express TGF–α protein and TGF–α mRNA.

EGFR expression in primary human breast carcinomas

Because TGF–α interacts through the EGFR system, a determination of the distribution and level of EGFR expression in primary human breast tumors becomes important. Approximately 40% to 50% of primary human breast tumors express detectable EGFR levels as measured by the binding of

^{125}I–EGF to isolated membrane fractions [33, 235, 236]. In addition, a large body of clinical data have demonstrated that there is an inverse correlation between ER and/or PgR status and EGFR expression in primary human mammary carcinomas [235–245]. High levels of EGFR expression are also correlated with axillary lymph node involvement and with poor prognosis [235, 242, 244, 246–248]. In some studies, EGFR expression in either primary or secondary lesions has been found to be associated with higher proliferation rates, suggesting that the growth of a subset of human breast tumors may be regulated by growth factors, such as TGF–α, which operate through the EGFR system [240, 249–251].

Expression of ras, *TGF–α, and EGFR in transformed human mammary epithelial cells*

A number of well-established human breast cancer cell lines have been used for in vitro studies to define the mechanism(s) by which various mammotrophic hormones and growth factors may be regulating mammary epithelial cell growth. These cell lines have been derived from either pleural effusions (e.g., MCF–7, MDA–MB–231 [252, 253]) or from solid tumors (e.g., Hs578T [254]). Some of these breast cancer cell lines are ER positive and estrogen responsive, such as MCF–7, whereas others are ER negative and estrogen unresponsive, such as MDA–MB–231. Most of these cell lines express EGFRs to some degree [133, 255]. Furthermore, in agreement with the clinical studies on primary breast tumors, an inverse relationship between ER and EGFR expression has been demonstrated for many of these breast cancer cell lines [255]. EGF appears to function as a mitogen for those breast cancer cell lines that express relatively few EGFRs (table 5), whereas breast cancer cell lines with a high number of EGFRs (above 70,000 sites/cell) are generally unresponsive to EGF [255]. However, one of the very high EGFR-expressing, ER-negative cell lines, MDA–MB–468, which also exhibits an amplification of the EGFR gene, is actually growth inhibited by EGF at concentrations that are normally growth promoting for other EGF-responsive cell lines [265]. Therefore, it is probably unlikely that the actual number of EGFRs per se is solely responsible for determining the degree of responsiveness to EGF. The amount of EGFRs may influence other biological characteristics of these cell lines. For example, the ER-negative, EGFR-rich MDA–MB–231 and MDA–MB–468 cell lines are considerably more tumorigenic than are any of the ER-positive breast cancer cell lines. In fact, in a study involving variants of the MDA–MB–468 cells expressing low levels of EGFRs, tumorigenicity was found to correlate directly with the amount of EGFR expression [257]. Therefore, these observations using various breast cancer cell lines tend to support the results that were obtained from the clinical studies, where EGFR expression is generally associated with a more aggressive tumor phenotype and with reduced ER expression.

Although TGF–α is expressed in a number of human breast cancer cell

lines, the role for TGF–α in regulating the growth of these cell lines is less clear (table 5). The ER-positive breast cancer cell lines that have been examined generally produce low basal levels of TGF–α, compared with ER-negative breast cancer cell lines [232, 258]. In the ER-positive breast cancer cell lines, TGF–α production can be increased severalfold after treatment with physiological concentrations of estrogen that are capable of stimulating proliferation in these cells [231, 232, 258]. In addition, anti-TGF–α-neutralizing antibodies can inhibit the anchorage-independent growth of MCF–7 cells in soft agar, suggesting that TGF–α may be functioning as an autocrine growth factor in vitro for these cells [231]. TGF–α expression in the estrogen-dependent MCF–7 tumors in nude mice was also found to be estrogen dependent, because estrogen withdrawal resulted in a gradual decline in TGF–α mRNA expression in the tumor xenografts [231]. From these results, it was postulated that TGF–α might mediate in part the mitogenic effects of estrogen. This may be the case, since anti-EGFR-blocking antibodies could partially attenuate the estrogen-induced growth of MCF–7 [231]. This hypothesis was further supported by the observations that EGF or medium conditioned by estrogen-stimulated MCF–7 cells could partially replace estrogen in promoting the growth of MCF–7 xenografts in nude mice [52]. To further evaluate the role of TGF–α in mediating the estrogen-responsive phenotype, overexpression of TGF–α in MCF–7 cells was studied following transfection with a TGF–α cDNA expression vector plasmid [259]. It was found that neither estrogen-dependent tumorigenicity, basal or estrogen-stimulated growth in vitro, nor ER and PgR status were significantly altered in several high TGF–α-expressing MCF–7 clones compared with the nontransfected MCF–7 cells. It was therefore concluded that TGF–α may be necessary but not entirely sufficient to replace the requirement of estrogen for growth and tumorigenicity of an estrogen-dependent breast cancer cell line. Taken together, these studies imply a supportive, rather than causative, role for TGF–α in determining the malignant phenotype of human breast cancer cells.

In contrast to some rodent mammary tumor cell lines that are overexpressing an activated c–Ha–*ras* proto-oncogene, there is no consistent association between the presence of an activated or overexpressed *ras* proto-oncogene and the absolute levels of TGF–α in a number of human breast cancer lines (table 5). For example, the ER-positive breast cancer cell lines MCF–7 and ZR–75–1 have been reported to have varying degrees of amplification of the N–*ras* gene that do not show any correlation with the basal levels of TGF–α in these cells [232, 260, 261]. In addition, the ER-negative carcinosarcoma-derived Hs578T cell line has been shown to carry a point-mutated c–Ha–*ras* gene but expresses no TGF–α mRNA [231, 258, 259, 262], whereas the ER-negative MDA–MB–231 cell line possesses a point-mutated c–Ki–*ras* gene and expresses high levels of TGF–α mRNA and TGF–α protein [231, 232, 258, 263]. Two groups have studied the effect of Ha–*ras* gene overexpression in MCF–7 cells. In one study, transfection and overexpression of

the v–Ha–*ras* oncogene in MCF–7 cells resulted in an estrogen-independent phenotype in vitro and in vivo and lead to enhanced TGF–α production [264–266]. In contrast, transfection and overexpression of either the point-mutated c–Ha–*ras* proto-oncogene or the normal c–Ha–*ras* proto-oncogene in MCF–7 cells did not affect their estrogen-dependent tumorigenicity in vivo, although their estrogen responsiveness in vitro was abrogated [267]. These results indicate that overproduction of $p21^{ras}$ can lead to an estrogen-independent phenotype in vitro and to a constitutive increase in TGF–α secretion in some human breast cancer cells and that under these conditions, estrogens are unable to further enhance TGF–α production [266].

The lack of well-defined populations of normal human mammary epithelial cells has impeded research into defining the mechanism(s) by which growth factors or activated proto-oncogenes might be involved in regulating the growth and transformation of these cells in vitro. These obstacles have been partially overcome with the development of a semi-defined, serum-free medium that is capable of supporting the limited in vitro growth of diploid, nonimmortalized human mammary epithelial cells derived from histopathologically normal reduction mammoplasty breast tissues [268]. Two of these normal human mammary epithelial cell strains, 184 and 172, express endogenous c–Ki–*ras* mRNA at levels comparable with levels of N–*ras* mRNA expression observed in MCF–7 cells [261]. The 184 and 172 cells do not possess ER or the estrogen-inducible pS2 protein [261, 269; and unpublished observations]. Both cell strains express TGF–α mRNA and EGFR mRNA at levels similar to or slightly above those found in some of the human breast cancer cell lines, such as MDA–MB–231 [261, 269]. These observations are not unique to the 184 cells and their derivatives, since primary cultures of human mammary epithelial cells secrete high amounts of immunoreactive and biologically active TGF–α [232, 269] and some normal human breast tissues display EGF binding at similar levels to tumor tissue [250].

An immortalized cell line was established from the 184 cells after benzo–a–pyrene treatment, and a subclone of the immortalized cells, designated 184AIN4, was subsequently isolated and studied, since the mammary epithelial cell strains normally senesce after 15 to 20 passages and are therefore difficult to use for protracted in vitro studies [270]. Both the 184 and 184A1N4 cells are EGF dependent for anchorage-dependent growth in vitro, express elevated levels of EGFRs, and secrete high levels of TGF–α protein [53, 269]. Neither of these cell lines is capable of growing in soft agar nor is tumorigenic in nude mice [270]. The 184A1N4 cells have been utilized as recipients for the introduction of various oncogenes, such as Ha–*ras* and SV40T, using recombinant retroviral vectors [271]. After overexpression of the v–Ha–*ras* oncogene in the 184A1N4 cell line, EGF dependency for growth was lost, and a low degree of tumorigenicity was induced [271]. Overexpression of both the SV40T antigen gene and the v–Ha–*ras* oncogene was necessary to yield a fully transformed, tumorigenic phenotype [271]. TGF–α production was not affected and the level of EGFR expression was

only slightly reduced in the v–Ha–*ras*-containing 184A1N4 cells or in the SV40T/v–Ha–*ras*-transformed 184A1N4 cells [269]. These results suggest that either additional genetic alterations are necessary to elicit a change in the production of TGF–α in this system or alternatively that other endogenous growth factor(s) can supplant the requirements for EGF that are lost following overexpression of the Ha–*ras* oncogene.

Conclusions

The overexpression of the c–Ha–*ras* proto-oncogene appears to be a fairly common occurrence in a subset of primary human breast carcinomas, which in some cases may be related to the loss of one of the c–Ha–*ras*–1 alleles and their accompanying regulatory elements [56, 57]. In addition, the well-characterized MCF–7 breast cancer cell line also exhibits varying degrees of amplification of the N–*ras* proto-oncogene, depending on the subline [260]. The presence of a point-mutated c–Ha–*ras* proto-oncogene or a point-mutated c–Ki–*ras* gene is limited to only two human breast cancer cell lines, Hs578T and MDA–MB–231, respectively [262, 263]. To date, no discrete point mutations, amplifications, or gross rearrangements in either the c–Ha–*ras*-1 or c–Ki–*ras*-2 genes have been detected in primary human breast carcinomas, suggesting that such an event occurs at a relatively low frequency in the population [56]. Alternatively, the use of more sensitive analytical techniques, such as the polymerase chain reaction, may be useful in being able to detect more subtle changes in these *ras* genes in breast tumors as has been demonstrated in primary human colorectal carcinomas [13, 14]. Activation of Ki–*ras* genes in the development of colorectal carcinoma occurs at a relatively early stage, because it can be detected in premalignant tubulovillous adenomas [13]. In contrast, high levels of c–Ha–*ras* mRNA or $p21^{ras}$ protein are generally found in more advanced stages of breast cancer, such as in invasive breast tumors from patients who have axillary lymph node involvement, and not in hyperplasias or in fibrocystic lesions [57]. However, the situation is not clearly defined, because varying levels of $p21^{ras}$ have been found in normal, hyperplastic, and malignant breast tissues [212, 214].

Whereas *ras* gene overexpression may be involved to some extent in the progression of human breast cancer, the occurrence of a point-mutated c–Ha–*ras* proto-oncogene seems to be more prevalently associated with the initiation and development of mouse and rat mammary tumors that arise after treatment with specific chemical carcinogens, such as NMU, and to a lesser extent with DMBA [10, 185, 194, 196]. Whether activation of c–Ha–*ras* or of other members of the *ras* gene family also occurs in other spontaneous, hormonally induced or MMTV-initiated rodent mammary tumors is not yet known. Nevertheless, it is clear that the presence of a point-mutated c–Ha–*ras* proto-oncogene that is driven by a strong constitutively active retroviral promoter in a previously immortalized population of rodent

mammary epithelial cells is sufficient to transform these cells in vitro and to allow for the development of carcinomas in vivo [201, 202]. In addition, if expression of $p21^{ras}$ can be elevated to very high levels, then *ras* can also transform primary cultures of rodent mammary epithelial cells. These results have been confirmed in vivo with respect to the frequency of breast cancer occurrence in transgenic mice that harbor activated *myc*, *ras*, or *myc* and *ras* genes under the control of a strong retroviral LTR [197–200, 202].

The mechanism(s) by which an activated (i.e., overexpressed or point-mutated) *ras* gene can transform a cell is still unclear. However, the isolation of rodent fibroblast revertants that are resistant to retransformation by the *ras* family of oncogenes may provide some clues to this enigma [272]. These *ras* revertants are apparently defective in a cellular target protein(s) that can potentially interact either directly or indirectly with the $p21^{ras}$ protein or that can function downstream in the transformation pathway. In this respect, Noda et al. [273] have recently cloned and sequenced a human gene, K–*rev*-1, which is partially related to $p21^{ras}$ in that it codes for a protein of 21 kD. Introduction of this dominantly acting gene into rodent fibroblasts confers upon these cells resistance to transformation by an activated *ras* proto-oncogene, suggesting that this protein may compete with $p21^{ras}$ for interaction with a common downstream effector protein, such as GAP [64]. It will be interesting to see if similar cellular revertants can be isolated from *ras*-transformed epithelial cells and, if so, to determine if the same K–*rev*-1 gene is involved. In addition to these genetic studies, there is also biological evidence to suggest that transformation by some of the tyrosine kinase oncogenes, such as *src*, *fes*, and *fms*, and by *sis* (PDGF B chain), which interacts with a tyrosine kinase receptor, can be blocked by an anti-$p21^{ras}$ antibody, suggesting that the transformation produced by these receptor-like, membrane-associated oncoproteins is through the same pathway that is used by $p21^{ras}$ and is upstream from $p21^{ras}$ [95]. Because $p21^{ras}$ has been postulated to function as a signal transducer for tyrosine kinase growth factor receptors, the proliferation of nonmalignant cells in response to these growth factors would therefore depend on the normal activity of $p21^{ras}$.

One consequence of activation of the *ras* family of genes as well as other activated cellular proto-oncogenes, such as *fes*, *fms*, and *src* in rodent fibroblasts, is a loss in the responsiveness of these cells to EGF, which may result from a reduction in the expression of EGFRs on these cells and/or to the simultaneous overproduction and secretion of a series of potential autocrine growth factors, including the EGF-related growth factor TGF–α [46, 107, 108]. Similarly, in rat and mouse mammary epithelial cells that contain a point-mutated c–Ha–*ras* proto-oncogene (NMU-induced rat mammary tumors and mouse NMuMg *ras* and NOG–8 *ras* cells) and/or an overexpressed c–Ha–*ras* proto-oncogene (DMBA-induced rat mammary tumors), there is evidence for a loss in EGF responsiveness and for elevated expression of TGF–α in these cells [115, 190, 203]. Likewise, the coordinate expression of $p21^{ras}$ and TGF–α in mouse mammary epithelial NOG–8 cells

containing an MMTV–LTR *ras* inducible expression vector plasmid suggests that the enhanced expression of TGF–α mRNA is a relatively early event following induction of the *ras* gene [203]. However, it is also apparent that while overexpression of TGF–α may be one prerequisite for maintaining some of the phenotypic changes that occur in *ras*-transformed cells, it is not entirely sufficient in mediating all of the biological effects of *ras*, because mouse NIH–3T3 *ras* revertants express TGF–α at a level that is equivalent to those levels observed in the parental *ras*-transformed NIH–3T3 cells [106, 108]. In addition, TGF–α is not expressed in a subset of rat mammary tumors that, in some cases, also possess a point-mutated c–Ha–*ras* proto-oncogene and that are metastatic and estrogen-independent [190]. The latter situation is in reasonable agreement with the data obtained from a small number of primary human breast tumors in which no clear association could be observed between the expression of c–Ha–*ras* mRNA and TGF–α mRNA, steroid receptor status, and axillary lymph node involvement and subsequent patient survival and relapse [230]. However, some ER-negative human breast tumors that are also expressing high levels of EGFRs do exhibit a higher tendency to express TGF–α mRNA than do some ER-positive tumors that do not express EGFRs, suggesting that a potential autocrine circuit may exist in vivo in a discrete population of human breast tumors [229].

The in vitro studies with an immortalized population of human mammary epithelial cells (i.e., 184A1N4 subline) into which various retroviral oncogenes have been introduced demonstrate that TGF–α expression is relatively ubiquitous in these cells and does not show any clear correlation with either the onset of transformation or the progression of tumorigenicity in this system [269]. Nevertheless, introduction and overexpression of a Ha–*ras* oncogene in the 184A1N4 cells does result in a loss in the response of these cells to exogenous EGF, which may be due in part to an absence of the high-affinity population of EGFRs even though these cells are not fully tumorigenic. These results suggest that such changes can occur at a relatively early stage in the transformation process. The levels of expression of TGF–α mRNA, TGF–α protein, and EGFR in the 184A1N4 cells and in the normal 184 primary outgrowths from which this cell line was derived is equivalent to or, in some cases, greater than the levels observed in some human breast cancer cell lines [231, 232]. These results demonstrate that normal proliferating mammary epithelial cells in vitro are capable of expressing TGF–α, since this growth factor is not found in nonproliferating organoids, and that this growth factor is not entirely limited to malignant populations of mammary epithelial cells [53, 261, 269]. The expression of TGF–α can be regulated in the 184A1N4 cells by itself as well as by EGF, demonstrating that an autoinductive regulatory process may be operative in these EGF-dependent cells [53]. The presence of multiple TGF–α species in human milk also tends to support the thesis that normal mammary epithelial cells in vivo may also be synthesizing and secreting this growth factor [142]. This has recently been confirmed by the in situ localization of TGF–α mRNA in the mouse, rat and

human mammary gland [274]. Using a specific 400 bp labeled TGF–α antisense riboprobe for RNA:RNA in situ hybridization, TGF–α mRNA expression could be detected in virgin, pregnant, and lactating ductal and alveolar epithelial cells in the mammary gland. Furthermore, the level of TGF–α mRNA increased approximately twofold to threefold in the lactating mammary gland compared with the virgin and pregnant glands, suggesting that production of this growth factor in vivo may be regulated by specific lactogenic hormones, such as estrogen or prolactin. This may well be the case, because estrogens can increase the expression of TGF–α mRNA and the synthesis of TGF–α protein in estrogen-responsive rat and human mammary carcinoma cells in vitro and in vivo [190, 231, 232]. It has not yet been formally demonstrated whether similar estrogenic control exists in normal rodent or human mammary epithelial cells, because it has been extremely difficult to show an estrogen-dependent growth response in these cells in vitro. However, if mammary epithelial cells are cocultured with appropriate stromal cells or grown on defined extracellular matrix components, then it may be possible to undertake these and similar studies [62, 138, 275]. Although a limited degree of TGF–α expression can be detected in vivo in normal mammary epithelial cells, chronic overexpression of this growth factor may occur in malignant mammary epithelial cells or in preneoplastic lesions that arise before the development of overt tumors (i.e., HANs).

It is still unclear whether the enhanced expression of TGF–α that is observed in some spontaneous or *ras*-transformed rodent mammary epithelial cells is etiologically related to the cause or is a secondary consequence of the transformation process. It is possible that a sustained elevation in the production of biologically active TGF–α at or beyond a threshold level may be sufficient to trigger and to maintain the transformation of a population of mammary epithelial cells. One would also predict that such a population of mammary epithelial cells might be more susceptible or responsive to the effects of TGF–α if they were already immortalized and possessed a critical level of functional EGFRs. These cells may be at an even higher risk for transformation if the activation of a proto-oncogene(s) also occurs in these cells. Some of these possibilities are apparently relevant, because overexpression of the human TGF–α gene in the NOG–8 mouse mammary epithelial cell line after transfection with an expression vector plasmid can result in the transformation of these cells in vitro and the development of invasive undifferentiated carcinomas in vivo [177]. It will be interesting and necessary to ascertain if similar results can be obtained with an immortalized population of human mammary epithelial cells. Primary mammary epithelial cells are notoriously resistant to conventional transfection procedures, such as calcium-phosphate precipitation for the introduction of foreign genes, thereby necessitating the use of retroviral shuttle vectors. Considering this point, it will be necessary to determine with apporpriate recombinant amphotropic, replication-defective retroviral expression vectors that contain the TGF–α gene whether infection and subsequent overexpression of this gene in

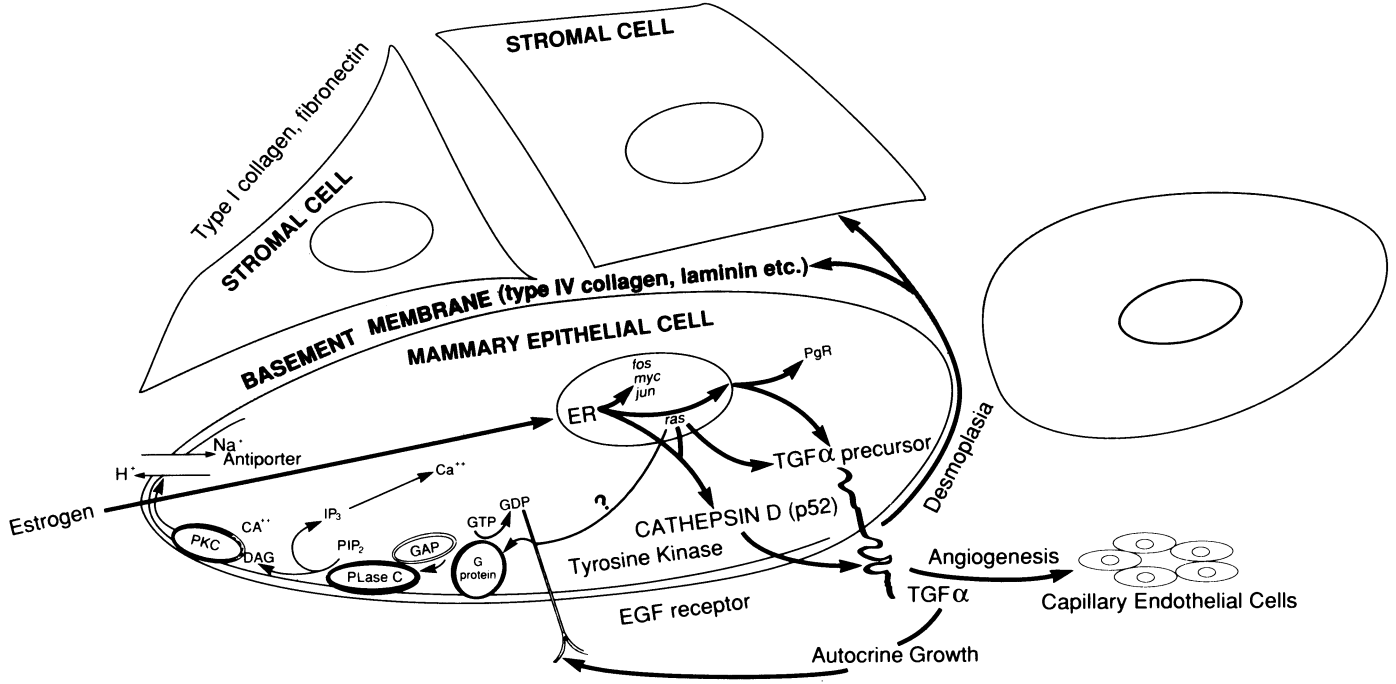

Figure 2. Potential interactions among estrogen, $p21^{ras}$, TGF–α, and EGF receptor in mammary epithelial cells, and the possible autocrine and paracine effects of TGF-α in the mammary gland.

primary rodent or human mammary epithelial cells can lead to their enhanced growth, immortalization, and/or transformation in vitro and in vivo in cleared mammary fat pads. Such an approach is also likely to uncover preexisting, or possibly novel, activated proto-oncogenes that may cooperate with TGF–α in these processes.

In summary, our current concept(s) of how estrogens, $p21^{ras}$, TGF–α, and EGFR might be interacting at the cellular and biochemical levels is presented in figure 2. This cartoon merely provides a framework from which various experiments might be initiated. In this regulatory scheme, it is important to keep in mind that the growth and differentiation of secretory (i.e., ductal and alveolar) mammary epithelial cells do not occur in an isolated environment but depend on an intimate association and interaction with adjacent myoepithelial cells, stromal cells, and adipocytes, which also reside within the mammary gland [138]. The basement membrane in the mammary gland provides a suitable in vivo scaffolding upon which the epithelial component of the gland can grow and differentiate as controlled by different hormones and growth factors [51, 136, 137]. TGF–α production can be regulated in mammary epithelial cells by two independent but not mutually exclusive pathways that involve induction by estrogens or by a *ras* proto-oncogene which has been previously activated either by a point mutation or by overexpression. TGF–α production is tightly regulated by the level of circulating estrogens and the expression of functional ER. In the latter situation with an activated *ras* proto-oncogene, TGF–α is constitutively elevated. In both cases, cell-associated or secreted forms of TGF–α (i.e., either the precursor or the low-molecular-weight peptide) may function as potential autocrine growth factors via interaction with EGFRs [161]. After binding to the EGFR, TGF–α can activate the EGFR tyrosine kinase, which in turn can lead to an enhanced turnover via phospholipase C of second messenger-like phosphoinositides, such as DAG and IP_3 [75]. Coupling of the EGFR to phospholipase C could be achieved through GAP and a G protein that may be $p21^{ras}$ or a closely related species. Secreted TGF–α is also likely to influence the growth and behavior of other cell types in the mammary gland in a paracrine fashion. These paracrine effects could include the neovascularization (i.e., angiogenesis) of capillary endothelial cells and the growth of surrounding stromal cells, which could contribute to the desmoplasia that is frequently observed in some breast tumors. Processing of the cell-associated TGF–α precursor to soluble *meso* forms may be accomplished by several distinct proteases, including cathepsin D (p52), which is itself an estrogen-inducible and growth factor-inducible, secreted protease [61, 276, 277]. The development and utilization of anti-TGF–α neutralizing antibodies, anti-EGFR blocking antibodies, and appropriate TGF–α antisense oligonucleotides or TGF–α antisense expression vectors will aid in the resolution of some of these issues. In addition, it will be important to determine whether other EGF-like peptides, such as amphiregulin and the pS2 protein that are produced by

MCF-7 cells, are also expressed in vivo in normal and/or malignant mammary epithelial cells [144, 278].

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7. The role of epidermal growth factor receptors in breast cancer

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Introduction

Epidermal growth factor (EGF) is a mitogenic polypeptide affecting proliferation of a variety of cells, including the mammary epithelium. The tissue-specific actions of EGF are mediated through binding to a transmembrane receptor glycoprotein (EGFR) [1]. The EGFR has been subject to intense study in relation to cancer biology because of its homology to the transforming protein encoded by the ovarian oncogene V–*erb* B [2]. Aberrant expression of the EGFR and c–*erb* B2, a closely related putative receptor, have been implicated in the malignant behavior of a number of tumor types, including mammary carcinoma [3]. In addition to EGF, the related polypeptide-transforming growth factor α(TGF–α) is also a ligand for the EGFR [4]. This review discusses the potential role of the EGFR and its ligands in the biology of breast cancer. Special emphasis is given to the utility of the MDA–MB–468 cell line model for mechanistic experiments in this area.

Structure, synthesis, and regulation of the EGFR

The structure of the EGFR has been deduced by protein chemistry and cDNA cloning [2, 5] and has been discussed in a recent review [6]. The mature protein (1,186 amino acids) is generated after cleavage of an amino terminal signal peptide. The domain structure of the molecule contains an extracellular EGF-binding region (621 amino acids) and an intracellular carboxyl-terminal region (542 amino acids) that has tyrosine kinase activity, separated by a transmembrane region. The core protein (135 kD) is modified during synthesis by the addition of 11 N-linked oligosaccharide chains to yield the mature 170 kD receptor.

The extracellular domain comprises alternating α–helix/β–sheet and cysteine rich regions. The three-dimensional features that facilitate EGF binding are in the process of elucidation [7]. Similar domain structures are present in the related receptor proteins, c–*erb* B2 and the insulin receptor,

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and it has been possible to construct chimeric receptor molecules [8]. An important structural role for the transmembrane domain is implied from the observation that the lipid environment can modify ligand binding and receptor kinase activity [9].

Much more information is available concerning the structure and regulation of the intracellular tyrosine kinase domain [6, 10]. Site-specific mutations have been induced to elucidate the obligatory role of kinase activation in initiation of intracellular signal transduction processes [11–14] and the role of the carboxyl-terminal autophosphorylation domain in receptor regulation [15]. A critical role in regulation has also been shown for Thr 654, which is a substrate for phosphorylation by protein kinase C [16].

The human receptor gene is located in the pl.1–pl.3 region of chromosome 7 [17]. Transcription results in two mRNA species, 10 kb and 5.6 kb, although the relationship between these transcripts is not known. The mature receptor glycoprotein has a half-life of approximately 20 hours. In the presence of EGF, receptor internalization is stimulated and half-life decreased, while the production of new receptor molecules is up-regulated by a posttranscriptional mechanism [18, 19].

General considerations of the role of EGFR in breast cancer

Subsequent to the elucidation of the relationship of the EGFR to the v–*erb*B transforming protein [2], a role for abnormalities of EGFR expression has been implicated in the biology of a number of tumor types, e.g., squamous carcinoma [20], glioblastoma [21], and mammary carcinoma [22]. There appears to be a relationship between these involvements in carcinogenesis and the important role that EGF plays in growth control and differentiation of the respective equivalent normal cell types, i.e., squamous epithelium, glial cells, and secretory mammary epithelium.

With specific reference to mammary epithelium, evidence for a role for EGF in growth control in vivo and in tissue culture has come from a number of sources (table 1) [Reviewed in 23]. In vivo, EGF plays a role in the development of the mammary gland during pregnancy and lactation [24, 25] and carcinogenesis of the breast in rodent systems [26]. EGF also acts as a mitogen and inducer of differentiation of primary cultures of rodent mammary epithelium [27–30] and for many human and rodent breast cancer cell lines in long-term tissue culture [31–34]. In addition, many breast tumors express high levels of the receptor protein as evidenced by ^{125}I–EGF binding or immunohistochemical staining [22]. It is of interest that there is an inverse relationship between such EGFR expression and estrogen receptor (ER) status [22, 35–38]; it has been suggested therefore that in ER–ve tumors, the EGFR may be involved in growth control.

The mechanisms underlying the overexpression of the EGFR in breast tumors has not been elucidated in detail. However, unlike other tumor types which overexpress the EGFR, such as squamous carcinoma and glioblastoma

Table 1. EGF: Role in mammary epithelial development and carcinogenesis.

1. Necessary for rodent gland development during pregnancy [24, 25].
2. Stimulates proliferation and morphological development of rodent tissue in in vitro systems [27–30].
3. Involved in rodent mammary tumor development [26].
4. Mitogen for a number of breast cancer cell lines in culture [31–34].
5. Level of EGFR in biopsies related to prognosis in breast cancer; over expressed in ER–ve tumors [35–38].

[20, 21], the predominant mechanism is not based on receptor gene amplification. Although approximately 20% of breast tumors express elevated levels of EFGR, only approximately 2% have documented EGFR gene amplification. Since the cell of origin of ER–ve breast tumors is not established and since the level of EGFR expression in differentiating normal mammary epithelium may be regulated at high levels in certain cells [39], it remains a possibility that the variability in expression may not be related to a mutational event in the carcinogenesis process but rather to the expansion of a rare cell population that normally expresses high levels of EGFR.

A cell line model to investigate the role of EGFR in breast cancer

Although EGF receptor gene amplification is not a common event in breast cancer, one such human breast cancer cell line has proven useful for mechanistic studies. This is the MDA–MB–468 cell line that we identified as expressing approximately $1–2 \times 10^6$ EGFR/cell, approximately 20-, to 100-fold higher than levels expressed in other human breast tumor cell lines or normal fibroblasts [34]. In this case the overexpression is based on an amplification of the receptor gene; Southern blotting indicates a 20-fold to 40-fold amplification, and in situ hybridization locates the amplified domain to an abnormally banding region on one copy of chromosome 7 [40]. No evidence of structural rearrangements in the EGFR gene have been detected. In addition, the properties of the overexpressed protein appear normal with respect to binding affinity, synthesis, turnover, and autophosphorylation [9, 40].

The utility of this cell line will be emphasized in the subsequent discussion of molecular mechanisms underlying the involvement of EGF receptor in breast cancer.

Mechanisms of EGFR involvement in breast cancer

Relationship of EGFR expression to tumorigenesis

Overexpression of the human EGF receptor in rodent fibroblasts causes an EGF-dependent transformed phenotype as evidenced by anchorage-independent growth and tumorigenicity [41, 42]. We have utilized the MDA–

MB–468 cell line model to investigate the relationship between tumorigenicity and EGF receptor expression in mammary epithelium.

Cell lines overexpressing the EGFR commonly can be growth inhibited in tissue culture by exposure to supraphysiological levels of EGF. The mechanism of this phenomenon has not been established, but it has been clearly related in some circumstances to receptor frequency and is not dependent on cell lineage. Using variants selected from A431 human epidermoid carcinoma cells expressing different levels of receptor, Kamamoto et al. [43] demonstrated a 'threshold' requirement, in terms of receptor number, to allow growth inhibition to occur, and in a series of squamous tumor cell lines, Kamata et al. [44] showed a direct relationship between growth inhibition and increasing receptor frequency. Despite these relationships, it is of interest that overexpression of the EGFR alone does not seem to confer the ability to be growth inhibited by high levels of EGF. In circumstances where expression of EGFR is imposed on normal fibroblasts after transfection with expression vectors, clones of transformed fibroblasts are generated that express high levels of EGFR but are not growth inhibited by supraphysiological levels of EGF [41, 42].

In keeping with the experience with squamous carcinoma cells, MDA–MB–468 is also growth inhibited in tissue culture by high levels (10^{-7}M) of EGF [34]. Clones selected for survival under these conditions were all found to express low numbers (approximately 2×10^4/cell) of receptor. In 12 selected clones the mechanism used to overcome the EGF-growth inhibition was loss of the copy of chromosome 7 bearing the amplified allele of the EGFR. This was demonstrated both by karyology and by the fact that the amplified allele of the EGFR in MDA–MB–468 cells has a useful restriction fragment length polymorphism [40]. Such variants exist within the parental MDA–MB–468 cell line at a frequency of approximately $1/10^6$.

Six of the 12 subclones (S1, S4, S5, S10, S11, and S12) were selected for investigation of EGF-mediated growth control in tissue culture and in vivo. In plastic-adherent tissue culture containing 10% fetal calf serum, all six clones could be moderately stimulated in terms of growth by the addition of EGF, and supraphysiological conditions did not cause growth inhibition. When suspended in agar-containing medium, clones S1, S5, S10, S11, and S12 displayed the same phenotype as in adherent culture. However, clone S4 is dependent on the addition of exogenous EGF for growth in agar [40]. Similarly, when the tumorigeneity of the clones was assessed, S4 displayed a phenotype separate from the other five clones. Clones S1, S5, S10, S11, and S12 are all tumorigenic, forming progressively growing tumors after the subcutaneous implantation of 10^6 cells in the flank or mammary fat pad of nude mice. Clone S4, however, is nontumorigenic under the same conditions. When the rates of tumor growth of clones S1, S5, S10, S11, and S12 were compared with parental MDA–MB–468 cells, a growth rate advantage could be detected for the parental cell line (doubling time of 6 days versus 10 days) [40].

The tumor growth experiments described above suggest that amplification of the EGFR gene and subsequent overexpression of the receptor protein provide a growth advantage in vivo. Similar conclusions have been reached through study of the in vivo growth of A431 and variant cell lines [45]. These experiments provide a biological rationale for the in vivo selection of tumor cell clones overexpressing the EGFR during tumor progression. Based on analysis of receptor expression in relation to tumor stage in a series of breast cancer patients [22], overexpression of EGFR is likely to be a late event in tumorigenesis. Our data are consistent with that view in that the predominant class of subclones of MDA–MB–468 (S1, S5, S10, S11, and S12) are still tumorigenic and therefore must possess other derangements of cell growth regulatory mechanisms. One such abnormality was described recently; MDA–MB–468 cells have a homozygous deletion of the recessive oncogene RB1, which confers susceptibility to retinoblastoma and possibly also to other tumor types [46].

The role of EGF receptor ligands in the malignant behavior of breast cancer cells

Conceptually, the discovery of the autocrine production of growth factors by tumors has led to the recognition of an important mechanism underlying the deregulated growth of transformed cells [47]. Of importance to the present discussion is the fact that transforming growth factor α (TGF–α), a growth factor frequently involved in autocrine regulation of tumor growth and produced during embryonal development, is an effective ligand for the EGF receptor [4]. Both EGF and TGF–α are frequently found to be secreted from rodent [48] and human breast cancer cells [49–51]. Importantly, estrogens have been shown to regulate expression of TGF–α in estrogen receptor-positive breast tumors of rodent [48] and human origin [52, 53] and in anti-estrogens shown to mediate growth inhibition through the regulation of TGF–β production [54]. This has led to the interesting and important hypothesis of a link between estrogen receptor and EGF receptor-mediated growth regulation of breast cancer cells [52]. However, the relative role of the EGF receptor in the complex regulation of mammary epithelial growth by steroids remains in doubt, since Arteaga et al. [55] demonstrated that blockage of the EGF receptor in vitro inhibits TGF–α-induced, but not estrogen-induced growth of MCF–7 breast cancer cells. Further complicating these issues is the knowledge that estrogens, progestins, and TGF–β can also regulate the expression of the EGF receptor [56–59], and activation of protein kinase C can regulate expression of both receptor and ligand [60].

Recently, important information has derived from the strategy of transforming normal mammary epithelial cells in tissue culture. Shankar et al. [61] demonstrated that transfection of human TGF–α cDNA could transform a normal mouse mammary epithelial cell line. In addition, in these circumstances TGF–α appears to act through an autocrine loop presumably utilizing

the EGF receptor. Valverius et al. [62] utilized normal and benzo–a–pyrene-immortalized human mammary epithelial cultures to investigate malignant transformation induced by transfection with oncogene sequences. In this model, alterations in TGF–α production are not implicated, but altered cellular responses to available ligands (EGF and TGF–α) are involved in the malignant transformation.

Signal transduction from the EGF receptor in breast cancer cells

The intracellular events contributing to signal transduction from the EGFR are not totally understood. A number of events subsequent to EGF binding have been implicated, including phosphorylation of a number of protein substrates, receptor autophosphorylation, activation of the Na^+/H^+ exchange system resulting in cytoplasmic alkalinization, mobilization of intracellular calcium stores, stimulation of phosphotidyl inositol (PI) turnover, and changes in gene expression (particularly induction of c–*myc* and c–*fos* genes) [reviewed in 1, 63]. Of these events, activation of the receptor kinase has been shown to be essential for signal transduction, since various mutant EGFRs devoid of kinase activity are unable to transduce EGF signals [11–14] and since specific pharmacologic blocking of receptor kinase activity abolishes EGF-mediated growth effects [64]. The requirements for other events, such as autophosphorylation, Na^+/H^+ exchange, PI turnover, and transcriptional regulation, are not general but are seen dependent on cell type. An important linkage between EGFR activation and second messenger generation has been suggested by the work of Wahl et al. [65, 66], who have demonstrated that in A431 cells, phospholipase C (PLC) is a substrate for the tyrosine phosphorylation catalyzed by the activated receptor. We have confirmed this finding in MDA–MB–468 breast cancer cells, by immunoprecipitating a phosphorylated form of PLC subsequent to EGF binding [Church, Pawson, and Buick, unpublished observations]. The functional significance of tyrosine phosphorylation of PLC remains to be established.

A potentially important example of receptor 'cross-talk' has been demonstrated in the SK–BR–3 human breast cancer cell line. King et al. [67] demonstrated that the activated EGF receptor is able to catalyze the tyrosine phosphorylation of the c–*erb*B–2 (*neu*) gene product. Since this putative receptor protein is overexpressed in a high proportion of breast cancers and the expression is linked to the aggressiveness of the disease [68], the potential for activation by EGF-receptor ligands in breast cancer could be very important. However, the generality of the observation remains to be established.

The growth properties of the MDA–MB–468 cell line and the previously described subclones provide an opportunity to assess differential mechanisms of signal transduction under circumstances of negative (MDA–MB–468) and positive (MDA–MB–468 S4) growth regulation. For example, in these cell lines, as in other cells responsive to EGF, an early consequence of EGFR

activation is the transient accumulation of transcripts of the c–*myc* and c–*fos* genes. It is considered possible that the products of these genes act to regulate transcription of other genes and thus play a role in the altered gene expression elicited by EGF. We found, however, that the magnitude and kinetics of the transient accumulation of c–*myc* and c–*fos* transcripts were similar in the parental cell line and subclones, despite the fact that EGF is acting as growth inhibitor and stimulator, respectively [69]. Therefore, the transient elevation in the level of transcript and the subsequent protein exspression from the c–*myc* and c–*fos* genes do not seem to play a role in the selectivity of the action of EGF.

Further evidence that dissociates c–*myc* and c–*fos* transcription from EGF-induced growth inhibition or mitogenesis came from experiments designed to assess the sensitivity of MDA–MB–468 and subclone S4 to pertussis toxin. We initiated these experiments because many hormone receptor systems are linked to the generation of second messengers by the interaction of G-proteins. This family of proteins has the property of linking receptor occupancy to activation of adenyl cyclase, phospholipase C, or ion channels [70]. A feature of certain G-protein α subunits is a sensitivity to ADP-ribosylation by pertussis toxin (PT); demonstration of an attenuation of hormone effect by PT has therefore been used to define the role of G-protein intermediates in the signal transduction of various hormones.

We demonstrated that PT could block both EGF-induced growth inhibition in MDA–MB–468 in anchorage-dependent or independent culture and the EGF-dependent growth of clone S4 in anchorage-independent conditions [71]. This indicated the possibility of a G-protein intermediate in EGF signal transduction. A role for such intermediates has also been suggested by the demonstation of EGF-mediated phosphorylation of a G-protein β subunit in human placental membranes [72], the PT sensitivity of proliferative responses of hepatocytes to EGF [73], and by tyrosine phosphorylation of the *ras* oncogene by activated EGF receptor [74]. The molecular characateristics of the G-protein intermediate in MDA–MB–468 cells has not yet been elucidated. It is of interest that the G-protein-mediated pathway of EGF signal transduction may have some specificity for this particular cell line, since we have shown that EGF growth inhibition of A431 cells is not PT sensitive.

Although PT is able to block the proliferative changes associated with EGF exposure in MDA–MB–468 and subclone S4, these same conditions did not block the increased accumulation of c–*myc* and c–*fos* transcripts [71]. This implies either (a) that the PT-sensitive event exits on a separate pathway of transduction from that causing the transcription of c–*myc* and c–*fos* genes, or (b) that the PT-sensitive event exists on the same pathway from that causing c–*myc* and c–*fos* transcription, but at a distal position.

One early consequence of EGF binding is cytoplasmic alkalinization caused by the activation of the Na^+/H^+ exchange antiport. Such early changes also occur in both MDA–MB–468 and subclone S4 in response to

EGF, despite the opposite proliferative response of the two cell lines [75]. However, we have ruled out an obligatory role for such activation in the EGF signal transduction process in these cell lines [75].

Conclusion

An important role for EGF receptor signal transduction in breast cancer is implied by the association of elevated expression of the receptor with estrogen receptor negative tumors. Useful cell line models have been derived from human tumors and, more recently, by transfection of transformation-related genes into normal rodent and human mammary epithelial cells. Studies in these systems have led to the suggestion that EGF receptor expression, ligand production, and/or transduction changes are related to progression of the tumorigenic state in mammary epithelium.

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8. Cell proliferation in metazoans: Negative control mechanisms

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> Two methods of attack are used most frequently against a new theory: the first one is to claim that the new theory is wrong; the second one is to claim that it is not new.
>
> [Ernst Mayr, *The Growth of Biological Thought*, 1982. Cambridge, MA: Harvard University Press.]

Control of cell proliferation is a topic of central relevance to the understanding of normal and aberrant development, inflammation, and tumorigenesis in multicellular organisms. Lack of reliable premises for assessing accumulated data is probably responsible for our fragmentary understanding of this subject. In this review, we will highlight areas where conflict among premises and data require novel approaches to reconcile obvious incongruities. Given the vastness of the subject, it would be unrealistic to claim comprehensiveness when assessing sources and topics. We apologize for unintentional misattribution of credit and for not referring to related and worthy topics.

Control of cell proliferation in normalcy and disease is not a neglected area of research. Periodically, hopes for a substantial understanding of this subject are generated. The advent of novel techniques calls for the reexamination of already explored hypotheses. This reiterative pattern implies that our collective outlook on strategies to resolve this puzzle is fundamentally sound; it would then follow that, serendipitously, someone may stumble into the solution of the cancer puzzle. We believe, instead, that our collective experience compels us to question the soundness of the premises adopted so far. Repeated failures in generating solid understanding of this subject may explain the skepticism surrounding the emergence of novel approaches in this area. This review is undertaken with full recognition of this malaise in the readership's attitude.

Normal and aberrant proliferative patterns (tumors) attract the attention of developmental and cell biologists, biochemists, and molecular biologists, who would like to integrate the diverse etiopathogenic aspects of this process in a coherent fashion. In achieving this goal, scientists would unravel a basic biological riddle and physicians would tentatively apply a more rational

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diagnostic, prognostic, and therapeutic approach to the disease called cancer. So far, as mentioned before, this goal has been elusive. Observers not fully familiar with the scientific traditions of this field are offered an almost chaotic impression when attempting to assess the validity of adopted premises and to weigh enormous volumes of data [1–7]. Despite the intensity and length of efforts in this area, we are still at the hypotheses-building stage; therefore, even those who appear fully committed to defend their interpretation of data make generous use of conditional tenses in prefacing and concluding remarks. We will not be an exception to such a rule.

Definition of the subject matter

During this century scientists have learned much about *how* cells proliferate [1–3]; they have yet to learn much about *why* a cell proliferates, or does not, in multicellular organisms. In other words, elaborate descriptions are available on the steps cells undertake during the cycle to generate daughter cells; however, little is known about which signals the cells recognize to enter or not to enter the cycle. The latter process represents the regulation of the initiation of cell proliferation. We highlight this definition in contraposition to those of signal transduction and regulation of steps dealing with events occurring *within* the cell cycle (e.g., phosphatidyl inositol metabolism, histone synthesis, DNA synthesis, tubulin assembly, cyclin synthesis, centriole cycle, etc.) (figure 1).

Three principal sources of confusion pervade the literature over this topic. The first is vague nomenclature to define cell proliferation parameters. For instance, cell growth and cell proliferation are used as interchangeable concepts; objectively, increased cell size (hypertrophy) need not be caused by or temporarily linked to increased cell number (hyperplasia) in metazoans. A second source of confusion is the lack of consensus over how to measure cell proliferation; this results in fuzziness about what constitutes evidence. The classical notion borrowed from microbiology that comparison between proliferation rates of two differently treated cell populations be done during the log phase is not applied consistently [8]. A third significant source of misunderstandings is represented, as mentioned above, by confusion between the notion of control of (a) initiation of cell proliferation and (b) steps occurring *within* the cell cycle. Spurious analogies between DNA replication in prokaryotes [9] and cell cycle control in unicellular eukaryotes [10] and in metazoans [8] have also contributed to the confusion [11]. In this context, Pardee's group postulated that commitment to a round of the cell cycle occurs at a specific point within the G1 period [12]; this point is the 'restriction point.' Data were interpreted as being compatible with the presence of a single protein labile in normal cells more stable in tumor cells (R protein) [13]. These experiments were done under the premise that starved cells in culture are in a state comparable to that of normal, quiescent cells

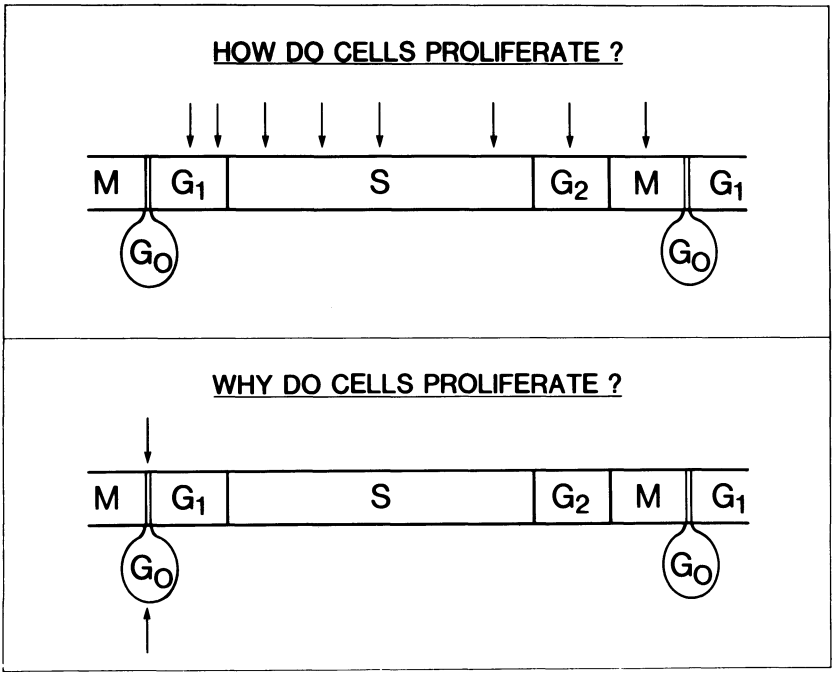

Figure 1. Schematic representation of the meaning of questions designed to learn about mechanisms dealing with intracycle parameters (how do cells proliferate?) and the initiation of the proliferative process (why do cells proliferate?). The latter deals with the decision to proliferate or remain quiescent; it is usually taken before cells enter the cycle proper. The former deals with the many discrete markers expressed during the successive, well-timed phases of the cell cycle. Intracycle markers should be efficiently expressed and regulated to successfully complete the proliferative event; no intracycle marker appears to have a hierarchical preeminence over any other. That is, a deffective DNA polymerase is not more deletereous than a deffective tubulin or histone assembly process. For details see text.

physiologically stopped from proliferating in animals. Whether or not the elusive R protein indeed plays a key role in determining the entrance of cells into the cell cycle is so far unclear.

Detailed genetic analysis of intracycle mutants has been described in yeast [14] and in multicellular organisms [15, 16]. However, little is known about how these organisms regulate the entrance of cells in the proliferative cycle; for yeast, a pattern similar to that in prokaryotes seems to be the most plausible option, i.e., nutrient availability is permissive for initiation of the proliferative event in a constitutive pattern, while nutrient starvation shuts off proliferation. To the contrary, cells in metazoans are subject to more complex regulatory mechanisms to control proliferation. Several questions address this issue: first, what kind of signals affect cells in metazoans to enter or not enter the cycle; second, when during the cycle are these signals read; and third, which are the cellular structures targeted for such signals. Complex answers for these questions are not yet available; however, attempts to deal

with them are starting to provide hopeful results. We have claimed that the G_0/G_1 boundary is the phase of the cycle where the signals to enter or not to enter the cycle are finally executed (figure 2) [11].

Under physiological conditions, cell cycle events are an unstoppable cascade of steps leading to the formation of two cells [1–3]; hence what happens along the cycle can be considered as a single omnibus marker amenable to quantification by merely counting cells as a function of time [11]. From our perspective, 'physiological conditions' imply assured sufficient nutrients present in the microenvironment where these cells reside. Extemporaneous starvation or intoxication prevent or delay the inevitable formation of daughter cells; however, these 'nonphysiological' experimental conditions are incompatible with events that cells face during normal development in living metazoans.

Cells in culture and in animals

'In culture' models represent a typical example of cartesian reductionism. The impossibility to extract ultimate answers from whole animal models has driven researchers to parcel questions using the welcomed ability of cells to proliferate in test tube conditions when supplied with a quasi-arbitrary set of

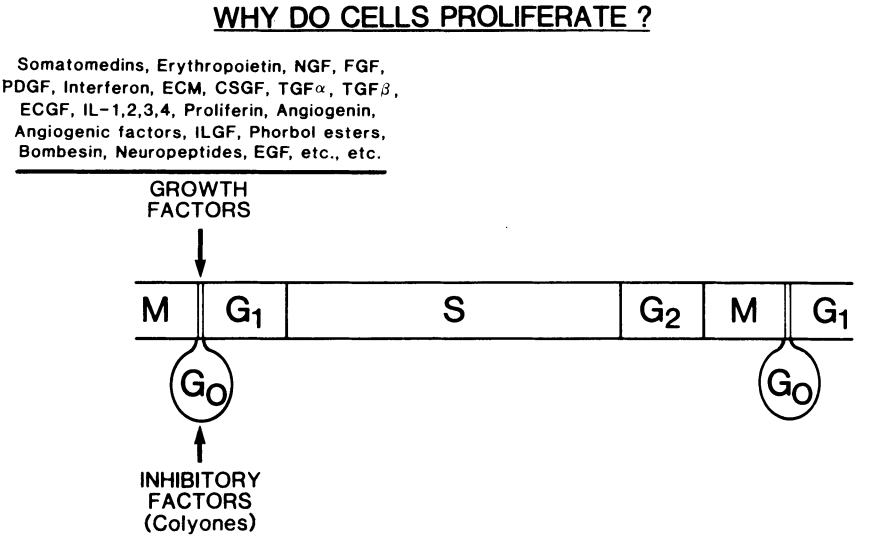

Figure 2. Schematic representation of choices available to study the control of initiation of cell proliferation. Positive control hypotheses are proposed under the general premise that cells are waiting for a signal to push them into the cycle; "growth factors" are the putative levers that carry these ultimate signals. Negative control hypotheses assume, to the contrary, that cells are always ready to proliferate; to implement this potentiality, inhibitory factors (colyones) ought to be neutralized, diluted, or not recognized by their target cells so they can enter the cycle. Under physiological conditions, the intracycle cell markers will be expressed unincumbered once the cell enters these cascade type series of events.

nutrients. Adoption of reductionist models implies an arbitrary selection of protagonists for the reaction under study. By now, after almost a century of experience, practitioners know that integrating cell culture and animal experiments is a sizable point of contention. Lack of agreement on premises adopted for experimental design and data analysis impair increasingly productive use of this powerful experimental tool. Cells in culture represent a complementary alternative to work done in whole animals.

The value of cell culture for understanding the control of cell proliferation is controversial [4, 11]. Organotypic cultures (primary cultures, organ cultures, etc.) are considered useful by some to study interactions between two or more tissues on a short-term basis; however, complex interactions among different cell populations in the explant preclude defining causality regarding the control of cell proliferation. Cloned established cell populations are considered valid experimental tools for studying the control of cell proliferation [1–3, 11]. However, some claim that in whole multicellular organisms, no cloned cell populations exist as such. Still a few question this experimental tool, because these cells either grow in monolayers, over plastic surfaces, or in suspension, conditions that can hardly be considered standard for cells in live metazoans. Finally, some question the use of cloned established cell lines that induce tumors when injected back into syngeneic hosts or nude mice; they argue that conclusions drawn with these cells cannot be extended to control mechanisms for 'normal' cells. These are the types of arguments raised against tentative generalizations on identifying controls affecting cell proliferation in metazoans. Are these valid objections? If the answer is 'yes,' one may surmise that work on this area is just a worthless exercise that provides employment to some skilled workers. If the answer is 'no,' it is probably incumbent upon the skeptics to demonstrate that, for instance, cloned established cell lines are not a valid reductionist approach to the challenge defined above. Resorting to the use of cells in culture grew out of the search for *ultimate* causes regulating proliferative events, which whole animal studies could not provide. Extrapolation is a legitimate scientific device. With hindsight, a case can be made for scientists having failed to properly extrapolate data gathered through in-culture models and through whole metazoans. We insist that the key for these failures has been the selection of premises for experimental design and interpretation of data.

Premises and working hypotheses

To unravel mechanisms by which cells decide to enter or not to enter the cycle, premises and working hypotheses are adopted to serve as intellectual constructs for designing experiments. Three main approaches represent a variety of premises regarding the proliferation of cells in culture. The first is (A) that cells proliferate in culture conditions because the 'defined' media in which they are placed contain nutrients necessary for their survival, while the supplemented sera merely carry hormones needed for triggering cell

multiplication. Proponents of this line of thought recommend substituting sera with a mixture of genuine and putative hormones ('growth factors') that may be exquisitely tailored for the proliferation of each cell type [17, 18]. Judging by the scarcity of cell lines that can be routinely carried in serum-free conditions for indefinite lengths of time, one may conclude, in hindsight, that this hypothesis has not been successful.

The second school of thought postulates (B) that cells in culture proliferate because of the above-mentioned reasons; however, data gathered using the methodology of the first school of thought appear to be erratic [19]. Hence, as a compromise between putative chemically defined media and the need to get reproducible, maximal cell multiplication rates, synthetic media are supplemented with serum at concentrations below the one needed for proliferation; then media are supplemented with more 'chemically defined nutrients' until proliferation is reestablished [19, 20]. This approach, like the previous one, has not been widely adopted either; nevertheless, periodic claims by independent researchers and commercial laboratories remind us about this still-open possibility [21]. Intended targets of this message are researchers wishing to minimize problems when purifying a secretory product from cell cultures. The usual result is a livable compromise whereby cells are presented with a *reduced* serum level supplemented to the basal media that allows for cell proliferation, acceptable viability, and reasonable yields of synthesis and secretion of the cell product of interest. These may be considered ephemeral pragmatic solutions, but clearly they end up begging the question posed above.

Finally, the third school of thought postulates (C) that cells proliferate exponentially and maximally, as predicted by the equation $Ct = C_o e^{\alpha t}$, only when they are supplied with a mixture of amino acids, sugars, vitamins, and salts, *plus* variable amounts of sera usually of heterologous species [22]. Hence, serum must carry yet to be defined essential, nutritive components that are necessary for maximal, steady, exponential cell multiplication.

Two opposing working hypotheses underly the above listed experimental approaches. While approaches (A) and (B) are based on the asumption that, ultimately, cells require positive signals to push them into the cell cycle (direct and indirect positive control hypotheses), approach (C) is founded on just the opposite assumption; that is, cells are always ready to proliferate and to do so they have to be free from inhibitory signals that prevent them from entering the cycle. These are diametrically opposed premises. Because these premises are mutually exclusive, data cannot be integrated to accommodate both hypotheses simultaneously [11, see below].

Strategic approaches

By adopting either the positive or the negative control hypotheses, one becomes committed to design experiments based on significantly different sets of premises. Those adopting the positive hypotheses would search for ever-increasing numbers of 'growth factors' and for minimal, chemically de-

fined media; instead, those adopting the negative control hypotheses would search for media full of nutrients in which, nonetheless, cells will not proliferate as a result of the action of specific inhibitors. Who will ultimately prevail in clarifying this issue, and when? Ideally, an unequivocal answer will emerge when 'growth factors' *and* 'growth inhibitors' become purified and tested in a milieu comparable to that bathing cells in multicellular organisms. While 'growth factors' are now available in purified form, genuine 'inhibitory factors' are not yet available. As a result, the current confusing state of affairs will probably continue, because none of the two competing hypotheses can be verified and determined acceptable by the practicing scientific community. On the one hand, proponents of the positive hypotheses have not marshalled compelling data favoring their arguments; this is mainly due to their inability to reproduce in culture the nonstarving, quiescent status experienced by normal cells in situ. Even more ominous is the paucity of evidence suggesting a physiologic role for *any* of the 'growth factors' described so far [1–5, 23–25]. On the other hand, those favoring the so far minority view (negative hypotheses) propose the hardly captivating option to define those components in serum that are true nutrients and those that have specific inhibitory roles on cell proliferation. At least this latter approach has provided for a semblance of physiological quiescence in nonstarving culture conditions [26].

The need for making an *exclusive* choice in selecting a working hypothesis for the control of cell proliferation becomes imperative. Positive hypotheses require that at some point a negative step be introduced; otherwise, constant positive stimuli will inevitably result in tumor formation for lack of a balanced population control. Therefore, even if one prefers positive control hypotheses, the coexistence of positive and negative signals becomes unavoidable. To accommodate this reality, the metaphysical notion of 'ying–yang' was introduced [27]. To the contrary, when adopting the premises of the negative hypotheses (direct and indirect), proliferation becomes a constitutive, integral property of cells; according to these premises, only negative signals will *ultimately* determine whether or not cells will proliferate or become quiescent. No positive signal is required when adopting the premises of the negative control hypotheses [11]. Summing up, depending on the premises one chooses, two paths may be followed to study the regulation of cell proliferation in metazoans: either (a) search for ever increasing numbers of 'growth factors' whose physiological roles are yet to be determined, or (b) plunge into the unappealing, but potentially rewarding, field of serum protein characterization and purification to define the role of their components under a physiological approach.

The nutritional requirements of cells in culture

Limited consensus exists on the important topic of cellular nutrition in relation to the control of cell proliferation in metazoans. Probably this is a reflection of our collective ignorance on the subject. In the last 30 years few

advances in understanding the general principles of cellular nutrition have been accomplished. The nutritional status of prokaryotes and of unicellular eukaryotes determines whether the cells will proliferate or enter a vegetative phase of their life cycle [8, 28]. On the contrary, when multicellular organisms in their natural environments are faced with extended starvation periods, they adopt a hierarchical order of proliferative responses at the cellular, organismic, and population levels. While this latter level have been discussed and documented since Darwin's publication of the *Origin of Species* in 1859, we know little about how metazoans cope with starvation at the cellular level [29, 30]. This paucity of knowledge extends to the topic of starvation of cells in culture; as a result, definition of essential nutrients and their role during cell proliferation in metazoans along upward levels of complexity (tissues, organs, systems, and the whole individual) is now vague.

Within this context, attempts to address the topic of control of cell proliferation in multicellular organisms have been hindered by lack of consensus regarding methodological approaches. The limiting factor when adopting the positive control hypotheses is the inability of having viable, nonstarving quiescent cells in 'chemically defined media;' the limiting factor for the negative control hypotheses is the inability of having exponentially proliferating cells in truly 'chemically defined media' on a long-term basis. Therefore, one may surmise that available culture media are not suitable for hypothesis testing, regardless of the hypotheses one may favor. Progress in this area will then be subject to successful designing of nutritionally adequate culture media. Recognition of this important stumbling block requires facing new challenges regarding the role and nutritional value of serum components.

A paradox emerges when one adopts the premise that serum is, according to the positive hypotheses, a mixture of nutrients (amino acids, salts, vitamins), hormones, and 'growth factors' (epidermal growth factor, platelet-derived growth factor, fibroblast growth factors, etc.) [17, 18]. The paradox is the following: while cells proliferate maximally in the presence of variable concentrations of serum-supplemented media, replacing serum by 'chemically defined' components supports cell proliferation only for a limited number of generations. This is then followed by (a) loss of the proliferative capacity of cells (senescence) even when they are returned to serum-supplemented medium, and (b) eventual cell death probably as a result of variable degrees of vital cell damage due to starvation [31]. From our perspective this indicates that serum carries unidentified essential nutritional components; they are unlikely to be the standard components of the 'richest' formulations nor the defined components generically known as 'growth factors' (see below). Instead, when adopting the negative control hypotheses for testing why cells proliferate, it is thought that nutrients merely play a permissive role and are irrelevant when signaling takes place in regulating cell proliferation [11]. This option, however, does not minimize the need to define the nutritional components of serum.

Strategies to evaluate cell nutrition during cell proliferation

Bettger and McKeehan claimed that 'the regulation of nutrient uptake is potentially a major site for control of cell processes such as survival, multiplication, and expression of differentiated function' [20]. Similarly, high-density lipoproteins have been considered essential nutrients in media where cells proliferate [32]; these normal sera components have become inducers of cell proliferation and indiscriminately referred to as 'mitogens' of many cell types from different species [17–20, 27]. As mentioned above, nutritional requirements have been defined using starvation in culture conditions as the procedural tool to identify macromolecular media components. Implicitly, this nonphysiologic set up has been repeatedly compared to the physiological nonstarving conditions that most nonproliferating 'resting' cells in metazoans are in. This unfortunate extrapolation has been responsible for the *operational* definition of 'growth factors.' Moreover, assuming for the sake of argument that cell proliferation is indeed regulated in embryos and adult metazoans through local nutritional restriction [20], this would require having in place a constantly updated mechanism whereby these restrictions are kept codified in each discrete microenvironment. The existence of such a code represents an extraordinary additional layer of complexity in the developmental plan. So far, no empirical evidence for such an option has been presented. Summing up, a combination of faulty premises and the misinterpretation of experimental results significantly contribute to confusion in discriminating among levers regulating cell proliferation in metazoans.

Hormones and 'growth factors' as regulators of cell proliferation

A regulatory role of hormones on cell proliferation has been predicated to occur through changes in plasma membrane permeability, intracellular compartition of nutrients, rates of nutrient uptake and metabolism, specific enzyme activity, etc. [20, 33–35]. Polypeptide 'growth factors' represent for some a novel form of hormones [36]; this proposition is based on data interpreted along the classic canons of endocrinology, whereby polypeptides are secreted by single or multiple organs, travel through the bloodstream, and have well defined target cells. Transferring properties of hormones to growth factors has not been entirely successful; first, some growth factors are apparently not secreted, nor are they blood-borne [37]; and second, given the broad target specificity of growth factors and the lack of plasma level fluctuations, it is now proposed that their effects are exerted through paracrine and/or autocrine mechanisms only [24]. In classic endocrinologic experiments, the removal of the putative source of a hormone and the subsequent replacement by an extract or the purified active molecule are compelling arguments for specificity of source and of target. Because growth factors are

synthesized by many tissues and there are many putative targets for each of these substances, a physiological definition of causality appears to be precluded; this peculiar feature requires explaining the role of growth factors as that of special cases of hormones [36]. In sum, increasingly convoluted, ad hoc arguments have been formulated for the last two decades to explain the elusive physiological role of growth factors.

Differentiated function and cell proliferation in animals and in culture

The misconception that differention status and proliferative capacity are inversely related is widespread in the literature [1, 2]. This is contradicted by the occurrence of normal and tumor cells expressing differentiated properties while proliferating during various developmental stages [38]. The experience with cells in culture sheds light on this topic. *The American Type Culture Collection* catalogue lists a good number of differentiated cell types from a variety of species and organs. Moreover, the actual ability of neurons to proliferate in situ during adulthood has now been documented in vertebrates [39, 40]; other so-called differentiated cell types are known to proliferate at a slow rate in situ as well [41, 42]. In sum, cells in culture routinely express 'differentiated' functions; these phenotypic properties do not preclude or anticipate their proliferative performance in culture conditions or during tumorigenesis.

Tumor cells as tools for studying the regulation of cell proliferation

The use of primary tumors or their metastases to establish cell lines offers the practical advantage of serving as natural enrichment sources of the cell types one may wish to study. Also, tumor cells in culture represent a natural source of genetic variants of the 'cell proliferation' marker. Lack of basic understanding regarding why cells in culture may become immortalized represents a significant obstacle for a more widespread and successful use of this approach. Nevertheless, tumor cells are suitable for the characterization of proliferative behavior from a somatic cell genetic perspective; the availability of 'in-animal in-culture' tumor cell models increases the relevance of this approach [11].

Sex hormones as regulators of cell proliferation

In metazoans, cells from one tissue communicate with those in other tissues through the internal milieu. These means of communication vary. Depending on the complexity of the metazoan, molecules traveling through diverse means (intercellular, lymph, plasma) regulate the proliferative capacity of

different tissues. In fact, the concept of hormone emerged from this background. For our purpose, estradiol has the advantage of being a relatively small molecule (MW272), is not significantly metabolized by target tissues, is secreted by mainly one organ (the ovary), and has phenomenological attributes that have been extensively verified in many species. Hence, estradiol represents a well-defined and uncomplicated lever for studying the proliferative regulation of its target cells [11]. To some extent this contrasts with the properties of androgens, which are extensively metabolized by their target tissues [43, see below]. However, compelling data suggest that 5α–dihydrotestosterone (DHT) appears to be the most active androgen and an effective regulator of the proliferative behavior of androgen target cells [43, 44]. Thus both estradiol and DHT fulfill the requirement of being physiologically relevant small molecules widely acknowledged to be the *proximate* cause for the proliferation of well-defined target cells. From our perspective, estradiol and DHT represent ideal case studies to unravel the regulation of entry of cells into the cell cycle and to finally identify *ultimate* causes of their mechanism of action.

Positive versus negative controls

The regulation of cell proliferation of estrogen-sensitive cells has occupied our attention for the last 20 years. The first five of them were spent exploring the most popular paradigm proposing that estradiol would directly and positively induce the proliferation of its target cells [45, 46]. Beginning in 1976, lacking objective success in verifying that estradiol induced directly the proliferation of its target cells, we began exploring alternative possibilities; that is, that the effect of estradiol was exerted indirectly (figure 3). This meant that, in the animal, estradiol would affect a first target cell that, in turn, would affect a second target cell (rat uterus, quail oviduct, rat pituitary, and mammary tumors) [35, 47–50]. After five years of testing indirect positive mechanisms of estradiol action, we concluded that this approach was equally unproductive [22].

Over a decade ago we began exploring the control of cell proliferation under the premises of the negative control hypotheses. Adopting these new premises required dropping those premises supporting the positive regulation hypotheses. This was due to the incompatibility of such premises; either cells are always quiescent until a positive signal thrusts them into the cycle, or else proliferation is a constitutive property of cells, which can only be regulated by inhibitors. Admittedly, adopting the negative control hypotheses requires disregarding an enormous body of literature generated under the premises of the positive control hypotheses; these include the whole fields of growth factors and those of oncogenes (see below). It should be clear that the outcome of the proliferative event remains unchallenged; only the mechanism of its initiation is being revised. That is, instead of searching for growth

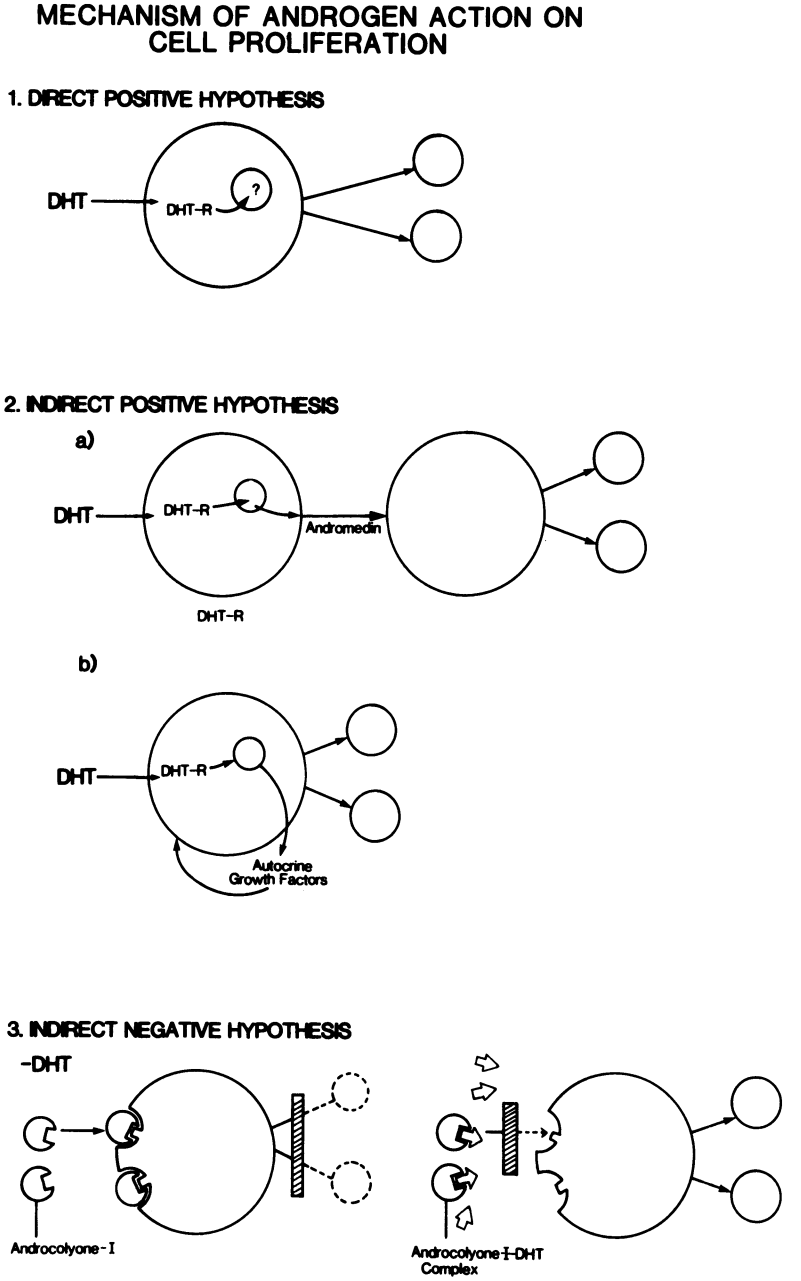

Figure 3. Schematic representation of the three main working hypotheses dealing with the control of cell proliferation in metazoans. For details see text.

factors and their mechanisms of action (positive control), defining when, where, and how the cancellation of inhibitory factors takes place becomes the new goal (negative control).

Estrogen and androgen-sensitive models

Toward the late 1970s we began formulating an exclusive negative control hypothesis for the control of cell proliferation [22]. Only when we verified a nonproliferative stage under *non-starving* in culture conditions did we figure out additional significant implications of negative controls for cell proliferation [26]. A realistic experimental context in which positive and negative hypotheses could be objectively tested then emerged. To determine the validity of the novel premises adopted, we explored a variety of experimental models, including rat pituitary and mammary estrogen-sensitive tumors, quail oviduct, Syrian hamster kidney tumor, and rat fibroblasts [49–52]. The empirical data collected refined our understanding of the regulation of cell proliferation in metazoans. Currently we are using human breast tumor cell lines as estrogen target cells [53–56] and human prostate tumor cell lines as androgen targets [57, 58] to explore the worthiness of negative control hypotheses. Crucial milestones in our work have been the realization that (a) homologous sera inhibit the proliferation of sex hormone target cells in a dose-dependent manner, and (b) sex hormones specifically cancel this inhibitory effect. These features satisfactorily explain the proliferation of target cells following the administration of estradiol–17β to oophorectomized females and testosterone to castrated males. We are now characterizing and purifying the human plasma-borne inhibitor of the proliferation of estrogen-sensitive cells. Generically, we call these inhibitors colyones (from the Greek κωλύω, meaning to inhibit); when dealing with estrogen- and androgen-sensitive cells, we have named their inhibitors *estrocolyones* and *androcolyones*, respectively.

The regulation of cell proliferation by sex hormones is exerted at distinctly separate hierarchical levels. The data gathered so far are compatible with the notion that estrogens and androgens regulate proliferation of their target cells in a two-step mechanism: first, they specifically *neutralize* plasma-borne colyones that lead to proliferation, and second, they *inhibit* proliferation by probably triggering the synthesis of indigenous cell proliferation inhibitors (shutoff effect) (see figure 4). The shutoff effect appears to be the one in which participation of intracellular-specific sex hormone receptors is definitively required [11, 53–58]. This shutoff effect completes a cycle of balanced cell proliferation under physiological conditions. More importantly, this combination of mechanisms anticipates a blueprint of possible aberrant proliferative patterns responsible for the emergence of sex hormone-sensitive target cell tumors and their respective insensitive variants. At this time our interpretation of the data suggest that (a) sex hormone target cell tumors

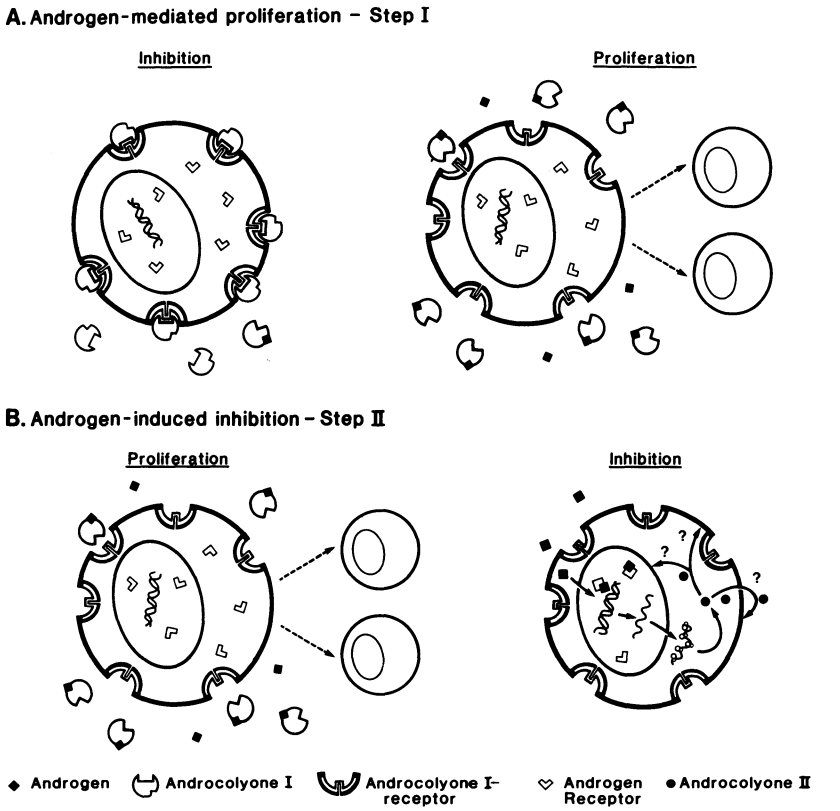

Figure 4. Schematic representation of the mechanism of action of sex hormones on the control of cell proliferation according to the negative control hypotheses. Even though the scheme mentions androgens exclusively, its general properties can be extended to cover estrogen effects on their own target cells as well. A two-step mechanism is proposed; during the first phase, sex steroids cancel the inhibition exerted by plasma-borne inhibitors that prevent their respective target cells from proliferating. During the second step, sex hormones specifically induce the synthesis of indigenous inhibitors that prevent proliferation of their target cells; this latter step would require the mediation of the respective sex hormone receptors to elicit the expression of genes (colyogenes) coding for these specific inhibitors or their receptors. This scheme is compatible with the data collected so far. For details see text and suggested references.

develop when the shutoff mechanism is partially or totally upset and (b) sex hormone sensitivity is lost by these tumor cells when they no longer recognize specific inhibitory signals by their respective plasma-borne colyones.

The adoption of these novel hypotheses necessarily entails the characterization of the missing protagonists in the proliferative reaction. They include at least two inhibitors of the proliferation of the human estrogen-sensitive cells we are using: the plasma-borne *estrocolyone–I* and the shut-off protein induced by estradiol, *estrocolyone–II*. Similarly, when dealing with

androgen-sensitive models, the plasma-borne inhibitor *androcolyone–I* and the one induced by androgens intracellularly, *androcolyone–II*, should be characterized as well. Plasma-borne inhibitors probably act through specific plasma membrane receptors. Full definition of these protagonists and their interactions are inherently difficult tasks because they require identifying small quantities of regulatory proteins from a complex mixture of components present in plasma and intracellularly, respectively.

The LNCaP human prostate tumor cell lines [59] are useful tools for studying the mechanism of androgen action on cell proliferation. Data compatible with the notion of inhibition of cell proliferation by homologous charcoal dextran-stripped serum in a concentration-dependent fashion and cancellation of this inhibition by androgens and 'antiandrogens' are now available [57, 58, 60]. Androgens also specifically trigger a shutoff effect in these LNCaP human prostate cells. Variants of the original LNCaP cells that are not inhibited by charcoal-stripped human serum have also been characterized [59]; this suggests that cells in prostate carcinomas resistant to castration may show a defective androcolyone–I receptor [Sakabe et al., submitted]. The study of variants of the regulation of estrogen- and androgen-sensitive cell proliferation through the perspective of the negative control hypotheses will help in the comprehensive understanding of these biological and molecular phenomena [11].

Negative controls in non-sex hormone target cells

How are cells other than sex hormone-sensitive cells regulated to proliferate within the context of the negative control hypotheses? We have published data compatible with the notion that negative controls are responsible for the proliferation of rat fibroblasts [51]. Also, the presence of a blood-borne inhibitor for the proliferation of hepatocytes has been proposed by data analysis from partial hepatectomy studies [61]. These are important clues to controls over the proliferation of non-sex hormone-sensitive cells. Reports suggest the presence of putative inhibitors of the proliferation of a variety of cell types [62]. Assessing data on the physiological context in which some of these putative inhibitors have been defined requires caution. For instance, transforming growth factors–β (TGF–β), the most popular example of a putative inhibitory factor, have been purified and sequenced [63–65]; however, no empirical biological evidence is so far available to consider them genuine physiological inhibitory factors. Lack of (a) specificity of source and targets and (b) significant differences between proliferative rates in control versus experimental hinder reliable claims that TGFs–β are genuine inhibitory factors. Similar considerations may be raised about other putative inhibitors [62]. Also, *chalones*, probably because their putative paracrine mechanism of action, so far have not lived up to expectations regarding their precise characterization and biological effectiveness [66].

Forward and backward approaches

From the negative control hypotheses perspective, defective steps in the receptive process of the inhibitory signals at the plasma membrane and intracellularly are probably responsible for unscheduled proliferative events. Characterization of these important regulatory steps and the cell products responsible for the normal behavior represent a formidable research enterprise to investigate with the tools available at present; this is so despite significant advances in the fields of genetic recombination and protein purification. However, promising inroads in this direction are being made using a forward approach in Drosophila and in human tumors [7, 67–72, see below]. This approach centers on identifying 'antioncogenes' and the gene products they code for. The best known current example of this approach is that of the putative 'retinoblastoma gene' tentatively located in the long arm of chromosome 13 in the human genome [7].

In contraposition to the forward approach, we adopted a backward approach. A discrete function (cell proliferation) in a well-defined model was selected with the purpose of identifying a discrete molecule responsible for its regulation [11]. Once the purification of the colyones is completed, by working our way back the identification of genes coding for specific inhibitors and their respective receptors will become a more manageable task. The backward approach that we adopted and prefer because of its more physiological bent and the forward approach adopted by molecular biologists are likely to provide complementary clues for a definitive understanding of the control of cell proliferation in normal and tumor cells (see below).

Carcinogenesis in the context of competing hypotheses on the control of cell proliferation

Enormous amounts of data on carcinogenesis have been compiled during the last hundred years. Most of the data are based on a variety of hypotheses whose premises have never been verified. No practical purpose would be accomplished if we reviewed them all; suffice it to say that no greater phenomenological and mechanistic understanding of this process has been attained today than when Peyton Rous in 1908 and Yagashima and Ishikawa in 1918 first suggested a viral or chemical etiology for tumors, respectively. Contrary to widely repeated perceptions, not even the molecular biology revolution has solved this puzzle, even though we now know much more about many aspects of physicochemical reactions that correlate with carcinogenesis (DNA synthesis, transcription control, xenobiotic metabolism, etc.).

That genetic damage plays a significant role in carcinogenesis is supported by the hereditary predisposition to cancer [38, 68, 72]. The mechanistic understanding of multiple steps in this process are unclear so far despite the extensively documented familial cancer trait. For the most part, data on this topic reflect interpretations compatible with the premises of positive hypoth-

eses for the regulation of cell proliferation, with selective reference to the interlocking of differentiation and proliferative processes (criticized above). Lately a trend towards combining positive and negative hypotheses is evident [7, 24, 71–73].

Defining tumors and tumor cells

Defining what a tumor and tumor cells are has proven to be a frustrating exercise. Over 15 years ago, F.F. Becker reflected on the relevance of experimental approaches faced with 'our inability to define the malignant cell' [74]. This is still a difficult and sobering task. Adopting a hierarchical criterion in defining the lowest common denominator within all tumor properties may be a productive approach. In a biological context, no tumor can be defined without an aberrant, time-dependent, localized increase of cells in a living host. From such a perspective, the central, inescapable issue in understanding carcinogenesis is to define regulatory mechanisms of cell proliferation at a cellular level in metazoans.

During the last hundred years an almost unending list of markers has been causally associated to the ability of cells to produce tumors (chromosomal aberrations, plasma membrane changes, enzymatic profiles, etc.); it was just a matter of time to see this wishful correlation vanish when normal (non-tumor) cells were shown to carry those very markers or patterns at some point in the developmental process. Oncogenes are but the latest example of 'unique' properties carried only by tumor cells; by almost any measure of reliable evidence, this is one more example of the elusive, putative uniqueness of the tumor cell when indiscriminately compared with normal cells.

Because of these acknowledged difficulties in defining tumor cells, it may be productive to consider them in a dual scenario: i.e., in biological and clinical contexts. In the latter, tumor cells retain their 'classical' properties; that is, cells may likely kill the host as a result of their increase in number and invasiveness. Biologically, instead, it is difficult to identify a tumor cell because of the lack of a discrete, consistent marker with which to reliably tag it; it should be remembered that clinically verifiable tumor cells may switch their invasive and proliferative pattern, stop, or even disappear [38, 68, 70, 75–79]. This often unpredictable pattern makes it unwise to insist in defining *the* biological uniqueness of clinically definable tumor cells. summing up, we propose that the proliferative behavior of cells in metazoans be considered at two independent hierarchical levels: a biological one and a clinical one. Failing to do this inevitably leads to a byzantine discourse lacking clarifying purpose. By defining the control of cell proliferation from a strictly biological context, one may concentrate on describing normal and aberrant patterns of cell proliferation and determining their underlying mechanisms; this can be done without engaging in clinically germane but epistemologically irrelevant concerns.

The oncogene-antioncogene theory of carcinogensis

The oncogene theory originally formulated by Huebner and Todaro [80] was revived in a slightly modified version by Weinberg [81] and quickly adopted by many others [82, 83]. Weinberg's modification as well as the original oncogene theory was based on the premise that the role of the oncogenes or their products was to signal positively to a cell waiting to enter the cycle. Also, the modified oncogene theory implied that there were viral genes that were responsible for tumor formation, and when they were transfected into 'normal' cells, they transformed them into tumor cells (the 'cancer phenotype' meant transformation *and* tumorigenicity). When it was found that 'normal' cells carried DNA sequences homologous to viral oncogenes, alternatives were considered to remedy this obvious lack of fit [6, 84]. By the late 1980s, it had become clear that while we knew more about viruses, viral genes, and gene expression in somatic cells, the foresight assessment of their relationship with the carcinogenic multistep processes might have been oversold [7]. Two short, recent quotations by active participants in this field provide pragmatic assessments of current epistemological arguments. J.M. Bishop stated, 'There is as yet no direct evidence that activation of proto-oncogenes by insertional mutagenesis is tumorigenic... nor has a point mutation of a given proto-oncogene been consistently implicated in the genesis of a specific tumor....' But according to him, it is 'the considerable logical force' of the argument for oncogenes that makes it compelling [85]. Along comparable lines, H.E. Varmus candidly admitted that 'monumental list of oncogenes and proto-oncogenes has been assembled, but the biochemical activities crucial to growth control and neoplasia awaits discovery' [86].

Carcinogenesis and negative control of cell proliferation

The premises of the negative control hypotheses suggest that cell proliferation is a constitutive property of all cells; therefore, cell proliferation must be subject to very stringent regulation in order to thwart extemporaneous cell proliferation that may eventually result in tumor formation. Available evidence is compatible with the existence of genes responsible for the synthesis of proteins that prevent proliferation of these cells [11, 72]. Based on our proposition to separate the clinical context from the biological context, we proposed a nomenclature that dealt with these inhibitors and the genes that coded for them. *Colyogenes* are genes coding for circulating and intracellular inhibitors of the proliferation of particular cell types (colyones).

During ontogenesis, inhibitory mechanisms would be in place to allow cells to proliferate only on a selective, temporary bases. These proliferative bursts would come to an end whenever colyogenes were transcribed, their mRNA was translated, and the colyones were in place and activated. One can easily

visualize the multiple sites at which regulation of the synthesis, post-translational events, and localization of colyones and their receptors may take place. Cell-to-cell contact sites represent additional loci where negative regulation of proliferation may take place in metazoans [74, 87].

Accurate, rigorous nomenclature will significantly contribute to a swift, increasingly complex understanding of this subject. This is now becoming of paramount importance. The terms 'antioncogenes' and 'suppressor genes' are used in expediency, disregarding the teleological implication that cells have in their normal repertoire genes that preclude their becoming tumor cells. Instead, calling genes for the control of cell proliferation 'colyogenes' is predicated without prejudice on the two hierarchical levels mentioned above (biological and clinical). To further dramatize this distinction, an analogous situation will be recalled here. We define the inborn error of metabolism, called phenylketonuria, as a genetic defect involving a mutation in a gene coding for a discrete enzyme [88]. It would be unacceptable to characterize such a gene as a 'mental retardation' gene. Mental retardation occurs in these patients only when the enzyme does not properly perform on its substrate and when many other requirements and conditions are satisfied. In the same context, colyogenes are genes that control cell proliferation without prejudice as to whether they are carried by normal or tumor cells; their meaning is directed at defining a physiological cell function executed with variable degrees of success by both normal cells during all stages of development and by genuine tumor-generating cells. The term colyogenes is free of any unwarranted implication regarding the clinical entity called cancer.

In sum, proliferative patterns during development are probably regulated by specific negative control mechanisms at cellular, tissular, and organ levels. Colyogenes would be expressed constitutively. A 'spontaneous' or an induced somatic mutational event affecting colyogenes or the reception of inhibitors they code for would represent the original genetic defect leading to eventual tumor formation. Additional miscues of an epigenetic nature also play significant roles in tumor formation (see below).

The multistep nature of carcinogenesis

The multistep nature of the carcinogenetic event is now well documented [4–7]. However, this process should not be construed as a precise, sequential series of mutations. Mutational events may represent a necesary but not sufficient requirement for the formation of a clinically detectable tumor [11, 84]. Tumor formation is probably due to a combination of (a) germinally-carried aberrant colyogenes coding for an aberrant protein whose function could either be that of an inhibitor of cell proliferation (colyones) or its receptor, plus (b) a temporary or permanently imbalanced microenvironment in which the inhibitory process of cell proliferation cannot take place [26, 68]. This latter step may be the one that can be experimentally manipulated to

better understand the epigenetic control of tumor progression. The lethal giant larvae [1(2)gl] mutant of Drosophila is the best example described so far that fits the above mentioned scenario [67–70]. Teratocarcinomas are comparable examples of this interpretation of available data [76]. Other instances where genuine tumor cells (i.e., cells that develop tumors when injected into syngeneic hosts) can be restored to be part of normal individuals have been documented for animals and plants [77, 78]. It is not their genomes that have been restored to normalcy; instead, these cells have found optimal temporal circumstances for processing a normal inhibitory signal (colyone) produced by proximal and/or distal cells from the host in which they were introduced. Equally important, these interpretations do not require linking differentiation properties of cells and their proliferative capacities; their respective regulatory mechanisms are not necessarily linked but just temporarily overlapped. The negative hypotheses for the regulation of cell proliferation can also account for other experimental tumors in a comparable fashion [89, 90].

Equally provocative questions are raised by studies relating to the hereditary nature of retinoblastoma, osteosarcomas, Wilms' tumors, and others [71, 72]. The evidence presented so far fits best when adopting negative control hypotheses [7, 11]. Unfortunately, the lack of experimental models where cause-effect relationships can be defined impair further understanding of the subject [72].

Conclusions

Analysis of data already available and of those generated by formulating the regulation of cell proliferation under the premises of the negative control hypotheses indicates a fertile source of promising options. It was failure to generate an increasingly complex understanding of this regulatory process using the premises of the positive control hypotheses that compelled us to search for significantly different alternatives aimed at answering the question, Why do cells proliferate in metazoans?. Negative control hypotheses generously fulfill needs for a comprehensive understanding of normal and aberrant (tumor) patterns of cell proliferation.

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III.

Estrogen and Antiestrogens

9. Steroid modulation of the expression of growth factors and oncogenes in breast cancer

K.S. McCarty, Jr., and K.S. McCarty, Sr.

Overview

Breast cancer, as with most cancers, is the consequence of a multistage process dependent on a number of independent mutational events. One of the critical events is an escape from cell cycle control. In normal breast cells the initial signal for cell cycle progression is estrogen modulated by progesterone. This signal modulates gene expression by the interaction of estrogen with its receptor (ER). The estrogen-receptor complex functions as a *trans*-acting factor targeted to specific *cis*-acting estrogen responsive elements (ERE) located either within or around specific target genes. Detailed analysis of this process provides insight into possible mechanisms for the hormone independence of breast tumors. Estrogen receptor is part of a superfamily of hormone receptors (HR). The interrelations of these receptors with other *trans*-acting factors, including growth factors and oncogenes, appear critical to understanding neoplastic transformation of the breast, and its relation to hormone control.

The consequences of estrogen independence must be considered in relation to growth and differentiation that is modulated by altered ER, growth factors, proto-oncogenes, normal oncogenes, and genetically altered oncogenes. This chapter examines some of the interrelations of these factors. There are a number of general reviews on oncogenes [1–3], oncogenes and growth factors [4, 5], specific discussions of estrogen receptors and growth factors [6–10], and specific mechanisms of hormone effects on growth control in breast tissue [11–13].

Our objective is to propose that it is the steroid activation of the estrogen receptor (ER) that accounts for the primary 'permissive' event required for cell cycle activity. The balance between growth factors and activator and suppressor oncogenes is essential for the competence, progression, and commitment needed for this process. It is only those cells that are in the 'permissive' state that respond. The increase frequently seen in cell cycle activity has been termed 'cell immortalization.' Cell immortilization is the first step in the complex multistage process of malignant transformation that is likely to require three to ten separate genetic mutations [14]. These steps may in-

M. Lippman and R. Dickson (eds.), REGULATORY MECHANISMS IN BREAST CANCER.

clude gene activation, translocation, or point mutation of activator growth factors, or the functional loss as a result of gene deletion or translocation of suppressor growth factors.

Not only do terminally differentiated cells appear to be more refractory to the process of malignant transformation, but it is also apparent that through genetic selection they are uniquely protected. This selection aganist oncogenesis is likely to be the consequence of the precision and complexity of the multiple events required to regulate the cell cycle. This also implies that cells that are rapidly proliferating are at greater risk of oncogenic mutations.

We are still in the stage of cataloguing growth factors and oncogenes, with a tendency to ignore the normal while concentrating more on the terminally malignant cell. Both growth factors and oncogenes must be considered together as competence and progression factors that are both involved in normal cell cycle activities. A key to understanding some of the mechanisms of oncogenesis will first depend on our concept of the normal cell cycle as a balance between activator and suppressor genes. Then with this perspective, we will be in a better position to consider the consequence of the effect of multiple complex genetic mutations.

The normal mammary gland

The normal breast is a self-renewing tissue consisting of cells at different stages of growth and differentiation. This organ, in its simplest form, consists of epithelial, myoepithelial, adipocytes, fibroblasts, and endothelial cells as well as circulating mesenchymal elements (e.g., platelets). Breast tissue responds to a series of regulatory signals from these cells, which are essential for its orderly development and progression through the cell cycle. The primary permissive signal is the level of estrogen that interacts with the ER of the target epithelial cell. In the mature breast the individual epithelial cell progresses from a resting to a secretory state on a 28 day normalized menstrual cycle. Estrogen as an endocrine signal is insufficient by itself to induce cell proliferation and requires additional paracrine signals. These signals consist of a series of growth stimulatory, inhibitory, and auxiliary factors that both amplify and repress the cell cycle. During embryogenesis and possibly during 'normal adult cyclic differentiation,' these signals consist of growth factors elaborated by cells other than the target cell. Normally, these growth factors function as paracrine secretions limited to a narrow window of time and specific not only for the tissue, but also for the stage of maturation and the level of receptors on the target cell.

It has become increasingly evident from the epidemiology and pathology of breast cancer that breast cancer, as is true with most cancers, is the consequence of a multistage process dependent on a number of independent mutational events. The best evidence of the molecular genetic alterations associated with tumor progression is provided by colorectal cancers. With

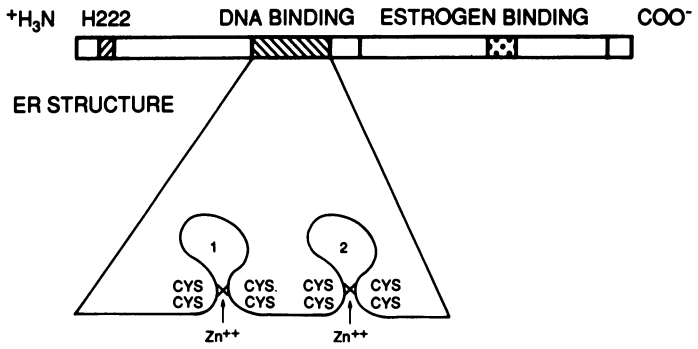
this tissue the stages of progression from hyperplasia, to benign adenoma, to carcinoma in situ, and finally to metastasis can be easily detected. Using this system Vogelstein has been able to follow the genetic alterations during colorectal tumorigenesis and has concluded that the number of these mutational events may be anywhere from three to ten [15]. This is in agreement with Callahan's studies of the genetic alterations in primary breast cancer [16]. In breast cancer a full appreciation of this process must be considered in light of the fact that its progression is initiated within a phenotypically heterogeneous population of cells. Tumors arise from a normal breast epithelial population that ranges from differentiated inactive G_0, early G_1, late G_1, and actively proliferating cells. This raises three basic questions. What is the mechanism of tumor cell progression? What are the genes that are involved? And is this progression influenced by control mechanisms inherent in different stages of the cell cycle?

Evidence has accumulated that the primary signal for cell cycle induction in breast epithelial cells is the presence of estrogen modulated by progesterone. In the absence of estrogen, breast cells remain quiescent in the G_0 phase. Since breast cancers are fundamentally a disease of uncontrolled cell proliferation possibly also with aberrant cell (programmed) death, our attention can be focused on the determinants of the process by which normal postembryonic cells alter their proliferation rate. This will allow the comparison of the defects observed in breast cancer cells and their interrelation with the large component of 'normal cells' that may be present in most breast neoplasms.

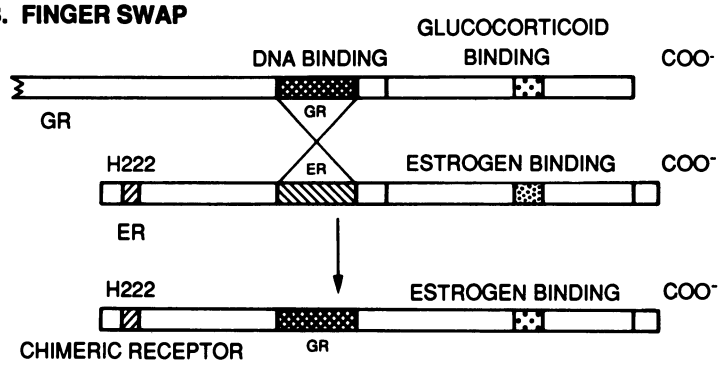
Estrogen receptors

Estrogen hormone receptors are a class of nuclear proteins that upon activation by estrogen increase transcription of regulated genes via direct interaction with specific DNA elements. The identification of the DNA hormone-responsive elements (HRE) in the vicinity of the genes regulated by steroid hormones has been a significant advance in understanding estrogen action [17]. Cloning and sequencing of cDNAs for several nuclear receptors indicate that they all share a similar structure [18–20]. The steroid or ligand-binding domain (approximately 25 kD in size) is composed of a large number of hydrophobic residues confined to the carboxyl terminal of the receptor [17] (figure 1A). Next to this is the DNA-binding domain, which consists of a highly conserved core of 66 to 68 amino acids with two 'zinc fingers' [21]. The DNA-binding residues contain a motif whose homology resembles that of the Xenopus 5S rRNA TFIIIA transcription factor [22], which has six zinc fingers, each of which is tetrahedrally coordinated with two cysteines and two histidines. This DNA-binding protein is known to regulate RNA polymerase III transcription of 5S RNA genes. The repetitive nature of the TFIIIA fingers suggests a structure in which the amino acids at the tip

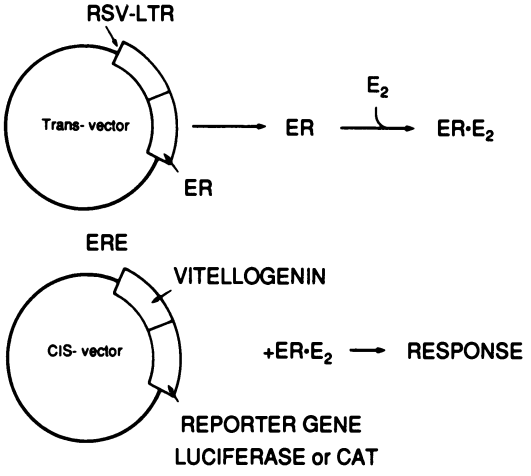
A. Zn FINGERS



B. FINGER SWAP



C. COTRANSFECTION ASSAY TO DETECT ERE



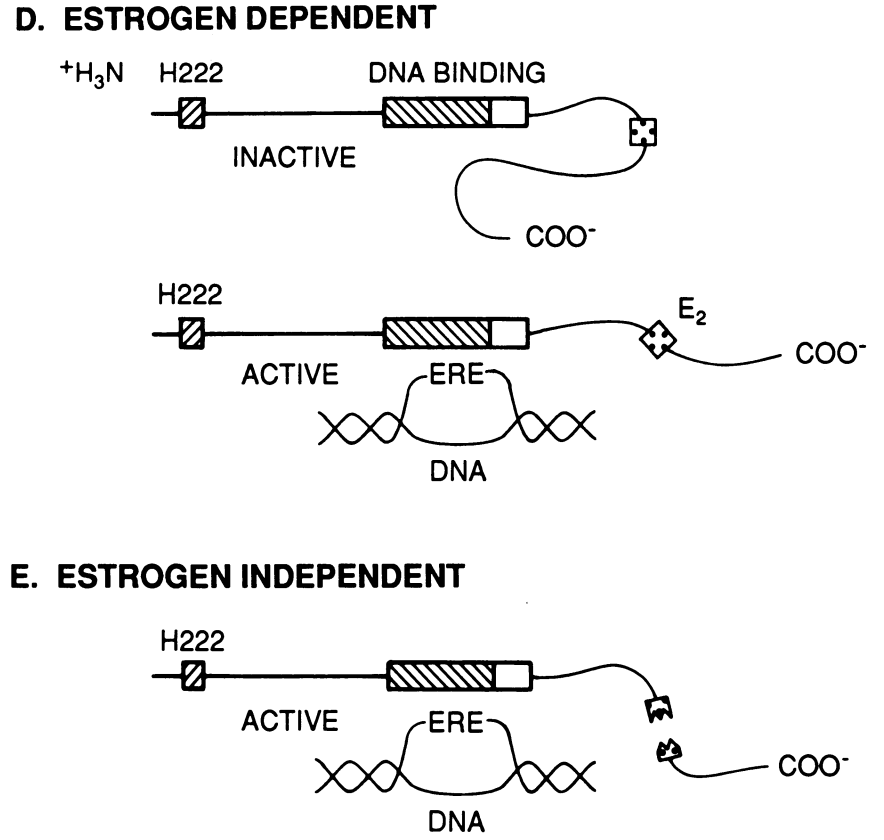


Figure 1. (A) Zn Fingers. (B) Finger Swap (c) Cotransfection Assay to Detect ERE. (D) Estrogen Dependent (E) Estrogen Independent.

of each finger loop interact directly with the DNA helix in the hormone-responsive region of a specific gene. In the presence of zinc, the four cysteines in the ER form a tetrahedral coordinated complex that interacts with a half-turn of the ERE region of the DNA. Although point or deletion mutations in this DNA-binding domain of GR prevent specific DNA binding, there is no loss of steroid-binding affinity [23, 24, 18]. The two zinc fingers of the ER are distinct [18]. The first finger appears to be relatively more hydrophilic, with few amino acids that would be expected to interact with DNA [21]. The second finger is likely to have a tighter DNA-binding affinity, since it is rich in both arginine and lysine residues. The presence of more than one zinc finger suggests the potential for multiple-binding specificity. The 'finger swap' experiments [18, 25] clearly establish the template specificity of the DNA-binding domain (figure 1B). This proof of function was obtained by substituting the putative DNA-binding domain of the ER with that of the

glucocorticoid receptor (GR), resulting in the predicted switch in template specificity [26].

Cotransfection assays have been most useful in these studies that define the function of each of the steroid-receptor domains [25, 27]. These analyses use cell cultures that normally fail to express ER. To induce the synthesis and expression of ER, they are cotransfected with two plasmids (figure 1C). The first plasmid is referred to as an expression-or transactivation-vector (trans-vector) that codes for the efficient synthesis of ER. The second plasmid is referred to as a *cis*-vector, that contains both an ERE and a strong promoter gene coupled to a signal or reporter gene. A number of genes with strong promoters and ERE(s) have been useful in these studies. Examples are vitellogenin, prolactin, and ovalbumin genes, in that they have EREs and strong promoters [28–30]. The strong promoters from these genes are then coupled to what are called reporter genes. The reporter genes are used to detect and quantify the estrogen response in the activation of ER. An example of a reporter gene is luciferase, which when activated causes light to be emitted from cell extract on the addition of ATP [31]. The chloramphenicol acetyltransferase (CAT) gene is another reporter gene that in this case, demonstrates its response by the acetylation of chloramphenicol [32]. Only those tissue cultures that have been cotransfected with both plasmids respond to estrogen induction.

These assay systems have played and will continue to play a role in the study of the effect of deletion mutations in specific regions of ER [33]. One significant conclusion from these studies is that the carboxyl terminal estrogen hormone-binding domain interacts with the DNA-binding domain to prevent its functional binding to the estrogen responsive element of the DNA [34] (figure 1D). This inhibition is relieved by the presence of steroid. Thus it appears that estrogen functions by its interaction with the carboxyl terminal domain that allosterically prevents its interaction with an ERE [18]. The experiments outlined in figure 1B demonstrate that when estrogen is bound to the carboxyl terminal of the ER, it modifies its interaction with the DNA-binding domain, permitting it to bind DNA at one or more EREs. This binding is essential for transcriptional activation. It is reasonable to propose that single point mutations or a deletion in the carboxyl terminal of the ER could be at least one mechanism that accounts for estrogen-independent tumors (figure 1E).

When compared with other HRs, the functional or amino terminal domain of estrogen receptors (ERs) has the least-conserved homology or size [20, 18]. The H222 antibody-binding sites of ER are located on the amino terminal [33, 35, 36]. In spite of these differences in the amino terminal regions, the HRs, and the thyroid receptors, they have been classified as a superfamily. On the basis of many structural similarities, it is possible to include ER [29], estrogen receptor related genes 1 and 2, ERR1, ERR2 [37], progesterone receptor PR [38], mineralocorticoid receptor MR [39], thyroid receptor T3Rα, T3Rβ and c–*erb*–A [40], retinoic acid receptor RAR [41, 42], vitamin D3 receptors

[43–45], and androgen receptors [46] as members of this superfamily [47]. These structural similarities implicate a common evolution of hormone receptors with specific DNA-binding domains that function to direct RNA transcription. Of particular interest is the observation that estrogen related ERR1 and ERR 2, in that their function may represent an additional permissive cell cycle entrance control. More information is needed to determine if and how these, the ERR1 and 2 receptors, are related to mammary tissue-specific expression. Of particular value would be to learn if these estrogen-related receptors might function constitutively in response to ligands other than estrogen.

Estrogen hormone dependence

Significant levels of ER have been detected in more than 50% of human breast cancers. Of these, approximately 60% respond to hormone manipulation as primary therapy compared with only 6% of ER-negative tumors [48]. In addition, the response probability in ER-positive tumors is proportional to the quantitative ER values [9]. The majority of ER and/or progesterone receptor (PrgR)-negative tumors have been reported to show aneuploid DNA populations [48] indicating multiple DNA damage (amplification, translocation, etc).

To put these observations in perspective, we must account for the activation of those factors that respond to the sex steroid signal required for the induction of the cell cycle and the growth of breast tumors. One proposed mechanism for this steroid-mediated growth is the concept that hormone-dependent cells both secrete and respond to a collection of autocrine and/or paracrine growth factors [49]. Based on this concept, hormone-dependent cells will both secrete and respond to specific growth factors. This response requires that the initial signal be a permissive event attained only when the concentration of circulating steroid reaches a critical level. The response depends on the steroid entering the nucleus of the target cell, binding its receptor, and recognizing its hormone-responsive element (HRE) or estrogen-responsive element (ERE) to activate specific promoters for proteins that alter the response to growth factors. Important to these studies is the steroid interaction with other trans-activating and inactivating proteins (like the heat shock 90 proteins), positive cooperative binding [50], and phosphorylation of ER [51–54]. It will be a complex task to determine the precise role of each of these factors and the sequence of events that results in estrogen hormone independence, or that steroid receptors can function as oncogenes as suggested by Fuller [55].

Estrogen hormone independence

The critical area that demands an explanation is the mechanism for cell cycle control demonstrated by ER-negative neoplasms. At the outset this appears

contradictory to the concept that estrogen is essential as the initial permissive signal. Critical examination of the mechanism of transcription control exerted by nuclear oncogenes is likely to provide important insights. For example, one can propose at least two possibilities that could provide a plausible explanation. The first would be the consequence of a point mutation or even total deletion of the carboxyl terminal hormone-binding domain of the estrogen receptor (figure 1E). The consequence of this would be a loss of estrogen binding, leaving the DNA-binding capacity intact, although an estrogen ligand-binding affinity assay would be reported as negative. The observations of Green et al. [19] and those of Walter et al. [20] both show, however, that the epitope recognized by monoclonal antibody H222 interacts in close proximity to the DNA-binding domain on the amino terminal end opposite to the steroid-binding domain [33] (figure 1E), This suggests that even though steroid-binding affinity would be lost, the antibody detection would remain intact.

A second possible mechanism of estrogen independence might be based on serendipitous observation of the sequence homology of the human estrogen receptor cDNA to that of the c–*erb*–A oncogene [40]. This gene demonstrates a striking sequence homology with the thyroid hormone receptor TR3 [40]. The thyroid hormone has two receptors; two TR3s have been identified at this time, suggesting a tissue specificity or a differential response to thyroid hormone metabolites. The thyroxin requirement is bypassed in *erb*–A, since this receptor lacks the thyroid ligand-binding site but still retains its activity. This suggests at least one example of how the loss of allosteric control of DNA could confer pathogenicity on the product of an oncogene. An oncogene equivalent for ER would bypass the ligand-binding requirement.

Activator growth factors

Even in the absence of estrogen, normal breast cells continue their function as differentiated cells in G_0 (figure 2A). Our concept is that it requires the presence of a functional ER, elevated levels of estrogen, and PDGF growth factor for the cell to enter the commitment phase of the cell cycle of the cell designated as G_0*. To leave the differentiated state designated as G_0* requires a complex cascade of both peptide growth factors and oncogenes. One of these growth factors, platelet derived growth factor (PDGF), has been characterized as a 'competence growth factor' in that it allows a 3T3 cell [2] to pass the G_0 nonpermissive state (figure 2A). The next stage requires 'progression growth factors,' which include epithelial growth factor (EGF) and transforming growth factor–α (TGF–α) [6, 56, 57]. These function to regulate intracellular events required to pass from G_0 through early G_1 to mid G_1. Further progression appears to require fibroblast growth factors (FGF), insulin growth factors (IGF–I) [58–63], and perhaps colony-stimulating growth factors (CSF) [64] to pass from mid G_1 to late G_1. The final restriction point (R) to DNA synthesis is confined to late G_1 phase of

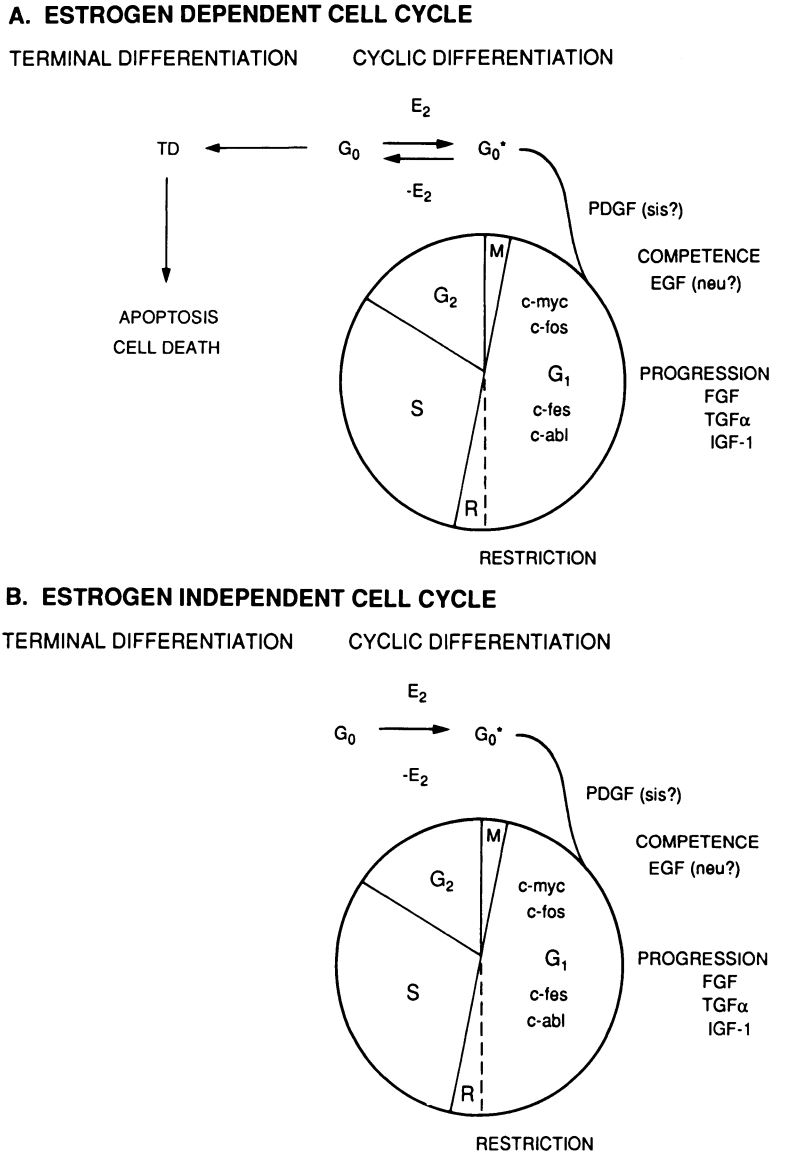


Figure 2. (A) Estrogen Dependent Cell Cycle. (B) Estrogen Independent Cell Cycle.

the cell cycle and differs in that it irreversibly commits the cell to the S phase [65, 66] (figure 2A). This progression through the R point in late G_1 requires the synthesis of specific proteins.

Differentiation versus proliferation

Considering the delicate balance between differentiation, proliferation, and cell motility required for the maintenance of the structural and functional

integrity of a specific tissue, it is reasonable to anticipate that these interrelationships involve many levels. If we consider that the initial cell cycle response is an all-or-none phenomena signaled by a gradient of growth factor(s), then one must determine not only which of the factors are involved, but at the same time the threshold concentration required for this signal [67]. Considering that there are growth factor genes, such as TGF–β, precise titrations with other activating growth factors, such as TGF–α, are also critical. It is also probable that the cell cycle requires a stepwise response to a specific extracellular signal to achieve a critical level for the activation of the next phase of the cell cycle. Since interactions with both growth factors and oncogenes play seminal roles, we will consider some of these factors and their interactions.

Human platelet-derived growth factor

The human platelet-derived growth factor (PDGF) appears to represent the major signal found in human serum for growth. This factor is stored in the α–granules of platelets and as such normally circulates in the blood. Much of our initial understanding of the function of growth factors comes from the study of tissue cultures of 3T3 fibroblasts and peripheral lymphocytes. In these studies a mitogen or growth factor was found in clotted sera that supported the growth of cells and was defined as PDGF because it was released from platelets [68]. The growth of 3T3 cells in culture is regulated by PDGF, which makes these cells competent to leave the G_0 phase of the cell cycle. These cells that are now in a competent activated G_0* state have the capacity to respond to TGF or EGF to progress to early G_1 [69]. Cells that have progressed to early G_1 then have the capacity to respond to insulin-like growth factors (IGFs) to progress to late G_1, past the restriction point (R), to the S phase of the cell cycle [66] (figure 2).

There are at least three mechanisms to explain how PDGF growth factor receptor interactions exert diverse pleiotropic effects without the intervention of oncogenes. First is the activation of protein kinases, the hydrolysis of phosphatidylinositol phosphate (PIP) to diacylglycerol (DG) and inositol 3 phosphate (IP3) stimulated by the growth factor PDGF. Second, this increase in DG in turn activates protein kinase C, stimulating in addition a sodium-to-hydrogen ion exchange and an increase in the intracellular pH. Third, the IP3 protein acts as a second message to mobilize calcium.

Epithelial growth factor (EGF)

The epithelial growth factor (EGF) is best characterized as a 'progression factor' [69] and functions via tyrosine phosphokinases to phosphorylate specific DNA-binding proteins, which in turn modulate the transcription of specific mRNAs [6]. The EGF–α is synthesized via a prepro EGF 1200 amino acid residue polypeptide that is then processed to 53 amino acids. The re-

ceptor to which it binds is composed of three identifiable domains: the extracellular domain, which is highly glycosylated and to which the EGF growth hormone ligand binds; the transmembrane domain, to which the receptor is anchored; and intracellular domain, which has tyrosine kinase activity. The *erb*–B oncogene product gp68 is a truncated form of the EGF receptor that lacks most of the extracellular ligand-binding domain for the EGF growth hormone. Thus the EGF receptor provides direct support for a relation between growth factor receptors and oncogenes. This represents a partial explanation of why the product of the *erb*–B oncogene might be independent of hormonal growth control.

Transforming growth factor (TGF–α)

Some transformed cells produce factors that (in addition to PDGF) can reversibly allow cell surface-adhered 3T3 fibroblasts to grow in suspension. Since this characteristic is often associated with malignant transformation, these factors are called transforming growth factors (TGF), of which there are two types: TGF–α and TGF–β. TGF–α is structurally related to the epidermal growth factor and in fact even binds to the same receptor [70, 71]. TGF–α is a mitogenic autogenic growth factor encoded by a 4.5–4.8 kb mRNA with approximately 30% homology with EGF [72–79], which probably functions by interaction with the EGF receptor.

TGF–α mRNA is synthesized by approximately 70% of human breast tumors. A number of breast tumor cell lines produce TGF mRNA and TGF–α mRNA [6, 69, 80]. Studies of TGF–α production and EGF expression in normal and oncogene-transformed mammary epithelial cells support the concept that TGF–α may not be a tumor-specific growth factor [69].

Insulin-like growth factor (IGF)

Insulin is required by virtually all cell types for optimal cell growth and proliferation. Two structurally related polypeptides, IGF–I and IGF–II, can substitute for insulin in this role [81]. The observation that 17β–estradiol is required for tumorigenesis of MCF–7 human breast cancer cells in nude mice suggests that estrogen might be coupled to the synthesis of one of the IGFs. The observation that IGF–I might be regulated by estrogens has lead Huff et al. [82] and Lippman [49] to conclude that estrogen may contribute to an autocrine and/or paracrine regulation in breast cancer mediated by IGF.

Suppressor growth factors

Not all growth factors exert a direct positive effect on the cell cycle or tumor progression. Although the existance of negative regulatory genes has been suggested by early observations that showed a loss of tumorigenicity as a consequence of fusing normal with tumor cells, hybridization has proved to

be a cumbersome tool [83]. More convincing is the ability to detect germline structural deletions, using cDNA probes, in fibroblasts from some patients with bilateral retinoblastoma [84].

TGF–β

TGF–β is an example of a suppressor growth factor that was first identified by its ability to enhance the growth of fibroblasts in soft agar. This growth factor turned out to be a potent growth inhibitor for many types of cell cultures, including human breast cancers [15, 85]. The TGF–β1 complex purified from human platelets consists of three peptides, including an active 12.5 kD subunit, a 40 kD amino terminal lacking the signal sequence, and a 125–160 kD-binding protein sequence. Significant are reports on the TGF–β1 effect on tissue formation and repair, as shown from the laboratory of Massague et al. [86, 87], who demonstrated that TBF–β1 regulates the synthesis of β2 and β3 integrins, which are part of a superfamily of cell surface receptors that mediate cell-to-cell adhesion [88] and, as such have a potential to participate in the regulation of metastatic behavior.

Thus TGF–β not only appears to be a negative effector on cell growth, but it also has the potential to alter cell adhesion. It is responsible for the synthesis of both receptors, but it also appears to regulate the expression of members of the αβ2 and αβ3 families of integrins [89]. This growth factor (transforming factor) elevates the expression of vitronectin receptors and thus is likely to have a role in the regulation of cell–matrix interactions mediated by vitronectin receptors, and cell–cell interactions mediated by intercellular adhesion receptor, such as LFA–β1 [90].

Mammastatin

A recent report by Ervin et al. [91] demonstrates the presence of a polypeptide, designated as mammastatin, that specifically inhibits the growth of 5 transformed human mammary cell lines but does not affect the growth of 11 nonmammary cell lines. Mammastatin is heat labile and appears to be distinct from TGF–β.

Retinoblastoma gene (Rb–1)

When the wild type Rb–1 gene, which was first cloned in 1987, was introduced into retinoblastoma cells that lacked a functional Rb gene, their transforming capacity was suppressed [84]. This suggests that in this tumor the loss of a single suppressor gene (in this case from chromosome 13) can be responsible for tumor progression. Other examples of this type of malignancy have also been seen in breast cancers [83, 85].

Thus it is becoming clear that there is a class of genetic elements that have been termed turmor suppressor genes. When these genes are inactivated

by deletion, point mutation or methylation, the cells frequently acquire a malignant phenotype [83].

Oncogenes

Although neither their role nor their precise interaction with growth factors is clear, there is little question that oncogenes have defined functions in cell growth. This response depends on the delicate balance between cell cycle, growth, differentiation, and apotosis. This interaction includes the oncogenes, which evolve from normal cellular proto-oncogenes. Genetic alteration and chromosome translocation indicate that oncogenes not only have the potential to modify growth factors, but they may even bypass their function. The role of oncogenes in the progression to the malignant state is complex and is also likely to involve different combinations of events.

Overexpression, chromosome translocation, or genetic alteration of oncogenes represent events that may account for the progressive loss of cell cycle and/or growth control of breast tissue. Such a loss of control in breast cancer is expressed in the full range from hormone-dependent lobular carcinoma in-situ, to locally invasive malignancies, to metastatic disease, and finally to total estrogen independence.

One class of genes whose products are rapidly induced after growth stimulation are the oncogenes derived from proto-oncogenes [2]. More than 40 oncogenes have now been identified and have shown to code for proteins that appear to have proliferation-related regulatory functions at the level of either the plasma membrane, the cytoplasm, or the nucleus.

Plasma membrane-associated oncogenes

Lippman et al. [11] have demonstrated the presence of c–*sis* transcripts in several human breast cancer cell lines. The activation of the *sis* oncogene, which has striking homology to PDGF, is associated with the autocrine stimulation of cell growth [49]. Using in situ hybridization, Ro et al. [92] have demonstrated *sis*/PDGF–B expression within the epithelial cells of malignant as well as benign breast lesions. These studies suggest that the epithelial cell components of nonmalignant as well as malignant breast lesions may be partially responsible for the stromal reaction. Thus the excessive proliferation of stromal fibroblasts in breast tumors with desmoplasia may be the result of the expression of such oncogenes as c–*sis*. These observations may help to explain the report of Sariban et al. [68] that PDGF receptors were undetectable in malignant epithelium that expressed PDGF–1 and PDGF–2 mRNA. This would suggest that growth factors produced by one malignant cell have the capacity to act through a paracrine mechanism to influence the cell cycle activity within a microenvironment of normal cells.

The *erb*-B oncogene encodes a truncated form of EGF plasma membrane receptor that is active even in the absence of EGF [93–98]. Both the *ras* and

the *sarc* oncogenes are also attached to the plasma membranes and exhibit tyrosine kinase activity [98].

Gene amplification appears to be a more common event than might have been anticipated, although it appears to be limited to malignant cells and some germ cells. One of the growth factors related to EGF is the c–*erb*–B–2 oncogene and Her2 *neu* gene [99]. The *neu* gene was first identified in rat tumors that had been induced by the carcinogen ethyl nitrosourea. The human homologue of the *neu* gene has been designated as c–*erb*-B–2 and is located on chromosome 17, q21 [98]. This oncogene specifies a transmembrane receptor-like phosphoglycoprotein that is closely related to the EGFr (c–*erb*-B–1). Although the specific ligand is still not known, a significant proportion of adenocarcinomas of the breast have been demonstrated to overexpress this gene and demonstrate its amplification [100]. The precise quantification of gene amplification has been less than precise, is time consuming, and requires that the amplified gene be quantitated against a single DNA copy as a standard obtained from the same tumor.

Changes in plasma membrane-associated oncogenes result in the modification of growth hormone response, subverting the normal differentiation and cell cycle-mediated events of G_1 progression, G_0 arrest, and apoptosis. The concept that cancer results from the accumulation of several genetic alterations within a single cell has in large part been based on the fact that in the absence of other factors, the activation of a single oncogene is insufficient to induce a tumor. Early evidence to support this view was obtanied by the observation that activated *ras* genes have often been found to be present, regardless of whether DNA was obtanied from premalignant, benign papillomas, or malignant carcinomas, when these tumors were induced by an initiator (DMBA) followed by a promoter (TPA). It is clear, at least in cell cultures, that additional events are required for the *ras* oncogene-containing cell to attain its full malignant potential. Many of the early studies of the presence of c–H–*ras*–1 genes detected in NMU-induced and DMBA-induced rat mammary carcinomas suggest that *ras* activation may in fact only represent one early step in the development of these mouse tumors [101].

Probably the best evidence of oncogene cooperation to produce a malignant transformation has been the observation that activated H–*ras*–1 bladder oncogenes alone are incapable of transforming baby rat kidney cells, whereas when complemented with *myc*, *myb*, and/or the SV40 and polyoma large T, successful transformation is achieved. Thus these genes all have in common the fact that they encode nuclear proteins and share the ability to induce immortalization of primary cell cultures.

Cytoplasmic oncogenes

The cytoplasmic oncogenes include *abl*, *dbl*, *erb* A, *fps*, *mos*, *raf*–1 and *sis*. These code for cytoplasmic protein kinases that most likely function as cytoplasmic effectors of signal transmission [98].

The *sis* oncogene is the transforming gene of the simian sarcoma virus. The c–*sis* product is the only known example at this time of an oncogene-encoded growth factor. Kraus et al. [56] have reported that the precursor molecule p28 (*sis*) is synthesized by cell membrane-bound polyribosomes, which are then dimerized in the endoplasmic reticulum and processed at the cell periphery to yield structures analogous to biologically active PDGF.

Nuclear oncogenes

The *myc* family genes encode nuclear protein that may be associated with the small ribonucleoproteins. The *fos* gene also encodes a nuclear protein, appearing within 1 hour of stimulation of quiescent cells by mitogens. These and other members of the nuclear proto-oncogene group probably play important roles in gene expression, by regulating RNA processing and transcription.

Of the several oncogene products found in the nucleus, the c–*myc* oncogene almost certainly plays a role in cell division. It is expressed in normal cells as well as in tumor cells. Evidence for this role is the observation that *myc* mRNA is greatly reduced in cells whose growth has been arrested [2]. Also, if cells are stimulated by mitogens such as PDGF, the *myc* mRNA appears within 1 to 2 hours. Thus mitogens like PDGF have the capacity to set off a chain of events best described as the induction of 'competence' to prepare the cells to respond to additional growth factors. It should be noted that both n–*myc* and c–*myc* are induced by estrogen in the rat uterus [102, 103]. The c–*myc* is frequently rearranged, amplified, and often over-expressed in primary tumors compared to normal breast cell controls [98, 104, 105].

Within 1 to 3 hours after the addition of PDGF growth factor to fibroblasts, there is a 10 to 40 fold increase in c–*myc* mRNA [106, 107]. This illustrates two important concepts. The first is that a linked response exists between the function of PDGF as a growth factor associated with the plasma membrane and c–*myc* as an oncogene that is located in the nucleus. The second concept is that this linked response is likely to be involved in the activation mRNA transcripts that are involved in cell cycle control. This response is not limited to fibroblasts, since it has also been demonstrated in an epidermal carcinoma cell line [68]. On this basis, the c–*myc* is strongly implicated as an important oncogene in cell control, where its expression has been shown to be altered in other cell types.

C–fos

C–fos is still another oncogenic growth factor that also encodes a nuclear protein that is shown to rapidly increase in response to steroids and peptide growth factors [108–111].

While sex steroids are suggested to function at the permissive level of the cell cycle, the interaction of both growth hormones and oncogenes should be considered at the determination and progression level. As a consequence of their interaction wiht growth factors, oncogenes modify the cell cycle and influence both cell proliferation and differentiation. At least three oncogenes code for cellular proteins that are relatd to growth factors or their receptors (figure 1).

Normally, the response to PDGF, EGF, IGF, and TGF appears to be sensitive to multiple complex feedback control and limited to paracrine induction of neighboring cells. From this viewpoint it is probably best to consider oncogenes as second messages modulated in response to growth hormone induction. Furthermore, it would appear that in the immortalized-transformed-cell progression there is a loss of this feedback response. This multistage progression occurs as a consequence of (a) point mutations or deletions of oncogenes [112]; (b) overexpression of oncogenes by gene amplification [113–115]; (c) translocation and/or insertion of oncogenes into new active promoter regions [116]; and (d) autocrine rather than paracrine response to both growth factors and oncogenes [49]. Any combination of these genetic events would present with a different phenotype [117].

The future

There is little question that oncogenes play an essential role in normal cell growth and development and in the transduction of cell signals required for these activities. Observations remain at the descriptive level, however, with a paucity of information concerning the disruption of those normal functions that lead to the development of breast neoplasms. The future will require our attention to the interaction of oncogenes and suppressor genes in relation to growth factors, and the transacting factors that stimulate estrogen receptor activation. Suppressor genes normally function to block the cell cycle and tumor development and drive the cell towards terminal differentiation. It is most likely that it is the balance between activating oncogenes and tumor suppressor genes that determines whether the behavior of a cell will be normal or aberrant. This balance is essential to maintaining a functional cell cycle. Only a few cell cycle suppressor genes have been described, for example retinoblastoma, TGF–β, and mammastatin. One might predict that more suppressor genes will be found, some of which will be cell and/or tissue specific. We must always bear in mind that cell immortilization (escape from cell cycle control) is only the first step in the multistage progression to the malignant phenotype.

Even though we are inclined to accept the notion that oncogenes play a primary role as normal second messages required for cell cycle progression,

the sequence and details are lacking. In fact, one might conclude that the complexity is intimidating, but we are continuing to define more about the normal cell cycle control. The role of only a few genetic modifications of oncogenes, oncogene receptors, or growth factors have been assigned to specific phase(s) of the cell cycle [7, 12, 82]. An obvious example is that v–*sis* oncogene encodes a peptide that is highly homologous to the growth factor PDGF [85]. The PDGF induces the expression of c–*fos* [11] and c–*myc*, and it appears that the c–*myc* product is an intracellular messenger of the PDGF mitogenic signal. The close homology of *erb*–B gene to the inner membrane-associated domain portion of the EGF receptor is another example.

If we define the concept of 'competence' as that condition that allows a cell to proceed through the cell cycle, then we can designate it as the initial barrier that must be passed. If we consider the complex nature of the multiple feedback controls that must be involved in the progression through the cell cycle, we can conclude that multiple growth factors with both positive and negative controls must be involved. Thus 'progression' factors are defined that by their nature must regulate specific intracellular restriction points in the cell cycle itself. Using these definitions, PDGF can be designated as a competence factor, with EGF and IGF assigned the role of progression factors. Scher et al. [106] demonstrated the function of PDGF as a competence factor by a pulse treatment of 3T3 cells in one S phase, which was found to render them competent to initiate DNA synthesis extended to the following cycle. Pardee [60] further defines what he calls the point of 'commitment' as an 'R' or restriction point that occurs only in late G_1 phase. This scheme allows multiple controls; the first at competence, the second at progression, with the final barrier R, representing multiple levels of control by these definitions.

Gene amplification

To our knowledge, there is no convincing evidence that normal cells amplify their genes. Credence is lent to this concept by the observation that normal bone marrow and gastrointestinal tract progenitor cells fail to become resistant to cytotoxic agents. In contrast, oncogene amplifications have already been detected in a variety of human cancers [104, 113, 114]. This observation implies possible 'prognostic significance' of application and has been suggested by some for this purpose [8, 9, 16, 115, 116, 117]. The techniques presently used to assign a gene as amplified at the DNA level and the level of expression, however, are still evolving with the advent of such methods as differential polymerase chain reaction [118–120]. This PCR technique should prove to be more sensitive than are our present RFLP methods [121, 122], providing reproducible quantitative values. The sensitivity should permit the detection of point mutations using a single section of paraffin-embedded material.

Oncogene germline transmission

Future directions must be to provide tissue-specific delivery of single and/or multiple activated oncogenes in order to detect the effect of rare homologous recombination events. A recent demonstration of the feasibility of germline transmission of a mutated oncogene introduced into a nonselectable autosomal gene by homologous recombination has been demonstrated by Schwartzberg [123]. This technique has the potential to replace normal cellular genes in the mouse by mutant alleles with defined sequence alterations [124]. The cells used for these experiments were cultured embryonic stem cells (ES) obtained from preimplantation mouse embryos that were subsequently reintroduced into mouse blastocysts by microinjection to form chimeric mice. Critical to these studies will be the feasibility of germline transmission of a mutated oncogene replacement of normal cellular genes combined with the ability to generate chimeric mice [125].

Estrogen receptors, growth factors, and activator and suppressor oncogenes

Many short term benefits will accrue in terms of estimating prognosis and improving treatment gained from an analysis of the mechanism of steroid hormone independence and its relation to growth factors, target gene receptors, and oncogene products. This should take us beyond the stage where we depend only on pathology, node status, the quantification of steroid receptors, and DNA content to characterize breast tumors. We must be committed to evaluate a great deal of data on growth factors, activator and suppressor oncogenes in terms of gene amplification, point mutations, deletion, gene product expression, and chromosome translocation. The most promising direction will be to concentrate on those early in vitro events that can be made reversible by altering the genome by homologous recombination. This information will provide much of the essential background needed to begin to define the status of a given breast tumor that is a part of an overall complex multistage process.

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10. Antiestrogen therapy for breast cancer: Current strategies and potential causes for therapeutic failure

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In 1958 Lerner and coworkers [1] described the biological properties of the first nonsteroidal antiestrogen, MER25. This discovery stimulated interest by the pharmaceutical industry in developing novel agents to control or to block estrogen-dependent events. A number of drugs have been studied in the laboratory and in the clinic [2–7]; however, research has focused upon the application of antiestrogens as antitumor agents for the treatment of breast cancer. There are several reasons for this. The knowledge that some breast cancers are dependent on ovarian hormones [8, 9] and the description of the estrogen receptor system in the estrogen target tissues and tumors [10, 11] naturally lead to the testing of drugs that would block estrogen binding in breast tumors. Several compounds were evaluated, but only tamoxifen was developed further because of the low incidence of side effects and the demonstrated efficacy in phase II/III clinical trials [12, 13].

The goal of this chapter is to provide the reader with the current view of antiestrogen therapy for breast cancer. The results from clinical trials in the past decade have provided the basis for an effective treatment strategy for the 1990s. Nevertheless, long-term chemosuppression of breast cancer cannot be considered to be a cure for the disease, even though treatment may be initiated during early stages. Resistance to therapy will inevitably occur, so it is important to consider various mechanisms whereby breast cancer cells no longer respond to tamoxifen to discover whether novel treatments can be developed in the future. Similary, new antihormonal agents are becoming available in the clinic, and it is possible that alternating treatments may circumvent early drug resistance.

Overall, the successful application of antiestrogens to treat breast cancer has been the result of a close collaboration between the laboratory and the clinic. Animal models have demonstrated the antitumor properties of the nonsteroidal antiestrogen tamoxifen [14–23]. Supported by the laboratory data, the drug entered clinical trials and now represents the treatment of choice for women with breast cancer [13].

The development of treatment regimens was greatly influenced by the mode of action of tamoxifen. The majority of data indicates that tamoxifen has a tumoristatic effect upon breast cancer cells. Thus, in both dimethyl-

M. Lippman and R. Dickson (eds.), REGULATORY MECHANISMS IN BREAST CANCER.

benzanthracene [16] and N–nitrosomethylurea [18] treated rats, initially suppressed tumors appear when tamoxifen treatment is stopped [24]. In addition, estrogen can stimulate the regrowth of residual breast cancer cells after up to six months of tamoxifen therapy in athymic mice with tumor implants [22, 23]. The drug does not demonstrate a tumoricidal action in these models.

Collectively, the laboratory observations suggest that adjuvant tamoxifen treatment will have to involve long periods (perhaps even the lifetime of the patient) to be effective in preventing tumor recurrence. The clinical information about two or more years of adjuvant tamoxifen therapy of stage I/II breast cancer will be reviewed.

Long-term adjuvant tamoxifen treatment of breast cancer

The prognosis of breast cancer is greatly influenced by early detection. It is therefore almost axiomatic that patients could derive the most benefit by early treatment of minimal disease. Although there have been extensive studies with a range of combination chemotherapies, tamoxifen, as a single agent, has been shown to have remarkable efficacy. The results of clinical trials using tamoxifen for at least two years (table 1) have shown a significant increase in patient survival [25] with limited side effects. As might be expected, most of the beneficial effects are observed in patients with estrogen receptor-positive tumors. Nevertheless, it is important to point out that some studies have reported similar improvement in patient prognosis independent of the receptor status. These include an improvement in overall survival and disease-free survival after tamoxifen treatment [26–28]. Current trends are to treat node-negative breast cancer with tamoxifen either as an unselected population [28] or, as in the United States, only estrogen receptor-positive patients [29]. Although improvements in disease-free survival are noted, it is important to point out that the majority of women will not have a recurrence of their disease. Side effects then become an issue for concern.

In the United States, tamoxifen is often combined with chemotherapy. The rationale is to destroy both the receptor-positive and receptor-negative cells. An early pilot study [30] that combined chemotherapy with long-term tamoxifen therapy reported an increase in the projected 4-year relapse-free survival for patients whose tamoxifen treatment continued after completion of the chemotherapy. Most importantly, this study indicated the safety of five years of tamoxifen therapy. Large trials have subsequently been organized [31] to test the combination of chemotherapy with up to five years of tamoxifen against stage II breast cancer. While benefit in terms of disease-free interval was observed in all tamoxifen-treated patients, no overall survival improvements have yet been observed.

The National Surgical Adjuvant Breast and Bowel Project has developed a series of interesting studies based on their evaluation of two years of adju-

Table 1. Adjuvant long-term tamoxifen treatment of breast cancer [25].

Trial	Number of patient	Object	Median follow-up (range) (mos)	Dose and duration (mos)	Statistical significance	Clinical side effects
Tormey, DC and Jordan, VC (1984)	124 None: 38 T: Stop: 43 T: Continue: 43	Post N+ Chemo+ DF	27.3 (1.9–73.1)	20 mg (60)	DFS (4 years)	No difference
ECOG (1985)	170 T: 86 P: 84	Post (≥65 years of age) N+ ER+ or unknown	41.0 (24)	20 mg distant first recurrence	DFS (4 years)	Similar except hot flashes
NSABP (1986)	1,858 PF: 924 PFT: 934	Pre, post N+	72.0 (24)	20 mg	DFI (DFS) (5 years) ≥50 years N+ (4 or more) Er+, PgR+ (≥10 fmol)	
NSABP (1987)	885 PFT: 443 PFT +: 442 additional year of T	Pre, post N+	60.0 (36)	20 mg	DFS SR (5 years) ≥50 years	
French Group (1986)	179 T: 89 C: 90	Post N+	41.0 (32–76)	40 mg (36)	1) DFS (5 years) 2) DFS SR (ER+)	
Stockholm (1987)	1407 T: 711 C: 696	Post	53.0 (18–110)	40 mg (24)	1) DFI 2) Loco-regional recurrence 3) reduction of treatment failure (ER+)	Gastrointestinal disturbances Hot flashes

Table 1. Cont.

Trial	Number of patient	Object	Median follow-up (range) (mos)	Dose and duration (mos)	Statistical significance	Clinical side effects
Scottish (1987)	1312 T: 661 O: 651	Pre (N−, unknown) Post	(30–96)	20 mg (60)	1) DFS distant-DFS 2) S 3) DFS (ER ≥100 fmol)	Stopped (4.4% in T)
NATO (1985, 1988)	1131 T: 564 No: 567	Pre (N+) Post ≤75 years of age	(66) (maximum 96)	20 mg (24)	1) events and deaths 2) local recurrence	unwanted effects: 25 patients (4%) Hot flashes, anorexia, nausea, depression, migraine, peripheral neuropathy, superficial thrombophlebitis
NSABP (1989)	2644 T: 1318 P: 1326	Pre, post N−, ER+ ≤70 years of age	(48)	20 mg (60)	1) DFS 2) local and distant treatment failure	1) T>P Hot flashes, vaginal discharge, irregular menses 2) stopped (5% in P and 7.1% in T) 3) Thromboembolism (T:0.9%, P:0.2%)

EGOG, Eastern Cooperative Oncology Group; NSABP, National Surgical Adjuvant Breast and Bowel Project; NATO, Nolvadex Adjuvant Trials Organization; T, tamoxifen; P, placebo; C, control; O, observation; No, no treatment; Post, postmenopausal; Pre, premenopausal; N+, axillary node-positive; N−, axillary node-negative; chemo+, given chemotherapy; DF, disease-free; ER+, estrogen receptor-positive; DFS (DFI), disease-free survival (interval); SR, survival rate; S, survival; PF, 1-PAM + fluorouracil; PFT, PF + tamoxifen.

vant chemotherapy with or without tamoxifen [32, 33]. The aim of the study was to evaluate extended tamoxifen therapy. A three-drug regimen [table 1] was administered to patients for two years. Then, upon completion of this therapy, a group of patients without disease recurrence was offered an additional year of tamoxifen. Women receiving a third year of tamoxifen had a better disease-free survival rate and survival rate through the fifth postoperative year compared to those patients receiving two years of tamoxifen. The benefit was restricted to women of 50 years of age or more.

Clearly, tamoxifen is effective in a minority of women and safe for the vast majority of women. The drug is very convenient to administer and patients maintain a good quality of life. Nevertheless, physicians need to remain vigilant for potential side effects in order to protect the future health of their patients.

Potential concerns with long-term adjuvant tamoxifen therapy

The 'risks' of taking a drug like tamoxifen for prolonged periods must be carefully weighed against the possibility of a recurrence of breast cancer that is fatal. The early detection of breast cancer has focused attention on the treatment of premenopausal women with node-negative disease, the majority of whom will be cured but will nevertheless be treated with tamoxifen for perhaps an indefinite period. The application of tamoxifen as a long-term therapy raises several important questions. What impact will years of therapy have on the patient? Will continuous therapy result in drug resistance? The first question will be addressed in this section, and the second question will be addressed in the next section.

Estrogen appears to be physiologically important for women to maintain bone, involving a stimulation of osteoblast proliferation and type I (bone type) procollagen gene expression [34, 35]. Additionally, estrogen is associated with a decrease in the incidence of heart attacks and strokes [36, 37]. Clearly, one could take the position that long-term therapy with an antiestrogen might precipitate osteoporosis and predispose patients to coronary artery disease. Clinically, bone density measurements and blood lipid analyses should be required on a regular basis. However, there is an alternative outcome that can only be evaluated during the long-term therapy with tamoxifen. Although tamoxifen is classified as an antiestrogen, the drug produces some distinct estrogen-like effects [13, 38–42]. This previously unwanted estrogenicity of tamoxifen may in fact be an advantage, and tamoxifen may prevent osteoporosis and atherosclerosis. Currently, there is only one report [43] to document that tamoxifen has no deleterious effect on bone density of patients after two years of adjuvant therapy. Furthermore, there are no adverse reports of the effects of tamoxifen on blood lipids to suggest an early development of atherosclerosis. Indeed, there is one report to demonstrate that tamoxifen may produce a beneficial profile of lipids in women treated for

breast cancer [44]. There is, however, a case report of a 61-year-old woman who developed hyperlipoproteinemia during tamoxifen therapy [45]. A further implication of the estrogenic effect of tamoxifen would be a decrease in circulating levels of antithrombin III, predisposing the patient to an increased risk of thromboembolic disorders. However, this is not clinically significant and appears not to be a problem in patients with no history of thromboembolic disorders [40, 46].

Tamoxifen shows estrogen-like effects upon various estrogen target tissues in women. Thus tamoxifen has the potential to be able to stimulate the uterus and to promote endometrial carcinoma. However, there are previously reported antiestrogenic effects upon the uterus [47], and tamoxifen has been used successfully to treat endometrial carcinoma [48–50]. The potential for tamoxifen to affect endometrial carcinoma adversely was illustrated by studies from the laboratory. Two steroid receptor-positive human endometrial tumors have been shown to be stimulated to grow in immune-deficient mice by either tamoxifen or estradiol [19]. Indeed, the growth of breast tumors can be controlled by tamoxifen in immune deficient mice, but the growth of endometrial tumors implanted in the same animal is stimulated by tamoxifen [19]. This experiment illustrates the possibility that occult endometrial carcinoma could grow during long-term adjuvant tamoxifen therapy for breast cancer. Interest in this phenomenon has recently been increased by the report by a Swedish clinical trials group that demonstrates that the longer therapy with tamoxifen is continued, the greater the risk of detecting endometrial carcinoma [51]. Nevertheless, it is important to point out that in the same study they demonstrated that the number of second primary breast cancers was significantly decreased in patients on tamoxifen. Therefore, the combined effect of tamoxifen controlling the recurrence of breast cancer and the development of second primary tumors should be weighed against the development of endometrial carcinoma. Breast cancer is fatal, but endometrial carcinoma has a good prognosis. Although the Swedish study has not been confirmed by data from any other clinical trials organization, it is prudent for physicians to monitor their patients to avoid the risks of a second estrogen-related malignancy during any long-term tamoxifen treatment schedule. This entire issue has been the subject of much discussion [52, 53].

A recent toxicological study with high-dose tamoxifen therapy in rats has shown an increase in malignant liver tumors [54]. There are, however, no indications from the clinical trials data that tamoxifen can induce liver tumors in women. Nevertheless, it is important to be aware of this possible side effect that might become evident decades from now.

Finally, the endocrinological effects of tamoxifen must be considered in women of childbearing age. Tamoxifen increases steroidogenesis in premenopausal women [55, 56], and long-term adjuvant tamoxifen therapy of premenopausal women with breast cancer also causes an increase in circulating estrogen [57]. The question, therefore, arises; Will an increase in circulating estrogen impair the antitumor actions of an antiestrogen? The NSABP [29]

and CRC [Cancer Research Campaign Adjuvant Breast Trial Working Party, 1988; 58] studies show that adjuvant therapy of premenopausal patients is effective. Nevertheless, we are uncertain about the overall effect on patient survival and about the long-term effects of ovarian hyperstimulation.

There is obviously another sociological issue that is important to consider when treating premenopausal women. We have no information about teratogenesis with tamoxifen. Patients may become pregnant during tamoxifen therapy for node-negative disease, and the moral issues of abortion will have to be confronted. Tamoxifen therapy is contra-indicated by the manufacturer during pregnancy, so physicians have the responsibility to recommend barrier contraception or voluntary sterilization.

Failure of tamoxifen therapy

As pleasing as the results can be following tamoxifen treatment [13], failure is a constant occurrence, even after long periods of remission. A number of explanations for this eventual failure have been suggested [59]. Disease recurrence under some circumstances can be identified and avoided by careful patient monitoring. Patient noncompliance during long clinical trials will result in fluctuating drug levels, leading to an estrogen receptor-positive tumor recurrence [60]. Changes in diet may also affect the potential activity of tamoxifen; as a lipophilic compound, it will deposit in body fat and biovailability to the tumor will be reduced. Equol and enterolactone (an intestinal bacterial product), both estrogenic compounds, can also increase during dietary changes and compete with tamoxifen and its metabolites [60].

These mechanisms of tumor reoccurrence, while easy to avoid, will result in the appearance of an estrogen receptor-positive tumor. The development of drug resistance to tamoxifen by tumor cells is more difficult to avoid and to treat successfully.

The development of drug resistance may involve a number of mechanisms, ultimately producing a hormone-independent phenotype. After long-term tamoxifen therapy of breast tumors in athymic mice, some tumors can be stimulated to grow by tamoxifen [23]. This form of tamoxifen resistance may involve the estrogenicity of the compound, the tumor burden, or an effect upon the immune system [61, 62], and it could represent a stage in disease progression to hormone independence.

Using serum-free culture systems and biological assays, previously well-characterized growth factors have been found to be produced by MCF–7 cells. These growth factors are thought to act as autocrine and paracrine growth stimulators [63]. These include EGF, PDGF, IGF–I, FGF, TGF–α and TGF–β [64–66]. Interestingly, highly malignant breast tumor cell lines and hormone-independent lines appear to secrete some of these growth factors at elevated levels in the virtual absence of estrogens.

The role of these growth factors (table 2) in malignant transformation and tumor progression to hormone independence has been studied using genetically altered MCF–7 cells [67]. The elevated expression of v–*Ha–ras* protein in these cells, correlates with the progression of the tumor into a more aggressive form. These altered lines, in the absence of estrogen, show features characteristic of hormonal stimulation (tumorigenicity in nude mice, elevated levels of EGF, TGF–α, IGF–I, and TGF–β) and are also resistant to antiestrogen inhibition. One of these growth factors, EGF, is known to down regulate the estrogen receptor [68] and reverse the antiestrogen-inhibited growth of MCF–7 cells [69], while antiestrogens appear unable to affect EGF-stimulated MCF–7 growth. Strategies directed against such growth factors or their specific receptors could alter the growth properties and the clinical progression of both hormone-dependent and -independent tumors. The possible interactions of hormone-dependent and -independent cells can be studied both in vitro and in vivo, and possible therapeutic strategies examined. When MCF–7 cells are cocultured in vitro with an estrogen receptor-negative cell line, the growth of the MCF–7 cells is significantly increased [70]. This paracrine-stimulated proliferation is not inhibited by antiestrogens. Since solid tumors are known to be a complex mixture of receptor-positive and receptor-negative cells, the possibility is raised that growth inhibition could be reversed by stimulatory growth factors originating from the surrounding estrogen receptor-negative cells. The opposing hypothesis has also been proposed. It is known that TGF-β is inhibitory for the

Table 2. Estrogen-stimulated proteins in MCF–7 cells: Their possible role in the growth of breast cancer epithelial cells [from 65].

Protein	Function
PDGF (platelet-derived growth factor)	Paracrine role, mediating the proliferation of stromal cells?
EGF (epidermal growth factor)	Mitogenic; immunosuppressive, countering host rejection of cancer cell.
TGF–α (transforming growth factor–α)	Mitogenic; angiogenic?
IGF–I (Somatomedin C)	Mitogenic
TGF–β (transforming growth factor–β)	Growth inhibitory for epithelial cells, mitogenic for stroma; angiogenic?
Anchorage Independent Epithelial Growth Factor	—
Plasminogen activator Collagenolytic enzymes	Influence tumor progression and growth by basement membrane digestion, process growth factors; direct interaction with receptor?
52 kD glycoprotein 7 kD protein, 24 kD protein	Mitogenic, protease.
39 kD glycoprotein complex, 160 kD glycoprotein	—
Laminin receptor	Attachment to basement membrane; invasiveness.

growth of estrogen receptor-negative cells [71, 72]. Since TGF-β can be induced by antiestrogen treatment of estrogen-dependent cells [71], this suggests that in a mixed tumor, estrogen-independent cells can be controlled by TGF-β from neighboring estrogen-dependent cells. Unfortunately, when mixed receptor-positive and receptor-negative tumors are grown in athymic mice, antiestrogens cannot control the growth of the tumor [73].

New antiestrogens

It is assumed that many patients carry micrometastases, present at the time of diagnosis of breast cancer; therefore, cure can only result from an effective systemic therapy. Antiestrogens are thought to exert their antagonistic actions by binding to the estrogen receptor, thereby competing with the natural ligand. The complex formed after antiestrogen binding to the receptor has minimal estrogenic properties due to the absence of secondary changes in the receptor that would normally occur after estrogen binding [7, 74].

However, almost all antiestrogens have been observed to be partial or weak agonists, stimulating uterine weight increases and progesterone receptor production [75]. This intrinsic estrogenicity of these compounds may be of importance clinically and may be the reason that failure is a constant occurrence [59]. This clinical failure has prompted a search for new antiestrogens; those compounds should display a clearly defined activity against hormone-dependent tumors, with insignificant toxicity. Several new compounds are currently being evaluated in the clinic.

Toremifene (Fc–1157a)

Toremifene is a triphenylethylene nonsteroidal antiestrogen (4 chloro–1,2–diphenyl–1,1,4,2–[N,N–dimethyl amino] ethoxy pheny1–1–butene), developed by Farmos Group in Finland [76, 77], that has shown promise in various laboratory tests as an antitumor agent [76, 78]. These tests have involved estrogen receptor-binding assays, uterotrophic tests in immature animals, and antitumor experiments against MCF–7 cells in vitro and DMBA-induced rat mammary tumors in vivo. It appears that toremifene binds specifically and competitively with high affinity to the estrogen receptor and displays a degree of estrogenicity (albeit lower than that of tamoxifen) that is organ and species specific. However, there is evidence that toremifene may accumulate in tissues in a nonspecific fashion due to the lipophilic nature of the compound [79, 80].

Although these results support a tumoristatic mode of action, there is evidence that toremifene may display tumoricidal actions effective against hormone-independent tumors [78, 81].

Supported by these encouraging results, toremifene has been the basis of several clinical trials. An overall response rate of approximately 50% has

been achieved, with visceral and soft tissue metastases responding equally well [77, 82, 83]. Toremifene appears to be well tolerated [77], with reported side effects, including sweating and hot flashes similar to those experienced with tamoxifen. Importantly, there have been no reports of renal, hepatic, pulmonary, or circulatory toxicity [77, 84]. This contrasts favorably with the reports of hyperplastic nodules in the liver of rats [85] and hepatocarcinoma [86] following high doses of tamoxifen, and liver damage and increased side effects in humans [87, 88]. This may reflect the higher estrogenicity of tamoxifen. The ocular changes (involving retinopathy, corneal changes, and a decrease in visual activity) observed after high-dose tamoxifen treatment and the hypercalcaemia reported in patients with bone metastases [89] have not been reported in toremifene-treated women. However, there is at present only limited clinical experience with this new antiestrogen.

Pharmacokinetically, toremifene is well absorbed after oral administration, with maximum serum concentrations achieved in four hours and an observed elimination halflife of five days [46]. This long elimination halflife should allow a significant accumulation of the drug following repeated administration. The majority of the drug is excreted in the feces due to enterohepatic circulation. The major metabolic pathways of toremifene are N–desmethylation and 4–hydroxylation, identified in the rat [90] and human [46]; the side chains are probably further oxidized to alcohols and carboxylic acids. The majority of the drug is found bound to serum proteins, including albumin [46], β_1–globulin and α_1 acid glycoprotein [90].

Physiologically, toremifene has a number of effects in postmenopausal women [77, 91]. These include a decrease in circulating estrogen, LH, and FSH, and an increase in SHBG. The latter two effects are thought to be the result of the estrogenicity of the compound upon the hypothalamus and liver, respectively. A decrease in circulating levels of prolactin could be an important facet of the antitumor properties of toremifene, as prolactin may play a role in tumor growth [92].

In summary, toremifene represents an antiestrogen with antitumor potential comparable to that of tamoxifen, the established treatment. Importantly, toremifene displays fewer side effects. The potential for the use of toremifene against estrogen receptor-negative tumors [81] may suggest a mode of action via a pathway other than the estrogen receptor. This would support the use of toremifene for patients that relapsed during tamoxifen therapy. Indeed, preliminary data have supported the role of toremifene in combination therapies, showing a synergistic effect with interferons [93]. However, the best clinical results should be obtained using patients with a history of no previous treatment and a small tumor burden.

Droloxifene (K–060E)

Droloxifene, or 3–hydroxytamoxifen, is a triphenylethylene derivative that displays a significantly higher binding affinity than tamoxifen for the estrogen

receptor [94]. This would suggest an increased biological activity. While antiestrogenic activity in vitro has been reported [95], some estrogenic qualities were observed, including progesterone receptor induction and rat uterine weight increases [96]. Overall, the powerful antiestrogenic and slight estrogenic qualities of droloxifene compare favorably with those of tamoxifen. Interestingly, some antitumor activity was observed against an ovarian-independent (estrogen-sensitive) tumor [94].

The major metabolite identified in rats is N–desmethyldroloxifene; this displays similar pharmacological properties to the parent compound. Unfortunately, the hydroxyl group present on the compound may render droloxifene susceptible to phase II metabolism upon passing through the liver after GI tract absorption [46]. This has been observed with similar compounds (e.g., 4–hydroxy tamoxifen) and would lead to a decrease in blood levels and a correspondingly lower antitumor activity.

No clinical trials have been reported for droloxifene, although these are progressing in Europe.

Zindoxifene (D16726)

Traditionally, nonsteroidal antiestrogens have triphenylethylene structures containing an alkylaminoethoxy or glycerol side chain [7]. This structure has been shown to be important for antiestrogenic action [97]. However, new compounds have been developed with a 2–phenylindole skeleton [98]. Zindoxifene, an impeded estrogen with high affinity for the estrogen receptor [99], represents the best-studied new 2–phenylindole antiestrogen.

Zindoxifene has been shown to be effective in antitumor tests in vitro and in vivo, with immature mouse uterine assays suggesting a weak agonistic quality [100].

The active metabolite of zindoxifene is thought to be the deacetylated derivative D15414, formed in vivo by esterase activity. Surprisingly, in vitro this compound displays no antiestrogenic properties. Indeed, D15414 induces the synthesis of both prolactin in cultured cells of the pituitary gland and the progesterone receptor in MCF–7 breast cancer cells in culture [101]. This is in contrast with previous reports of an inhibition of MCF–7 cell growth and proliferation [102]. However, the antitumor action of this suspected estrogenic metabolite may be due to mechanisms similar to those proposed for the inhibitory effects of high doses of estrogen [103, 104].

Summary

There is an enormous literature that supports the use of tamoxifen as the treatment of choice for breast cancer. However, preliminary evidence suggests that long-term treatment with tamoxifen *may* expose the women to an increased risk of liver and endometrial cancer. This is probably only of

concern to young women with stage I (node negative) disease. It remains to be seen whether any of the new strategies, including the new antiestrogens discussed here and novel endocrine therapies [105], will prove any better as an antitumor treatment. Treatments will probably be directed initially at treating patients after tamoxifen failure and ultimately at treating hormone-independent cancers. These therapies may involve the autocrine growth factors produced by breast cancer cells [65] as potential targets. Such development of novel treatments will be extremely beneficial to these women, who face a worrying lack of therapeutic opportunities, with the exception of chemotherapy.

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11. Steroidal pure antiestrogens

Alan E. Wakeling

Introduction: The rationale for seeking a pure antiestrogen

Classical antiestrogens, exemplified by tamoxifen (ICI 46,474; "Nolvadex" ['Nolvadex' is a trademark, the property of Imperial Chemical Industries PLC] have an important role in the therapy of breast cancer. It is well established that tamoxifen inhibits the growth of hormone-responsive advanced breast cancer [1, 2] and provides disease palliation with minimal toxicity. More recently an analysis of more than 16,000 patients receiving tamoxifen adjuvant treatment for primary breast cancer has shown that tamoxifen can prolong survival [3]. Disease response is largely [4] but not entirely [5, 6] confined to patients with estrogen receptor (ER)-positive breast cancer. Since ER status is a primary determinant of disease response to antiestrogen treatment, an observation entirely consitent with clinical studies of response to ovarian ablative procedures, it is highly probable that disease remission is due primarily to blockade by antiestrogens of estrogen-induced tumor cell proliferation. Studies with human breast cancer cells in vitro and with animal models of breast cancer are consistent with this conclusion [7]. The implication of this large body of experimental and clinical data is that tamoxifen blocks the mitogenic action of estrogens by competing directly with the natural ligand(s) for binding to ER. The further assumption implicit in this conclusion is that the tamoxifen–ER complex is essentially biologically inactive when compared with the oestradiol–ER complex. Reference to pharmacological studies in animals and man [1, 8, 9] shows that this is not the case, since tamoxifen can induce a full spectrum of hormonal effects from complete antagonism of estrogen action, through partial estrogen agonist/antagonist, to full estrogen agonist effects.

The complex behavior of tamoxifen highlights a number of uncertainties relevant to the medical applications of antiestrogens [10] and emphasizes the deficiencies in current perceptions of the molecular mode of action of estrogens. With particular reference to breast cancer, it is not clear whether *complete* estrogen withdrawal would result in a better therapeutic effect than that achieved with tamoxifen. It might be possible, for example, to achieve improvements in onset, completeness, or duration of tumor remission by

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complete blockade of estrogen action. Clinical evaluation of a pure antiestrogen (a compound that would bind to ER with a high affinity without itself activating *any* ER-responsive events) would provide a test of this hypothesis. An important additional advantage that a pure antiestrogen might have in comparison with tamoxifen is in the treatment of nonmalignant estrogen-responsive diseases, like endometriosis, benign proliferative disorders of the uterus and breast, and in the prophylaxis of breast cancer. Concerns about the use of antiestrogens with estrogenic activity in such conditions arise from long-term rodent toxicology studies that have shown that such compounds, including tamoxifen, can lead to abnormalities in the development of the reproductive tract [11, 12]. These effects were attributed to estrogenic activity, as was the induction of Leydig cell and ovarian tumors in a carcinogenicity test of tamoxifen in the mouse [13]. Because of the marked differences between species and target organ responses to tamoxifen, the relevance of these toxicological findings to man remains problematical. However, the potential advantage that might be obtained from elimination of estrogenic activity by the discovery of a pure antiestrogen should be clear.

At a more fundamental level, the absence of a ligand that binds to ER but prevents effectively and consistently the normal activation of gene transcription by ER is a major constraint on further elucidation of the molecular events that follow ligand recognition by ER. Tamoxifen (or 4–hydroxytamoxifen) does not have this property and displays partial agonist activity on estrogen-responsive systems in vitro. This is illustrated by comparative studies on the transcription of exogenous [14, 15] or endogenous [15, 16] estrogen-responsive genes, on the growth of human breast cancer cells [17, 18, 19], and on the display of invasive behavior by breast cancer cells in vitro [19], in which responses to tamoxifen are of a mixed agonist/antagonist type analogous to those described in many in vivo assays.

In the following sections the identification and properties of novel steroidal antiestrogens that fulfil all of the key elements defining pure antagonists are described.

Strategy for identification of pure antiestrogens

At the inception of this programme in 1980, the simplest concept of a pure antiestrogen was that of a ligand that would recognize the ER with a high affinity and specificity without promoting nuclear localization. Although assays for nuclear translocation of ER and DNA binding were available at that time, there was no information on either the structural nature of the ligand-binding site or on the nature of the ligand-induced transformation of ER, which precedes DNA binding. Further difficulties were associated with devising a chemical approach, since many attempts to synthesize novel antiestrogens of nonsteroidal [20, 21, 22] or steroidal [23, 24] structure had failed to provide a complete separation of estrogenic and antiestrogenic activity. It was therefore

necessary to adopt an empirical approach to both synthetic chemistry and assays of biological activity.

The biological test system of choice, because of its simplicity and reproducibility, was the uterotrophic/antiuterotrophic assay in immature rats. This assay compares the estrogenic activity of test compound(s) administered alone with that of a fully stimulatory dose of 17β–estradiol and measures antiestrogenic activity in animals dosed concurrently with test compound(s) and 17β–estradiol [25]. In vivo tests may not reflect concisely structure-activity relationships in respect of ER binding because of differences in bioavailability and metabolism. However, intrinsic potency was readily assessed by using a growth inhibition assay of MCF–7 human breast cancer cells in vitro [25].

We chose to explore the biological activity of 17β–estradiol analogues bearing an alkyl chain at the C7 position in the steroid nucleus, as a starting point for novel chemistry. This was considered a promising lead because previous work from Baulieu's group [26], following initial exploration by Raynaud et al. [27], had shown that 7α–estradiol derivatives with a long, unbranched alkyl chain retained a high affinity for ER. Compounds of this type were conceived to fulfil two essential criteria: to have high affinity for ER and to contain a substituent of sufficient size to interfere with secondary structural changes in ER necessary for activation.

Bioassay data for tamoxifen and a selected series of compounds bearing different functional groups linked to C7 of estradiol by a decamethylene bridge are listed in table 1. All of these compounds were biologically active, many with the classical partial agonist/antagonist profile of tamoxifen. However, in this initial series, the butyl secondary amide (compound 5; ICI 160,325)

Table 1. Activity of C7 derivatives of 17β–estradiol in the immature rat uterotrophic/antiuterotrophic assay.

C7 substituent $(CH_2)10^R$	Dose (mg/kg s.c.)	% Agonism[a]	% Antagonism[b]
1. R = $-COOH$ (mixed isomers)	25	23	30
2. R = $-CH_2OH$ (mixed isomers)	25	28	33
3. R = $-CH_2N(C_2H_5)_2$ (mixed isomers)	10	33	62
4. R = $-CONH(CH_2)_5COOH$ (mixed isomers)	25	19	33
5. R = $-CONH(CH_2)_3CH_3$ (7:3α/β)	10	−3	92
6. R = $-CONH(CH_2)_3CH_3$ (7α)	10	−3	100
7. R = $-CONH(CH_2)_3CH_3$ (7β)	25	0	0
8. R = $-CON(CH_3)(CH_2)_3CH_3$ (7α)	5	−5	102
Tamoxifen	10	41	59

[a]Percent agonist = C–A/B–A × 100.
[b]Percent antagonist = B–D/B–A × 100, where A, B, C, and D are mean uterine weight (mg/100 g body weight) in groups of animals (n=5) treated with vehicle alone (arachis oil), 17β–estradiol (0.5 μg/rat), compound alone, or together with estradiol, respectively.

demonstrated the desired profile of a pure antagonist, being entirely free of uterotrophic activity but also completely blocking uterine growth when co-administered with 17β–estradiol. It was shown subsequently that the activity of ICI 160,325, a 7:3 mixture of 7α–and 7β–isomers, resides exclusively in the 7α–isomer (table 1) [28], consistent with a more than hundred fold difference in relative affinity for ER of the two isomers [29]. The most potent pure antiestrogen was the methyl tertiary amide (compound 8, table 1), ICI 164,384, which was selected for further intensive study.

Pharmacology of the pure antiestrogen ICI 164,384

Effects in immature or ovariectomized rats and mice

Uterine effects of ICI 164,384 were measured in immature or ovariectomized adult rats and mice in the three day assay referred to above [25]. In all cases, ICI 164,384 alone was devoid of trophic activity and blocked completely the stimulatory activity of exogenous estradiol [30]. Additionally, in immature rats acute (6, 24 hours) or chronic (8 days) treatment with ICI 164,384 did not stimulate the uterus, and in ovariectomized rats and mice no cornification of the vagina could be detected after short- or long-term treatment. The contrast between ICI 164,384 and antiestrogens, like tamoxifen and LY 117018 [21], was particularly marked in the mouse where tamoxifen is a full agonist [31]

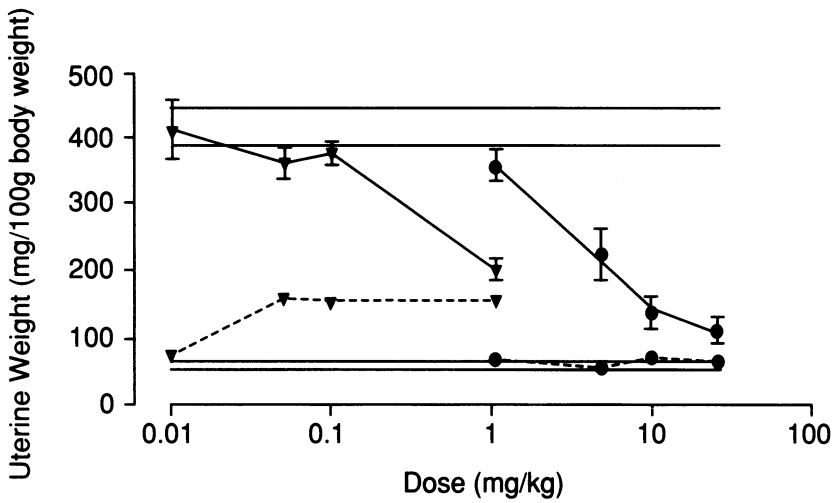

Figure 1. Uterotrophic and antiuterotrophic effects of LY117018 (▼) and ICI 164,384 (●) in mature ovariectomized mice. Animals ovariectomized three weeks previously received three daily doses of arachis oil vehicle alone (lower open bar), 0.5 μg estradiol benzoate s.c. alone (upper open bar), or increasing doses of LY117018 or ICI 164, 384 s.c. alone (dashed lines), or together with estradiol benzoate (continuous lines). Uterine weights were recorded 24 hours after the final dose and corrected for body weight. Each point represents mean ± S.E.M., n = 5. Where no bar is shown S.E.M. was smaller than the symbols.

and LY 117018 is a partial agonist [21]. A comparative study of ICI 164,384 and LY 117018 in the mature ovariectomized mouse is illustrated in figure 1. At the highest doses used in this study, the agonist/antagonist ratios were 24%/62% and 1%/88% for LY 117018 and ICI 164,384, respectively. Higher doses of ICI 164,384 blocked estradiol action completely, whereas a similar dose escalation of LY 117018 achieved a maximum 20% versus 80% balance between agonist and antagonist activity.

Two other observations in the immature rat serve to emphasize further the differences between tamoxifen and ICI 164,384. Firstly, coadministration of a fixed dose of tamoxifen and increasing doses of ICI 164,384 blocked the uterotrophic action of tamoxifen in a dose-dependent manner, and at a high dose ratio ($\geq$25) this was complete [28]. Secondly, treatment of neonatal female rats with tamoxifen caused premature vaginal opening, whereas ten-fold greater doses of ICI 164,384 were without effect; in this test the effect of tamoxifen was also blocked by ICI 164,384 [30]. Premature vaginal opening and subsequent abnormal development of the reproductive tract has been reported previously for tamoxifen [32] and other antiestrogens [33]. It was assumed that these effects were due to the estrogenic activity of these compounds; the absence of such effects in ICI 164,384-treated rats [34] provides powerful support for this conclusion. The absence of "toxicological" sequelae in ICI 164,384-treated neonates confirms an important potential advantage of pure antiestrogen over a partial agonist.

Comparative studies of the effects on mammary gland development of estradiol, tamoxifen, LY 117018, and ICI 164,384 in ovariectomized rats have further emphasized the potential importance of eliminating estrogenic activity. Postpubertal growth of the mammary gland in rats is characterized by a more than twofold increase in ductal length between 30 and 50 days of age. That this growth is largely due to estradiol can be demonstrated by comparison of intact with ovariectomized, estradiol-treated animals [35]. Mammary duct elongation is associated with a high rate of cell proliferation in terminal end bud (TEB) structures. In ovariectomized rats treated with tamoxifen or LY 117018 instead of estradiol, ducted elongation and mitotic activity in the TEBs was indistinguishable from that in intact or estradiol-treated animals. By contrast, ICI 164,384 alone had no effect on ductal growth, and when administered with tamoxifen, blocked the proliferative action of tamoxifen [35].

Surgical or chemical ovariectomy reduced the incidence of mammary tumors in rats treated with dimethylbenzanthracene (DMBA). This inhibition of tumor formation was reversed by treatment with estradiol, tamoxifen, or LY 117018 [35]. The effect of ICI 164,384 on tumor formation in DMBA-treated ovariectomized rats remains to be determined [35], but the differences between the conventional antiestrogens and ICI 164,384, predict that the pure antagonist would not itself support tumorigenesis and should block estrogen-dependent tumorigenesis. In this regard, a pure antagonist may have greater utility in breast cancer prophylaxis, an important and currently controversial area of therapy for breast pathology [13, 36, 37].

Effects in normal adult rats

Chronic treatment of normal adult rats with a pure antiestrogen would be expected to produce effects similar to those manifest in ovariectomized animals, namely involution of the accessory sex organs, an increased rate of growth, and a large increase of serum gonadotrophin concentrations. In fact, ICI 164,384 produced a dose-related reduction in uterine weight without affecting luteinizing hormone (LH) secretion or the rate of growth of intact adult female rats [29]. Compared with the effect of ovariectomy at the start of a 14-day treatment, a daily maximum dose of 5 mg/ICI 164,384/kg produced uterine involution equivalent to 90% of that found in castrated rats; in a similar study with tamoxifen, a maximum reduction of 67% was recorded [29]. Even at this high dose of ICI 164,384, there was no increase in serum LH or the rate of body weight gain, implying a selective action at peripheral but not hypothalamic ER [38, 39]. In contrast, 0.1 to 10 mg tamoxifen/kg daily reduced both LH and body weight gain, both effects being attributable to an estrogenic action of tamoxifen [12, 38, 40]. Consistent with this was the ability of tamoxifen, but not ICI 164,384, to reverse as effectively as estradiol the postcastration increase of LH in ovariectomized rats [30].

The effects of tamoxifen on the vaginal smear pattern in intact and ovariectomized rats were consistent with mixed estrogenic/antiestrogenic activity. The normal cyclical variation between predominantly leucocytic and fully cornified vaginal smears in intact rats was disrupted [30, 41]. In ovariectomized rats, ICI 164,384 did not induce vaginal cornification, and in intact animals at the maximum dose tested (5 mg/kg), it produced a fully leucocytic smear pattern presumably as a result of complete blockade of estrogen at the vagina. Interestingly, the threshhold for significant vaginal inhibition by ICI 164,384 appeared greater than that for the uterus, since at low and intermediate doses (0.5, 2 mg/kg) 100% and 60% of rats had proestrous smears during treatment, but uterine weight was reduced by 61% and 83%, respectively. Thus there is some evidence for selectivity of antiestrogenic action of ICI 164,384 between different peripheral organs as well as between central and peripheral effects. This is perhaps not surprising, since differential sensitivity between target organs is a feature of estradiol action [42]. The clinical implications of differential threshholds of anti-estrogen action are profound. For example, it may be possible to select a dose of a pure antiestrogen to treat uterine (or breast) pathology in pre-menopausal patients without affecting the hypothalamic-pituitary-ovarian axis, or bone metabolism, or causing hot flashes.

Antitumor activity

The effects of ICI 164,384 on the growth of established DMBA-induced rat mammary carcinomas was compared with that of tamoxifen and ovariectomy.

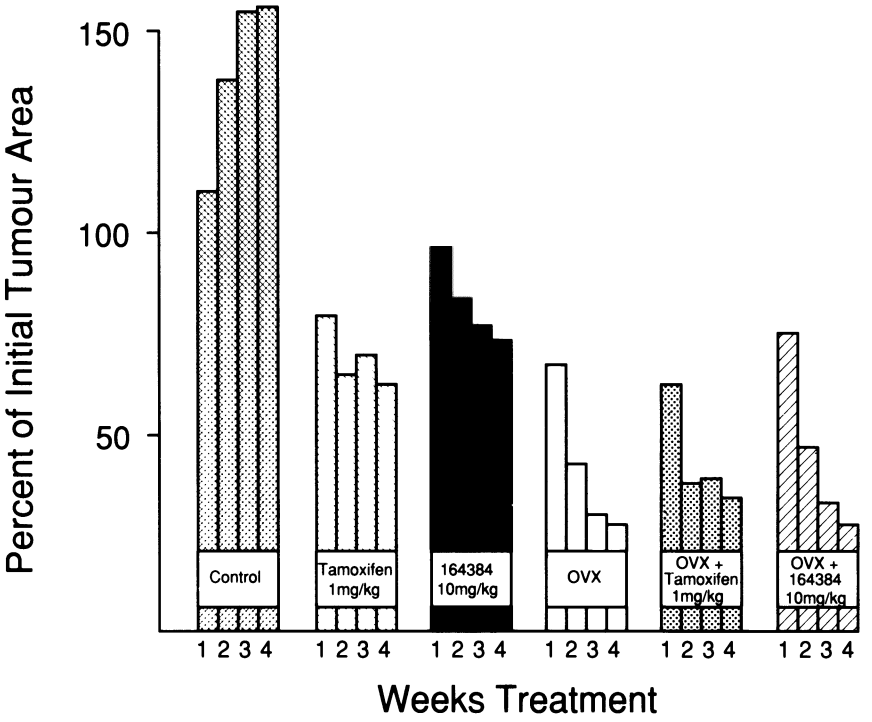

Figure 2. Effect of tamoxifen and ICI 164,384 on the growth of DMBA-induced mammary tumors in intact or ovariectomized rats. Groups of five or more animals, each with one or more tumors of minimum diameter 1 cm at the beginning of the experimental period, were treated as indicated for 28 days. Tamoxifen was administered orally and ICI 164,384 s.c., once daily. Tumor area recorded weekly was then summed for all individual tumors in which area was calculated as the product of the largest diameter and the axis perpendicular to it. Two weeks after the final drug dose, intact animals were ovariectomized and tumors that failed to regress thereafter (i.e., nonhormone-dependent) were excluded from the analysis.

The antitumor efficacy of ICI 164,384 was not superior to that of tamoxifen [29] (figure 2). This was surprising, since, as described above, ICI 164,384 produced a castration-like involution of the uterus, and previous studies had shown that ovariectomy provides a greater antitumor effect than tamoxifen in this model system (figure 2) [43]. It is possible that the sensitivity of the tumors to estrogen withdrawal is less than that of the uterus, but alternative mechanisms also play an important role. The effects of pituitary hormones, particularly of prolactin, whose secretion is positively controlled by circulating estrogens, may be predominant in regulating the growth of DMBA-induced tumors [44, 45]. Since ICI 164,384 treatment in normal intact animals did not affect LH [29] or prolactin secretion [Wakeling, unpublished studies], the absence of inhibition of prolactin may account for the failure of ICI 164,384 to produce an ovariectomy-like tumor remission.

Direct comparison of the antitumor activity of tamoxifen with that of ICI

164,384 is complicated by differences in host response. Tamoxifen treatment initially increases, then suppresses, prolactin secretion. However, this reduction is not as effective as that in ovariectomized animals [46]. Tamoxifen also desensitizes mammary gland lobular responses to prolactin [46]. As noted above, tamoxifen reduces the rate of growth of intact rats, probably because of an estrogen-like suppression of appetite. Since dietary restriction alone can also reduce the growth of DMBA-induced tumors [47], this could also contribute substantially to tamoxifen-induced remission. This mechanism is not operative in ovariectomized or ICI 164,384-treated rats. Finally, treatment of ovariectomized tumor-bearing rats with tamoxifen or ICI 164,384 (figure 2) did not change the rate of ovariectomy-induced tumor involution. Therefore, direct tumor stimulation due to the estrogenic activity of tamoxifen was not apparent [48], nor was there an indirect host-mediated effect despite the proven ability of tamoxifen to raise serum prolactin concentrations in ovariectomized rats [46]. It should be clear from this discussion that simplistic comparisons between the effects on breast tumor growth of a partial agonist and a pure antagonist antiestrogen, or between a pure antiestrogen and ovariectomy, are inappropriate. Complex differential actions on host mechanisms are apparent in the rat and are unlikely to predict accurately the therapeutic potential in man of a pure antiestrogen compared with that of tamoxifen. A less complicated picture emerges from studies with ER-positive human breast cancer cell lines in vitro, which are described below.

Effects on the proliferation of human breast cancer cells

During the screening of novel antiestrogens for intrinsic potency using ER-positive MCF–7 human breast cancer cells, ICI 164,384 emerged as a particularly active inhibitor of cell growth. A comparison of ICI 164,384 with tamoxifen revealed IC_{50} values of 2 nM and 1 μM, respectively, for MCF–7 cells grown in medium containing phenol red, insulin, and 5% charcoal-stripped fetal calf serum (CFCS) [30]. Although growth of MCF–7 cells under these conditions was not stimulated by addition of estradiol, the inhibitory effect of antiestrogens was fully reversible by estradiol [30]. Similarly, in ZR–75–1, ER-positive human breast cancer cells whose growth is stimulated by estradiol, ICI 164,384 inhibited the mitogenic effect of estradiol in a dose-dependent competitive manner [28]. IC_{50} values were 3 nM and 1.2 μM for ICI 164,384 and tamoxifen, respectively, in ZR–75–1 cells grown in the presence of 0.1 nM estradiol [30]. Thus the comparative potency of these two antiestrogens was similar in both ER-positive cell lines.

Further evidence of specificity of action is illustrated in figure 3, which compares the action of tamoxifen, the 4′–hydroxy metabolite of tamoxifen (4′–OHT), and ICI 164,384 in MCF–7 cells and in ER-negative MDA–MB–

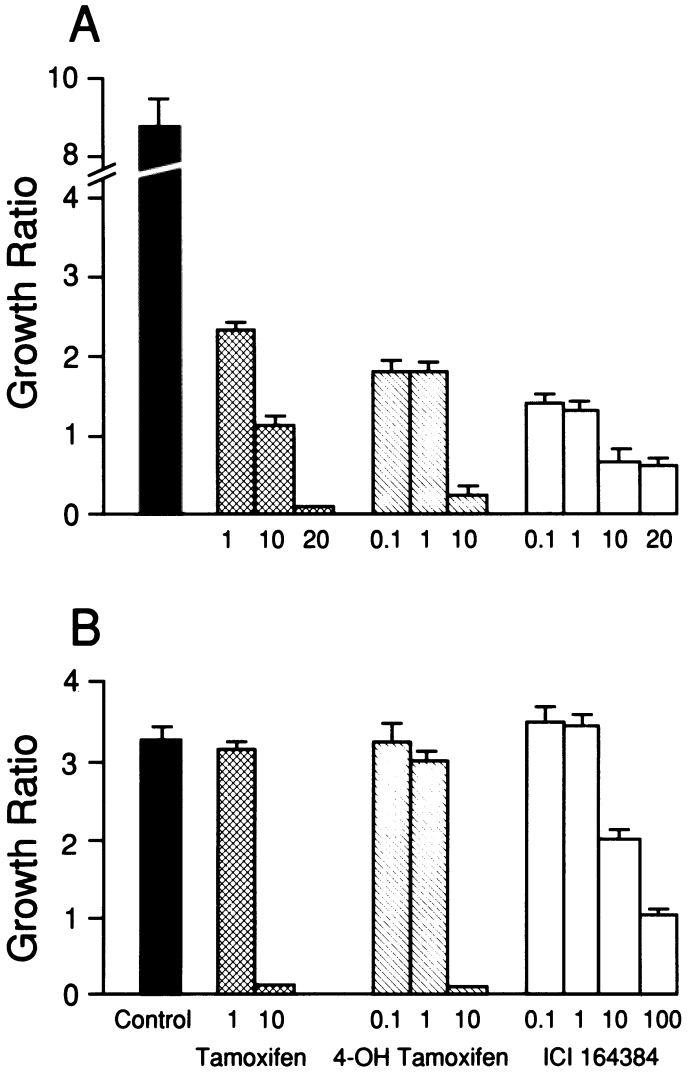

Figure 3. Inhibition of the growth of MCF-7 (A) and MDA-MB-231 (B) human breast cancer cells by tamoxifen, 4′-hydroxytamoxifen, and ICI 164,384. Cells were grown in monolayer culture in 12-well dishes for 5 (B) or 6 (A) days, in Dulbecco's MEM with 5% charcoal-stripped fetal calf serum supplemented with nonessential amino acids, glutamine, insulin (1 μg/ml), and 17β-estradiol (0.1 nM), in the absence (control) or presence of the antiestrogens at the indicated concentrations (μm). Growth ratio is defined as the increase in cell number compared with that at the start of the experimental period. Values are mean ± S.E.M., $n \geq 4$.

231 human breast cancer cells. At concentrations of 1 μM or less of each compound, which radically reduced the growth rate of MCF-7 cells in an estrogen reversible manner, there was no effect on the growth of MDA-MB-231 cells. At 10 or 20 μM, tamoxifen and 4′-OHT killed both MCF-7

and MDA–MB–231 cells, consistent with a cytotoxic effect reported previously [49, 50]. At similar high concentrations ICI 164,384 was less effective in killing cells than was tamoxifen of 4′–OHT. This may simply be a reflection of an inability to achieve a sufficiently high free-drug concentration to produce non-specific cytotoxicity, since ICI 164,384 is much more lipophilic and is thus much less soluble in aqueous media than is tamoxifen.

A detailed analysis of the mechanism of action of high-affinity hydroxylated antiestrogens, including 4′–OHT, had demonstrated a clear separation between estrogen-reversible and estrogen-irreversible effects on breast cell growth [51]. For the former mechanism, concentrations of 0.1–10 nM reduced cell growth in a dose-dependent manner, and this reached a maximum plateau value of 60% to 70% reduction of cell number. Potency paralleled receptor affinity in this concentration range but did not do so for the estrogen-irreversible cytotoxic effects at 1–10 μM, which led to a further reduction of cell number [51]. Although both low and high concentrations led to an accumulation of cells in the G_1-phase of the cell cycle and a corresponding decrease of S-phase cells, the mechanism of the cytotoxic effect remains unclear but may reflect convergence of both effects on common signalling pathways leading to cell division [10, 51]. It is unlikely that the high-affinity, antiestrogen specific-binding site [52] plays a direct role in either mechanism [53, 54]. ICI 164,384 does not compete with tamoxifen for binding to the triphenylethylene-specific antiestrogen-binding site [Wakeling, unpublished studies, and MA. Blankenstein, personal communication], an observation that supports the conclusion that this binding protein is not directly involved in the estrogen-reversible growth inhibitory actions of antiestrogens.

A particular feature of the estrogen-reversible growth inhibitory action of ICI 164,384 on MCF–7 cell growth is its apparent increase in efficacy compared with that of the nonsteroidal antiestrogens tamoxifen [30], 4′–OHT [51, 55], and hydroxyclomiphene [51, 56]. Initial studies [30] showing a maximum reduction of cell number by 80% in ICI 164,384-treated cultures compared with 50% in tamoxifen-treated cultures were subsequently confirmed [55, 56] and are not explained by the difference of ER-binding affinity between tamoxifen and ICI 164,384 [28 and see below]. Analysis of the effects of ICI 164,384 on cell cycle progression has failed to reveal a significant difference from those of nonsteroidal compounds that might account for the efficacy difference. Both types of compound block MCF–7 cells in the G_1-phase [56], as noted above; a similar action was seen on ZR–75–1 cells [57]. More detailed examination of the population dynamics of antiestrogen-treated MCF–7 cells showed that ICI 164,384 was more effective than tamoxifen in reducing th proportion of actively proliferating cells in an asynchronous population [18]. When cells were grown without phenol red or insulin but with estradiol, almost 50% of the cells passed through S-phase during a 48 hour DNA-labelling period. This was reduced

to 19% and 7% by tamoxifen and ICI 164,384, respectively, at maximum noncytotoxic concentrations. Correspondingly, in the presence of estradiol and insulin, cells grew more rapidly, with 85% passing through S-phase; this was reduced to 43% and 29% by tamoxifen and ICI 164,384 [18]. Thus a greater proportion of breast cancer cells appeared to be susceptible to blockade by the pure antiestrogen than by tamoxifen. Since extrapolation of these data to the clinical setting implies that a pure antiestrogen might have improved efficacy compared with tamoxifen, it is important to understand the underlying mechanism(s).

Although the estrogenic (partial agonist) activity of tamoxifen in vivo was readily demonstrated, similarly facile observation of mitogenic activity in vitro has proven to be more difficult [58, 59]. Complete removal of the influence of endogenous estrogens in serum-containing media was problematical [60, 61]. However, the recognition that phenol red [62] (or an impurity commonly present therein [63]), universally employed as the pH indicator in cell culture studies, is also estrogenic significantly ameliorated this problem and provided an opportunity to reevaluate the significance of partial agonist activity. It seemed that the absence of such activity in ICI 164,384 was the most obvious potential explanation for the efficacy difference in vitro between ICI 164,384 and tamoxifen. We chose to use 4′–OHT in place of tamoxifen because of its increased potency in vitro; it should be noted that the pharmacology of 4′–OHT in vivo is qualitatively indentical to that of tamoxifen [64], although it is less potent on chronic dosing. Furthermore, Katzenellenbogen et al. also had demonstrated that several nonsteroidal antiestrogens, including 4′–OHT, do stimulate the proliferation of MCF–7 cells under phenol red-free conditions [17]. The mitogenic action of 4′–OHT on MCF–7 cells was confirmed [18]. Under identical conditions, ICI 164,384 did not stimulate cell growth, and in combination with 4′–OHT, it blocked the stimulatory effect of 4′–OHT [18]. The cells and basal culture conditions used in these experiments were extremely sensitive to estrogenic stimulation; half-maximal effects were achieved with $\leq$0.01 nM estradiol and maximum stimulation at 0.1 nM [57]. However, the magnitude of the 4′–OHT stimulation was small (ca. 30% that of estradiol), and as reported previously, [17], it was highly concentration-dependent, being low at $<$0.01 nM, maximal between 0.01 and 1 nM, but decreasing at higher concentrations. The absence of an effect of 4′–OHT on the proliferation rate at concentrations $>$1 nM did not parallel the pattern of response to partial agonist/antagonist compounds in vivo, where stimulatory effects reach a dose-dependent stable maximum plateau value characteristic of a specific compound and a specific response. The reasons for this bell-shaped dose response to 4′–OHT are not understood but may reflect a change in balance between multiple concentration-dependent effector mechanisms, including what has been termed a "receptor-mediated cytotoxic" action of antiestrogens [65], as well as those described previously. In the presence of estradiol, 4′–OHT and ICI 164,384 reduced

cell growth in a dose-dependent manner, and complete blockade was obtained at >10 nM; 4′–OHT was fivefold more potent than ICI 164,384 [18].

In estrogen-free conditions insulin alone stimulates MCF–7 cell growth, but in combination with estradiol, a synergistic enhancement or growth rate was observed [18, 57]. This synergy provided an opportunity to reexamine the estrogenic activity of 4′–OHT. In combination with insulin (1 μg/ml), the stimulatory action of 4′–OHT was enhanced [18], and in contrast to the effect of 4′–OHT alone, cell numbers were greater than in insulin controls at all concentrations of 4′–OHT (0.001–1000 nM). The response curve was again bell-shaped and closely paralleled that recorded with 4′–OHT alone. The effect of ICI 164,384 in the presence of insulin appeared qualitatively similar to that of 4′–OHT but was greatly attenuated. A small enhancement was seen at 0.01 and 0.1 nM, but this degree of stimulation did not reach statistical significance in a series of experiments [18]. Thus it is possible that partial agonist antiestrogens are less effective inhibitors of cell growth than are pure antiestrogens, because of their synergistic mitogenic interaction with insulin or other stimulatory growth factors present in serum, a mechanism that is inoperative in the case of pure antagonists.

Finally, in this section previous studies have shown that antiestrogens can attenuate the actions of growth factors in vitro [66, 67, 68, 69]. As noted above, this was not the case for insulin and 4′–OHT, but it was observed with a high concentration of ICI 164,384 [18]. However, both 4′–OHT and ICI 164,384 did inhibit partially the mitogenic action of TGF–α and IGF–1, alone or together, on MCF–7 cells in estrogen-free conditions [18]. At concentrations equally effective against exogenous estradiol, ICI 164,384 was significantly more effective than 4′–OHT. The mechanism of this inhibition is not understood, but it could involve the induction by antiestrogens of inhibitory growth factors, like TGF–β [70]. Attempts to reverse the suppressive action of ICI 164,384 in MCF–7 cells stimulated with TGF–α and IGF–1 by coincubation with a neutralizing TGF–β antibody were unsuccessful [57]. This does not exclude the possibility that induction of negative growth regulatory factors plays a role in antiestrogen action. Clearly, much work remains to be done in elucidating the interrelationships between steroid and growth factor-mediated mitogenic pathways in breast cancer.

Mode of action

The cell biology and pharmacology studies described above strongly imply that ICI 164,384 exerts its actions through the estrogen receptor. Formal evidence for this derives from classical receptor competition assays in which ICI 164,384 displaced tritiated estradiol from rat uterus ER in a concentration-dependent manner [28] parallel to that recorded with estradiol and the classical antiestrogens tamoxifen and 4′–OHT (figure 4). Compared with estradiol, relative affinities for ER were 0.19, 0.025, and 2 for ICI

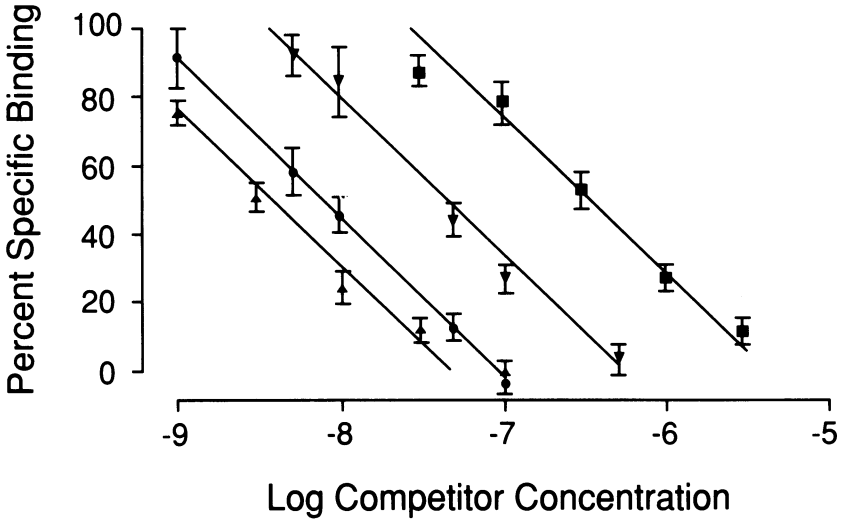

Figure 4. Competition of antiestrogens for rat uterus estradiol receptor. Cytosol was incubated with ^{3}H-labeled estradiol for 2 hours at 25°C in the absence or presence of increasing concentrations of estradiol (●), 4′-hydroxytamoxifen (▲), tamoxifen (■), or ICI 164,384 (▼). Each point represents mean ± S.E.M. for nine observations in three separate experiments.

164,384, tamoxifen, and 4′-OHT, respectively. Thus the rank order of relative affinities was the same as the order of relative potency in cell growth inhibition assays in vitro. The ratio of potencies between compounds was, however, different; for example, 4′-OHT has tenfold higher affinity than ICI 164,384 for ER but is fivefold more potent on cells; whereas for tamoxifen, the ER affinity ratio, compared with ICI 164,384 is approximately tenfold, but the potency ratio in cell culture is more than a hundredfold. The absence of a precise correlation in the two systems most probably reflects not only differences in experimental conditions but also differences in hydrophobicity between the antiestrogens [71]. Studies with tritiated ICI 164,384 have shown that in crude rat uterus cytosol preparations, more than 95% was nonspecifically bound [Wakeling, unpublished studies]. However, partial purification of ER from human uterus reduced nonspecific binding to less than 50% of the total and permitted direct measurement of specific binding parameters [72]. Dissociation constants were 0.44 ± 0.16 and 0.69 ± 0.18 nM for estradiol and ICI 164,384, respectively, and number of binding sites determined by Scatchard analysis was the same for both ligands [72]. The higher affinity of ICI 164,384 for ER in these studies compared with that in the rat cytosol assay may represent a closer approximation to intrinsic potency because of reduced interference from other proteins; but it must still be regarded as an approximation. Since both ^{3}H-estradiol and ^{3}H-ICI 164,384 were shown to bind the same number of sites, it may be assumed that the antagonist interacts with ER at the *same* site as estradiol.

Following receptor binding, steroid hormones transform or activate the

receptor to a form capable of recognizing specific DNA sequences termed hormone response elements (HREs) [73]. These HREs reside in the vicinity of hormone-responsive genes and control steroid-dependent activation of transcription. Comparative studies of ER–ligand binding to calf thymus DNA, using human uterine ER loaded with ^{3}H–estradiol or ^{3}H–ICI 164,384, demonstrated the activation of ER by estradiol but not by ICI 164,384 [72]. Similar assays of ER–DNA interaction with ^{3}H–4′–OHT had shown that 4′–OHT does activate DNA binding, albeit of a lower affinity than estradiol [74]. These in vitro assays of receptor binding to nonspecific DNA may not reflect in vivo recognition of specific HREs by antihormone receptor complexes [75, 76]. Detailed analysis of the ability of such complexes to bind HREs and to induce transcriptional activation, using native and chimeric receptors transfected into cells with native or synthetic hormone-responsive reporter genes, has shown that both agonists and antagonists can promote HRE recognition [76, 77]. However, in a specific comparison of the transcriptional activation of an estrogen-responsive promotor region of the human pS2 gene, it was shown that 4′–OHT acted as a partial agonist/antagonist, whereas ICI 164,384 was a pure antagonist [14].

The estrogen receptor is a large protein with multiple transcription regulation domains as well as distinct ligand- and DNA-binding regions [78]. Multiple secondary protein–protein interactions between DNA-bound receptor dimers and other transcription factors appear to contribute to efficient transcription. Qualitative and quantitative differences in the efficiency of these processes with different ligand receptor complexes may account ultimately for differential gene control by antiestrogens [78], but a complete account of these processes at the molecular level remains a major challenge to the ingenuity of the investigator. That studies of hormone response with synthetic recombinant promotor-gene constructs need cautious interpretation is illustrated by comparative studies of the effects of antiestrogens on the expression of endogenous pS2 and pS2–chloramphenicol acetyltransferase (CAT) fusion genes [15]. In MCF–7 cells, pS2 messenger RNA was induced 13-fold by estradiol treatment; 4′–OHT also induced pS2 weakly, and in combination with estradiol, it reduced pS2 RNA to a level consonant with its weak agonist activity. ICI 164,384 alone was devoid of stimulatory activity and blocked completely estradiol induction of pS2 RNA [15]. In the same cells transfected with the estrogen response element of pS2 fused to the CAT reporter gene, 4′–OHT *and* ICI 164,384 behaved as partial agonist/antagonists. Clearly, factors other than the primary sequence of the HRE are of dominant importance in signalling biological response [79].

Measurements of the effects of ICI 164,384 on six other endogenous estrogen-inducible RNAs in MCF–7 cells showed a complete absence of induction and complete inhibition of estradiol response [80], in agreement with the studies on endogenous pS2 [15]. ICI 164,384 also blocked tamoxifen induction of pNR–1 [80], which is induced by tamoxifen or 4′–OHT to 80% of the maximum response to estradiol [81]. Limited studies reported to date

on the effects of ICI 164,384 on the synthesis of estrogen-induced cellular and secreted proteins are also consistent with pure antiestrogenic activity. For example, ICI 164,384 did not induce the progesterone receptor [82] or the 46 kD cathepsin D–like acid protease in MCF–7 cells [80], and it also blocked estrogen-induced synthesis of the latter protein [80].

Summary and future prospects

The 7α–alkyl amide analogues of estradiol, exemplified by ICI 164,384, represent a new class of estrogen antagonists. Extensive comparative studies of the physiology of ICI 164,384, tamoxifen, and estradiol, both in vitro and in vivo have emphasized that ICI 164,384 is a pure antiestrogen. It is unique and different from tamoxifen in possessing the intrinsic capacity to block completely the stimulatory actions of estradiol while itself being entirely free of "estrogen-like" stimulatory actions. This has been demonstrated in studies of organ, cell, protein, and RNA responses. A particularly informative property of such pure antiestrogens is their capacity to block the proliferative actions of conventional partial-agonist antiestrogens. Thus ICI 164,384 blocks the uterotrophic and mammotrophic actions of tamoxifen and the mitogenic effect of 4′–OHT on ER-positive human breast cancer cells. Both partial agonist and pure antagonist antiestrogens bind to the high-affinity estradiol receptor; the actions of ligand receptor complexes formed by partial agonists are only attenuated partially, whereas the pure antagonist complex fails to support transcriptional responses. These pure antiestrogens hold out great promise both as tools to analyze further the molecular basis of steroid hormone action and as therapeutic agents for the treatment of estradiol-responsive benign and malignant disease.

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12. Estrogen-regulated messenger RNAs in human breast cancer cells

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Abstract

Estrogen-regulated RNAs have been cloned from cDNA libraries constructed from two breast cancer cell lines. Three RNAs (pNR–1, pNR–2 and pNR–25) were expressed and regulated only in estrogen-responsive cell lines, whereas the majority were expressed in all the cell lines tested but regulated only in those that were estrogen responsvie. Sequencing of the cDNA clones established that one mRNA (pNR–100) encoded cathepsin D and that another (pNR–2) corresponded to pS2 RNA. The majority of the mRNAs did not correspond to any sequence currently compiled in nucleic acid databases. Three patterns of regulation by the antiestrogen tamoxifen were identified. Tamoxifen was a full estrogen agonist for the pNR–1 RNA but a weak estrogen agonist for the pNR–2 and pNR–25 RNAs. For cathepsin D RNA, tamoxifen was an estrogen agonist, and there was synergism between estradiol and tamoxifen for the induction of the RNA over a limited range of concentrations of tamoxifen. The pNR–2 mRNA was measured in breast tumors. The pNR–2 expression was only detected in tumors expressing estrogen receptor mRNA, and pNR–2 expression was associated with response to tamoxifen in a group of patients receiving primary tamoxifen.

The estrogen responsiveness of a proportion of human breast cancers has been appreciated since the last century [1] and has provided the impetus for the development of compounds with antihormonal activity. The most widely used hormonal treatment for breast cancer is currently tamoxifen [2], which provides low toxicity palliation in approximately one third of unselected patients. The establishment of breast cancer cell lines [3] that retain estrogen receptors and whose growth is estrogen responsive has provided an amenable cell culture system for studying the mechanisms of action of estrogen and antiestrogens and for elucidating the mechanisms involved in estrogen-stimulated proliferation [4].

We have been interested specifically in the control of gene expression by estrogens in breast cancer cells and, in particular, the identification of genes whose activity is regulated by estrogens and antiestrogens. The long-term aims are, first, to generate cloned probes for estrogen-regulated genes

M. Lippman and R. Dickson (eds.), REGULATORY MECHANISMS IN BREAST CANCER.

with which to analyze the molecular mechanisms of estrogen and antiestrogen action in a cell culture system. The second aim is to identify potential clinical markers of estrogen responsiveness that might be useful for predicting the response of breast cancer patients to antiestrogen therapy. Finally, any estrogen-regulated gene may potentially be involved in mediating the effects of estrogen on cell proliferation, and the cloning of such genes may increase understanding of this process.

This chapter describes the isolation of regulated mRNA sequences and their regulation by estrogens and antiestrogens. It also describes some clinical data on the expression of one estrogen-regulated RNA in breast tumors and its relationship to response to tamoxifen.

Cloning of estrogen-regulated mRNAs from breast cancer cells

A number of strategies have been developed for identifying differences in the expression of genes in different cells or under different experimental conditions. Differential screening of cDNA libraries [5] has proved to be extremely useful because the resultant cDNA clones can be used directly in hybridization experiments to measure the corresponding RNAs or to clone the corresponding gene.

Two cDNA libraries were constructed from mRNA prepared from the MCF–7 and ZR–75 estrogen-responsive cell lines [6, 7]. These two cell lines were chosen because their proliferation is estrogen responsive and because estrogen-regulated proteins had previously been identified by one- and two-dimensional gel electrophoresis [8, 9].

Colonies containing recombinant plasmids were inoculated into the wells of microtiter dishes and grown overnight. Bacteria were then inoculated in a 96-well array directly from the microtiter dishes onto nitrocellulose filters placed on agar and grown up overnight. The plasmid DNA within these colonies was then denatured and immobilized on the filters. Duplicate filters were hybridized with radiolabled cDNA synthesized by reverse transcription primed with random oligonucleotides from mRNA extracted from withdrawn and estrogen-treated cells. The amount of radioactive cDNA hybridized was assesssed by autoradiograhy.

The majority of the recombinants in the libraries hybridized with equal intensity to cDNA from withdrawn and estrogen-treated cells. Any recombinant that hybridized differently to the two probes was rescreened. The technical problems associated with achieving reproducible replica screening resulted in many recombinants appearing to be regulated, and therefore following the second round of screening, recombinant plasmid DNA was extracted, labeled with ^{32}P–dCTP by nick-translation and hybridized to Northern Transfers of total RNA extracted from withdrawn and estrogen-treated cells.

This procedure resulted in the identification of 62 recombinants from

Table 1. Estrogen-regulated RNAs isolated by screening an MCF and a ZR–75 cDNA library by differential hybridization.

Cell line	RNA	No. of isolates	Size (kb)
MCF7	pNR–1	4	3.5, 3.1, 1.8, 1.2
	pNR–2	47	0.6
	pNR–7	3	1.1
	pNR–8	1	1.9
	pNR–13	1	3.6
	pNR–17	1	9.0
	pNR–20	1	3.9
	pNR–21	1	2.4
	pNR–22	1	2.9
	pNR–23	1	2.0
	pNR–25	1	9.5, 1.5
ZR 75	pNR–100	30	2.1
	pNR–101	4	1.7
	pNR–102	1	1.9
	pNR–105	10	0.6

the MCF–7 library and 45 recombinants from the ZR–75 library, out of 30,000 recombinants screened. The cDNA clones corresponding to the 13 regulated mRNAs identified are listed in table 1.

The recombinants were further characterized by cross-hybridization and restriction mapping. Many of the RNAs were isolated once only, while others, such as the pNR–2 and pNR–100, were isolated frequently. Two mRNAs (pNR–2/pNR–105 and pNR–8/pNR–102) were isolated from both libraries.

Expression and regulation of estrogen-regulated RNAs in different cell lines

The estrogen-regulated RNAs varied in abundance by up to two orders of magnitude. Some of them, such as the pNR–101 RNA, could easily be measured using nick-translated probes, while others were difficult to detect. The problem of detection was particularly severe in total RNA prepared from cells cultured in the absence of estradiol. The cDNA insert corresponding to each RNA was therefore subcloned into vectors that permit transcription of RNA from phage promoters flanking the cDNA insert. The radiolabeled RNA probes gave greatly increased sensitivity, which allowed the detection of unabundant RNAs and a more precise determination of the degree of regulation.

The abundance and regulation of the RNAs by estrogens and other steroids was measured in a series of breast cancer cell lines (estrogen responsive and unresponsive) as well as two cell lines (Hela and A431) established from other human malignancies (cervical and epidermoid vulval

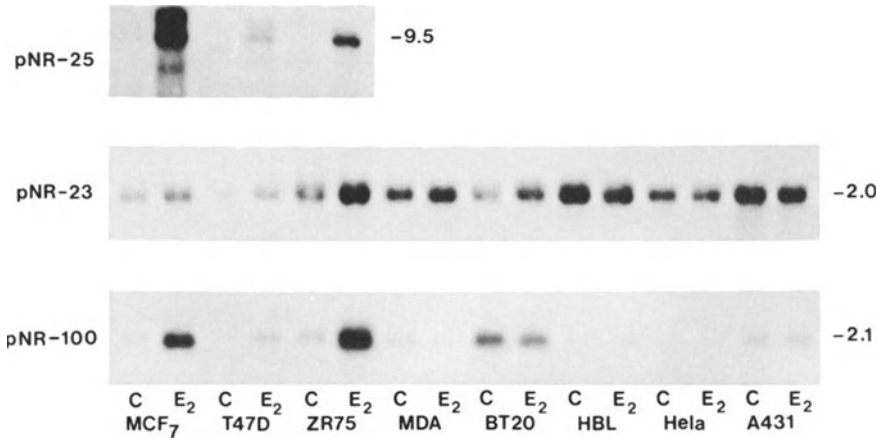

Figure 1. Regulation of pNR–25, pNR–23, and pNR–100 RNAs by estradiol in different cell lines. The cells were withdrawn for 5 days and then grown in the presence of 10^{-8} M estradiol for 2 days. Total RNA was prepared, separated by gel electrophoresis, transferred to a nylon membrane, and then hybridized with ^{32}P-labelled probe.

carcinoma, respectively). Three RNAs (pNR–1, pNR–2, and pNR–25) were expressed and regulated only in the three estrogen-responsive breast cancer cell lines (figure 1). Nine RNAs were expressed in all eight lines tested. The relative abundance in the cell lines varied and, in general, they were only regulated by estrogen in the three estrogen-responsive cell lines. For each RNA, the extent of induction varied in the different cell lines. For example, the pNR–100 (cathepsin D) RNA was least regulated in the T47D cell line, while the pNR–7 RNA was most induced in this cell line. The pNR–17 RNA was unusual in that it was not expressed in all cell lines, but its expression was not restricted to estrogen-responsive cells.

Thus the regulated RNAs were expressed at different levels and regulated to different degrees; and furthermore, these two parameters varied with the cell line studied. Interpreted in the light of current models of steroid action, this suggests that the strengths of the promoters for these genes and the effects of the estrogen-inducible enhancers vary considerably. It seems possible that there is an interplay between the estrogen receptor and other transcriptional factors, which determines the different regulation observed in the various cell lines.

Other steroids were tested for the induction of estrogen-regulated RNAs. On the whole, the induction was specific for estradiol. The most notable exception was the pNR–8/102 RNA, whose levels were induced maximally by dexamethasone. Dexamethasone reduced pNR–1 and pNR–17 but increased pNR–25 RNA. Dihydrotestosterone and progesterone both slightly increased pNR–1, pNR–2, and pNR–25 RNAs. The regulation by other steroids could be explained either by these steroids acting through the

estrogen receptor or, more probably, by acting through their own receptor to influence the activity of the estrogen or another response element.

Identification of estrogen-regulated sequences

Others have screened cDNA libraries of breast cancer cells for estrogen-regulated mRNAs [11, 12, 13]. Sequencing has shown that the pNR–2 cDNA cloned by ourselves is identical to the pS2 RNA. This mRNA is one of the most abundant estrogen-regulated RNAs in MCF–7 cells, and its expression is exquisitely sensitive to estrogen. We have detected its expression only in estrogen receptor-positive breast cancer cell lines, where its level is stimulated up to a hundredfold. It has been recognized that the encoded protein is related to porcine spasmolytic polypeptide, a peptide that controls gut motility and that it is expressed in human gastric mucosa [14]. Interestingly, its expression does not appear to be under hormonal control in the stomach. The biological function of the pNR–2 protein remains unknown, but it has a high cysteine content, a typical leader sequence, and is secreted from breast cancer cells [15]. It has been speculated that it might have growth factor activity.

Sequencing of the pNR–100 cDNA clones, which corresponded to the most abundant regulated mRNA in the ZR–75 cells, showed that this mRNA encodes the lysosomal aspartyl protease cathepsin D [10]. This protein has previously been identified as an estrogen-regulated secreted protein in MCF–7 breast cancer cells [9, 16]. Estrogen stimulates the synthesis of cathepsin D [17], but the reason why some is misrouted and secreted from breast cancer cells rather than being sequestered in lysosomes is currently unclear. The biological significance of cathepsin D expression in breast tumors is also unclear. Cathepsin D can apparently degrade extracellular matrix [18]; and cathepsin D expression, as assessed by a double-determinant cytosolic assay, has been found in one study [19] to be associated with poor prognosis.

An immunohistochemical study [20] on a series of breast tumors, using an antiserum against mature cathepsin D, has shown, however, that cathepsin D expression is associated with increased time-to-relapse and death. This study would suggest, therefore, that cathepsin D is a marker of good prognosis.

The reasons for the different conclusions of these two studies is not known but may be accounted for by the different methodology used to determine cathepsin D expression. In the first, cathepsin D was measured in tumor extracts, whereas in the second, cathepsin D was assessed immunohistochemically, and therefore, its expression in both tumor cells and inflammatory cells could be analyzed independently. This is of importance because inflammatory cells are frequently present in large numbers and can express high levels of cathepsin D.

Two other regulated RNAs (pNR–8 and pNR–13) correspond to human

homologues of two Drosophila heat shock genes. This is of interest in view of the chaperoning role attributed to heat shock proteins and their association with various classes of steroid receptor.

The other cDNAs do not correspond to sequences currently compiled in nucleic acid or protein databases.

Estrogen and progesterone receptor RNAs are regulated by estradiol

In addition to the estrogen-regulated mRNAs cloned by differential screening, cloned probes for the estrogen and progesterone receptor were provided by Professor P. Chambon (University of Strasbourg, France), and these were hybridized to RNA extracted from withdrawn and estrogen-treated cells.

It has been appreciated for some years that progesterone receptor levels are controlled by estrogens. In MCF–7 cells, estrogen increased progesterone receptor mRNA levels more than a hundredfold within 24 hours. The receptor RNA was also regulated in three other estrogen-responsive cell lines [21]. The antiestrogen tamoxifen was found to be a partial estrogen agonist for the induction of the progesterone receptor mRNA, and the induction by tamoxifen varied from 20% of the estrogen-induced level in T47D cells to less than 1% in EFM–19 cells [21].

The concentrations of estrogen receptor in breast tumors varies over a large range, although the factors that control the expression of the estrogen receptor gene are largely unknown. We observed that when estrogen-responsive cell lines were withdrawn from estrogens, the amount of estrogen receptor mRNA declined slowly [22]. This effect was most marked in the EFM–19 cell line in which the estrogen receptor mRNA levels dropped by about tenfold over an 18 day period. The observation that this decline could be reversed by estradiol treatment and that the reversal was inhibited by antiestrogens suggests that the expression of the estrogen receptor gene is under the control of estradiol in these cells. A similar effect was observed in other cell lines, although the differences between withdrawn and estrogen-treated cells were much less marked.

The observation that the estrogen receptor mRNA can be regulated by estrogens and antiestrogens has clinical implications. It is possible that the effectiveness of antiestrogen therapy may decrease with time, because it actively promotes the genesis of estrogen receptor-negative breast cancer cells by a chronic inhibition of the estrogen receptor expression.

Effects of antiestrogens on expression of estrogen-regulated genes

The generally accepted model of antiestrogen action suggests that antiestrogens inhibit the binding of estrogens to the estrogen receptor within target tissues. This model is conceptually simple and implies that the receptor complexed to an antiestrogen is biologically less active than the receptor

complexed to estrogen. We have measured the biological activity and anti-estrogenic potency of a number of antiestrogens, including tamoxifen and some of its metabolites [17] and ICI 164,384 (a steroidal pure antiestrogen, [23], on a number of estrogen-responsive mRNAs and on cell proliferation.

All these studies were performed using phenol red-free culture medium and charcoal-treated serum. Phenol red is a weak estrogen, and the weak agonist effects of antiestrogens are difficult to measure in its presence. For cell proliferation, tamoxifen had a small stimulatory effect on cell growth, which was approximately tenfold less than that of estradiol. Tamoxifen metabolites, such as N–desmethly tamoxifen and 4–hydroxytamoxifen, also showed slight stimulation of cell growth. The exception was compound E, a metabolite that was first identified in the bile of dogs administered tamoxifen. Compound E was fully estrogenic and maximally stimulated cell proliferation at concentrations as low as 1 nM [17].

Interestingly, the dose response curves for tamoxifen and all the metabolites except compound E were biphasic, with no or inhibitory effects being observed at higher doses. The agonist activity of the antiestrogen was not related to the affinity of the antiestrogen for the receptor, but it was related to the concentration at which agonist activity was observed. Thus the maximal agonist activity was observed at approximately 100 nM for tamoxifen but at approximately 1 nM for 4–hydroxytamoxifen. 4–hydroxytamoxifen has an affinity for the receptor, which is close to that of estradiol.

The agonist activity of tamoxifen and metabolites was measured on the expression of the estrogen-regulated mRNAs. The first important observation was that the effects of tamoxifen were specific for the RNA being measured. The induction of the pNR–2 and pNR–25 RNAs paralleled the effects on cell proliferation in the sense that the levels of these RNAs were increased slightly by concentrations of tamoxifen above 100 nM. In contrast, tamoxifen behaved as a full estrogen agonist for the induction of the pNR–1 RNA at concentrations greater than 100 nM. The magnitude of the induction varied with the MCF–7 subline. In the first subline to be characterized, tamoxifen induced the pNR–1 RNA to approximately 80% of the estrogen-induced level (figure 2) [24], whereas in a second subline tamoxifen increased pNR–1 levels to twice those of estradiol [17]. Tamoxifen was also markedly estrogenic for the induction of cathepsin D mRNA, which showed similar dose response characteristics to the pNR–1 RNA [17].

The potencies of tamoxifen and its metabolites as antiestrogens were examined by measuring their ability to inhibit the induction of the RNAs by estradiol. Three qualitatively different types of effect were observed with tamoxifen. Tamoxifen had little effect on the estrogen-induced level of pNR–1 RNA. For pNR–2 and pNR–25, tamoxifen inhibited the induction by estradiol at concentrations of 1 μM and higher [17, 24].

For cathepsin D mRNA, there was a superinduction of the mRNA levels over a narrow concentration range of tamoxifen that increased the mRNA levels to six to eight times those found in estrogen-stimulated cells [17].

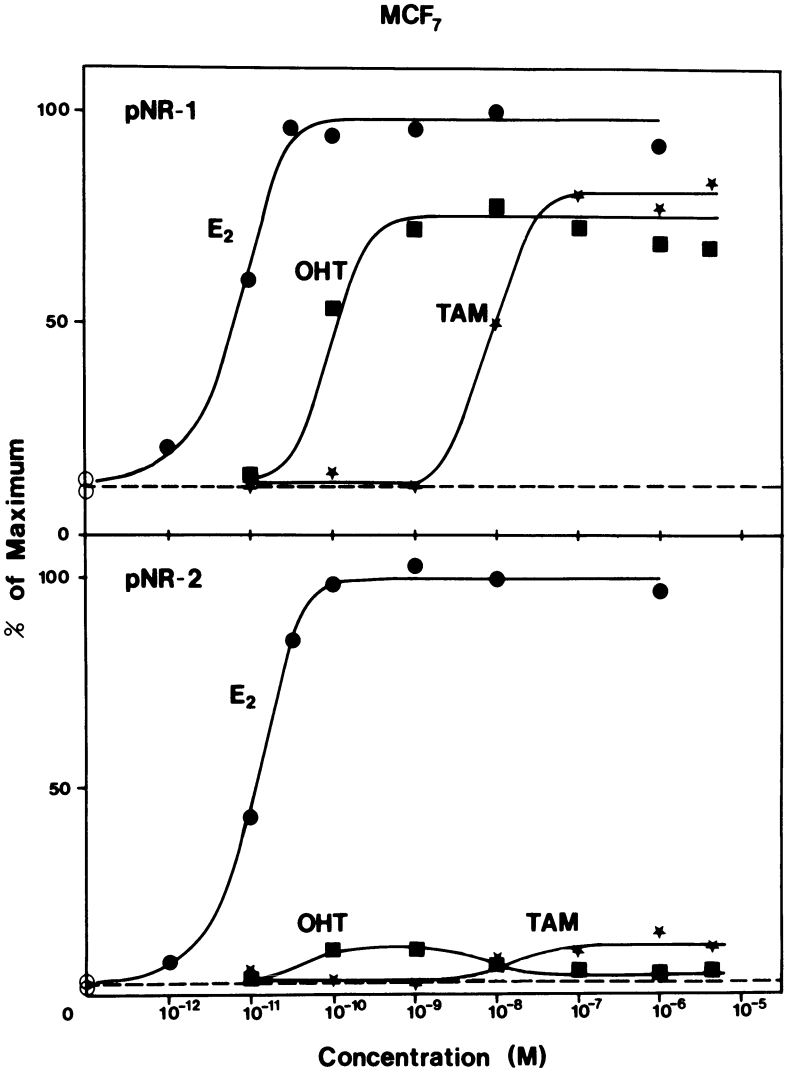

Figure 2. Induction of pNR–1 and pNR–2 RNAs by different concentrations of estradiol (E_2), tamoxifen (TAM), or 4–hydroxytamoxifen (OHT). MCF–7 cells were withdrawn and then treated with the indicated concentrations of estradiol (●–●), tamoxifen (★–★), or 4–hydroxytamoxifen (■–■) for 2 days. Total RNA was prepared, and 2 micrograms separated by gel electrophoresis and transferred to nitrocellulose. The filters were then hybridized with ^{32}P-labeled pNR–1 or pNR–2 RNA probes. The amount of radiolabeled probe hybridized was measured by scanning autoradiographs obtained using preflashed x-ray film exposed at −70°C. The amount of radiolabeled probe hybridized is plotted as a percentage of the maximum value for each RNA.

N–desmethyl–, 4–hydroxy–, and 3–hydroxytamoxifen all had qualitatively similar effects to those of tamoxifen; however, the effects were seen at different concentrations. Thus, 4–hydroxytamoxifen was a more potent inhibitor than was tamoxifen, and the superinduction of cathepsin D mRNA occured at lower concentrations. Compound E had no inhibitory effects on the induction of any of the RNAs.

The analysis of several different responses to antiestrogens has suggested that a simple model of antiestrogen action in which the receptor is inactive when complexed to antiestrogen is an oversimplification. Given that the estrogen receptor is thought to act by binding to regulatory sequences within responsive genes thereby modulating their transcription, these results suggest that the way in which the response elements within genes are able to interpret antiestrogen receptor complexes differs. For instance, the response element of the pNR–1 gene may not be able to discriminate between an estrogen and an antiestrogen receptor complex, whereas for pNR–2 the response element cannot interact with the antiestrogen receptor complex in a way that stimulates gene transcription. It is hoped that the cloning of the control regions of the estrogen-responsive genes should provides more direct evidence for this type of model.

The stimulatory effects of tamoxifen and its metabolites on the growth of breast cancer cells has implications for the treatment of breast cancer with tamoxifen. The major metabolites of tamoxifen that were detected in patients are N–desmethyltamoxifen and 4–hydroxytamoxifen, with intratumor concentrations of approximately 50 ng/mg protein for N–desmethyltamoxifen and 50% and 10% of this concentration for tamoxifen and 4–hydroxytamoxifen, respectively. As these concentrations are inversely related to the relative binding affinities of these compounds for the estrogen receptor, they could all affect the growth of estrogen-responsive tumor cells. In the absence of endogenous estrogen, the relative concentrations would be expected to determine the growth rate of tumor cells, while in the presence of estrogens, growth inhibition could be influenced by the concentration of each compound.

Effects of the steroidal pure antiestrogen ICI 164,384 on the expression of estrogen-regulated mRNAs

The experiments described in the above section clearly show that tamoxifen has significant estrogen agonist activity in MCF–7 cells. A series of 7–alpha derivatives of estradiol have recently been developed by ICI plc., some of which are pure antiestrogens (i.e., have no estrogenic activity) when tested in vivo and on the proliferation of MCF–7 cells [25]. The effects of ICI 164,384 on the expression of six estrogen-regulated mRNAs (including the pNR–1 RNA, which is induced dramatically by tamoxifen) have been measured [23]. Overall, ICI 164,384 did not increase the levels of the estrogen-regulated

mRNAs (including the pNR–1 RNA) and did not increase cell proliferation. When the antiestrogenic potency of ICI 164,384 was measured, it was 50 to 100 times as potent as tamoxifen in inhibiting the induction of the RNAs by estradiol (200 pM). ICI 164,384 also inhibited the induction of the pNR–1 RNA by tamoxifen. These results confirm that antiestrogens, such as ICI 164,384, are potent pure antiestrogens and emphasize the potential value of compounds of this type in the treatment of estrogen-responsive breast cancer.

Expression of estrogen-regulated mRNAs in breast tumors

One important long-term aim of these studies is to identify estrogen-regulated RNAs that might be useful for identifying patients with estrogen-responsive breast cancer that will respond to antiestrogen therapy. The advantage of using recombinant DNA techniques to isolate estrogen-regulated genes is that the cDNA clones can be used directly to monitor gene expression in RNA extracted from tumors. The problem with this prospective approach, however, has been that clinical data takes a long time to accumulate. Thus far the expression of the pNR–2 mRNA has been assessed [26].

RNA was extracted from a series of breast tumors. The levels of estrogen receptor mRNA and pNR–2 RNA were measured by hybridization of radiolabeled RNA probes to Northern Transfers of total RNA. Estrogen receptor mRNA was detected in 90% of primary tumors, whereas the pNR–2 RNA was detected in 57%. The levels of the estrogen receptor and pNR–2 mRNAs in this series of tumors is shown in figure 3. None of the primary tumors that were estrogen receptor negative expressed pNR–2 mRNA, and pNR–2 mRNA was more likely to be present in tumors expressing higher levels of estrogen receptor mRNA. We have shown previously that there is a significant correlation between the levels of the estrogen receptor and its mRNA [27]. There was a significant correlation between the levels of the estrogen receptor mRNA and pNR–2 RNAs for all tumors (Spearman's rank correlation coefficient = 0.42, $p < 0.0001$).

The observation that the pNR–2 protein is expressed only in tumors expressing estrogen receptor mRNA is consistent with the view that this protein might be useful for predicting the response of patients to antiestrogen therapy. The tumors in the above series have been collected since 1986, and there is as yet insufficient clinical follow-up data to determine the predictive value of pNR–2 levels.

In Newcastle, it has been common practice since 1984 to use tamoxifen as first line therapy for elderly patients with primary breast tumors. Assessment of response to tamoxifen is relatively easy in this group of patients, as the size of the primary tumor can be monitored. Estrogen receptor and pNR–2 mRNAs have been measured in a series of 21 such patients who received salvage surgery following disease progression or relapse on tamoxifen. Ten patients had responded to tamoxifen for periods of 9 to 30 months, ten

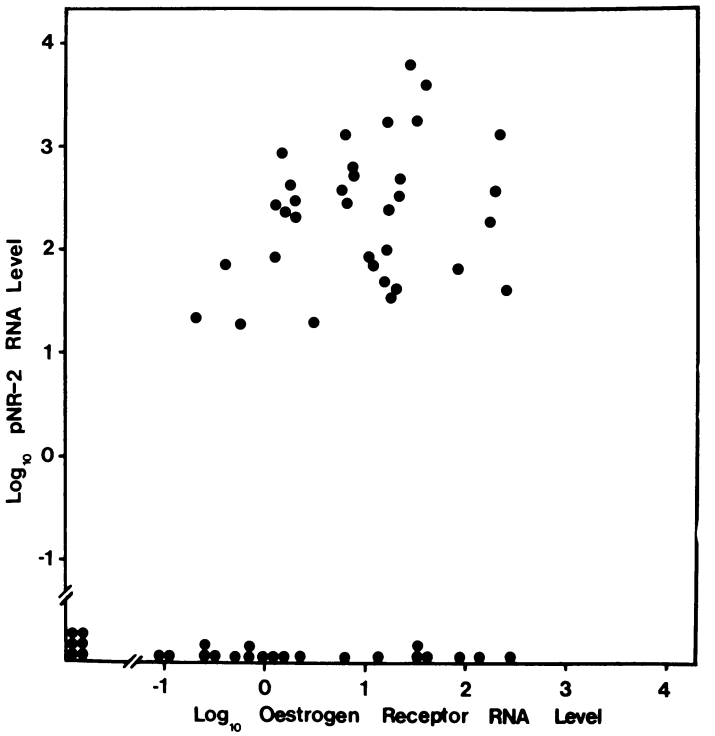

Figure 3. Correlation between estrogen receptor mRNA and pNR–2 RNA levels in primary breast tumors. RNA levels were determined by hybridization of the pNR–2 probe to Northern Transfers of extracted RNA and are plotted on a logarithmic scale. The RNA levels were compared using Spearman's Correlation Coefficient, $r_s = 0.42$, $p < 0.001$, $n = 60$.

patients had not responded, and one patient received surgery despite a continued response to tamoxifen.

A significantly higher proportion of tumors from the 11 patients who had responded expressed pNR–2 (8 of 11) than did tumors from the 10 patients who had not responded (1 of 10, $p < 0.025$). In contrast, estrogen receptor mRNA showed no significant association with response to tamoxifen. Thus pNR–2 mRNA expression was significantly associated with response to tamoxifen. The feasibility of measuring pNR–2 expression in tissue samples obtained at diagnosis using nonsurgical techniques is now being investigated together with retrospective immunohistochemical studies using antisera against the pNR–2 protein.

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13. Estrogen and progesterone receptors

Mary Beth Martin, Miguel Saceda, and Ralph K. Lindsey

The Role of estrogen and progestins in human breast cancer

One of the most prevalent of all cancers, breast cancer, is characterized by hormonal control of its growth. In cell culture, the control of proliferation and differentiation of human breast cancer cells involves steriod hormones, peptide hormones, and growth factors [1–6]; however, epidemiological and clinical findings suggest that the major stimulus for growth of breast cancer is estrogen. Epidemiological studies suggest that endocrine status is an important underlying factor in the prediction of risk. These risk factors include sex, age, age at menarche and menopause, age at first pregnancy, dietary factors, and family history of breast cancer, [7]. In addition to epidemiological studies, clinical observations that support the role of estrogen in the growth of breast cancer are the marked decrease in tumor growth following ovariectomy and remission following treatment with antiestrogens and progestins [7]. Consequently, the estrogen receptor (ER) is used to predict those patients who benefit from hormonal therapy.

Although the presence of estrogen receptor is employed to predict the hormone dependency of a tumor, the response to endocrine therapy is not absolute. Human breast cancers display a considerable heterogeneity in estrogen receptor concentration. Significant levels of estrogen receptor have been detected in more than 60% of human breast cancers, and approximately two thirds of these ER-positive tumors respond to endocrine therapy [8–11]. In addition 5% to 10% of the ER-negative patients respond to therapy [12, 13]. If it is assumed that the estrogen receptor is important for growth, then the presence of an estrogen-regulated protein may reflect the presence of an intact estrogen-responsive pathway. In normal tissue, progesterone receptor (PR) expression is regulated by estrogen [14, 15]. In addition to the presence of the estrogen receptor, the presence of progesterone receptor improves the predictability of hormone dependency of a tumor; however, this relationship is not perfect. Retrospective studies suggest that 70% of PR-positive and 25% to 30% of PR-negative tumors respond to hormone therapy. The reasons for the discrepancies between the level of receptors and their predictive value are not clear but may be attributed to the ability of ER to bind to nuclei in the

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absence of ligand [16], to the ability of ER to bind ligand but not bind to nuclei, or to the synthesis of estrogen-regulated proteins in the absence of a functional ER pathway. Additional assays, such as the binding of the ER to an estrogen-responsvie element (ERE) or the mutational analysis of ER by the polymerase chain reaction, may be necessary to accurately predict tumor response to endocrine therapy.

To fully appreciate the prognostic value of the estrogen and progesterone receptors in breast cancer, a clearer understanding of their mechanism of action is important. Cloning of the estrogen receptor [17–19] and progesterone receptor [20–22] has provided the opportunity to define at the molecular level the mechanism of action of steroid hormones, the structure-function relationship of steroid receptors, the regulation of receptor expression, and the role they play in tumorigenesis.

Mechanisms of hormone action

Steroid hormones enter cells by simple diffusion through the plasma membrane, where they bind to intracellular receptors. It was generally believed that steroid hormone receptors were cytoplasmic proteins; however, recent evidence suggests that this may not be true for all steroid receptors. The estrogen receptor [23] and the progesterone receptor [24] may, in fact, be nuclear proteins. Subsequent to binding, however, the hormone receptor complex undergoes a transformation. At physiological temperatures, the hormone receptor complex is altered or activated such that it acquires an increased affinity for nuclear components. Upon activation, the hormone receptor complex binds in the nucleus to the promoter region of steroid-responsive genes to regulate the level of transcription. In addition to enhancing the level of transcription [for review see 25], it is now known that steroid hormones also suppress the level of gene transcription [26–28].

Prior to hormone binding, steroid receptors in the 8S form are found associated with the 90 kD heat shock protein (hsp 90) [29, 30]. Although other proteins associated with steroid hormones have been identified [31], the hsp 90 is the best studied. It has been suggested that hsp 90 binds to the ligand-binding domain of receptors when the hormone is absent [32, 33]. In addition, it has been hypothesized that hsp 90 blocks the DNA-binding domain of the receptor possibly through the interaction of the negatively charged amino acids of the alpha helical 'region A' and the positively charged amino acids of the second zinc finger of the DNA-binding domain and the carboxyl-terminal of the receptor [34]. This mechanism would prevent the binding of the 8S receptor to DNA. Binding of ligand would induce a conformational change in the hormone-binding domain, resulting in the dissociation of hsp 90.

Steroid hormone receptors represent a class of transacting factors that stimulate transcription by binding to specific DNA elements in the promoter

region, termed hormone response elements (HRE) [for review, see 25]. It has been demonstrated that response elements have the properties of enhancers in that they exert their action in an orientation-independent manner when placed at variable distances upstream from homologous or heterologous promoters. Since the stimulation of gene transcription is strictly dependent upon hormone binding to its cognate receptor, these receptors represent inducible enhancers.

It has become increasingly evident that steroid hormone action also involves posttranscriptional regulation of mRNA stability [35]. The nature of the posttranscriptional event and the role of steroid receptors in the regulation of mRNA stability are not well understood. In the case of Xenopus laevis, where vitelllogenin and albumin are synthesized in the same cell, estrogen stabilizes the mRNA for vitellogenin [35] and destabilizes the mRNA for albumin [36–38]. It has been proposed that a cytoplasmic middle-affinity estrogen-binding protein mediates the stabilization of vitellogenin mRNA [35]. However, a similar mechanism does not appear to be responsible for destabilization of albumin mRNA [36]. In addition to transcriptional and posttranscriptional regulation, there is also evidence to suggest that hormone receptors play a role in the regulation of translation [39].

The structure-function relationship of steroid receptors

Steroid receptors represent a family of closely related genes that include the glucocorticoid, progesterone, androgen, mineralocorticoid, estrogen, thyroid, retinoic acid, and vitamin D receptors [for review see 40]. All receptors in this gene family appear to be structured in a similar way. The estrogen receptor, a 66 kD protein, was originally divided into domains termed A through F, based on regions of sequence homology between the human estrogen receptor and the chicken oviduct estrogen receptors [16]. Region A/B is the variable N–terminal region of the receptor that has a modulatory effect on the transactivation of transcription [16]. Although the N–terminal region appears to play a role in activation of transcription, the mechanism of activation is not well defined. The central domain, region C, is a short well-conserved cysteine-rich region that corresponds to a DNA-binding domain. The cysteine residues in the central domain are capable of forming two zinc fingers [41, 42]. The DNA-binding fingers are formed when the cysteines tetrahedrically coordinate with a zinc ion, allowing the intervening amino acids to form a finger that could interact specifically with DNA. Interestingly, each of the zinc fingers is encoded by separate exons [43]. It appears that one finger alone is unable to bind DNA or activate transcription. The N–terminal zinc finger determines gene specificity as shown by finger swapping experiments [44]. A single amino acid at the root of the finger is responsible for specificity. The N–terminal zinc finger contacts the major groove of one half of the palindrome of the HRE, whereas the C–terminal zinc finger contacts

the sugar phosphate backbone of the flanking sequences. In addition to a role in DNA binding, the N–terminal zinc finger plays a role in transactivation. A mutation of a lysine to a glycine at the root of the finger blocks transactivation but does not effect DNA binding. The most structurally and functionally complex region of the receptor is the C–terminal domain that corresponds to regions D, E, and F. Region E is the hormone-binding domain and it is mainly hydrophobic. It has been proposed that it may form a hydrophobic pocket for the estradiol ligand. In addition to binding hormone, region E also appears to contain an estradiol-inducible transcription activation function [45]. The original function of region D was believed to be a hinge between the hormone- and the DNA-binding domains [45]. In the absence of steroid, the DNA-binding domain is masked by the hormone-binding domain. Binding of hormone relieves this putative masking effects. Recently it has been suggested that region D also mediates the inhibitory effects of the estrogen receptor on gene transcription. The C–terminal region also plays a role in the hormone-induced dimerization of the receptor [46]. In addition, a weak constitutive dimerization domain is found in the DNA-binding domain.

Steroid receptors can be classified based on the HRE to which they bind. The estrogen receptor binds to a palindromic 13–mer consensus sequence, GGTCANNNTGACC [40]. The consensus sequence for the thyroid and retinoic acid receptors is similar to the consensus sequence for the estrogen receptor but with a different internal spacing. Although the thyroid receptor binds to the estrogen receptor element (ERE), it does not activate transcription. In the case of the glucocorticoid receptor, the consensus sequence is a palindromic 15–mer, GGTACANNNTGTCT [40], which also binds and is activated by the progesterone, androgen, and mineralocorticoid receptors. Although the same sequence can mediate responses to different hormones, the interactions are not identical. Glucocorticoid receptor and progesterone receptor show similar but different patterns of binding [47–49]. Mutations of the sequences in the HRE give different responses to glucocorticoids and progestins.

It is becoming increasingly evident that hormone receptors can act as transcriptional repressors as well as transcriptional activators. Several mechanisms have been suggested for transcriptional repression [50]. It has been proposed that steroid hormones may function as repressors by competing with upstream activating factors or general transcription factors for binding to DNA, by binding to adjacent nonoverlapping DNA sequences to prevent transcription factors from interacting with the transcription complex, or by binding to negative hormone-responsive element such that the bound hormone receptor is inactive. In addition, overproduction of hormone receptor could result in the sequestration of other transcription factors, resulting in the suppression of transcription. Prolactin expression is repressed by a glucocorticoid receptor binding to a negative glucocorticoid-responsive element (nGRE) [26]. The nGRE has some sequence homology to the GRE, but there is no apparent consensus sequence. Because the nGRE also binds

positive transcription factors, it has been suggested that glucocorticoids repress transcription of prolactin by competing with these factors for binding to DNA or by binding to adjacent, nonoverlapping DNA sequences to prevent the interaction of factors with the transcription complex. However, these results do not rule out the possibility that when bound to an nGRE, the glucocorticoid receptor is inactive. In the case of human glycohormone alpha subunit, the glucocorticoid receptor mediates repression by competing for DNA binding [27]. The promoter region of the glycohormone alpha subunit contains two glucocorticoid receptor-binding sites that overlap two cAMP response elements. Repression by glucocorticoids occurs only when the cAMP response elements are active.

Regulation of estrogen and progesterone receptor expression

Expression of the ER in human breast cancer appears to be a complex process involving multiple steps subject to hormonal regulation by estrogen. In addition to ER, autoregulation of hormone receptor concentration by the homologous ligand has been demonstrated for steroid hormones, such as progestins [22, 51], glucocorticoids [52–54], and thyroid hormones [55].

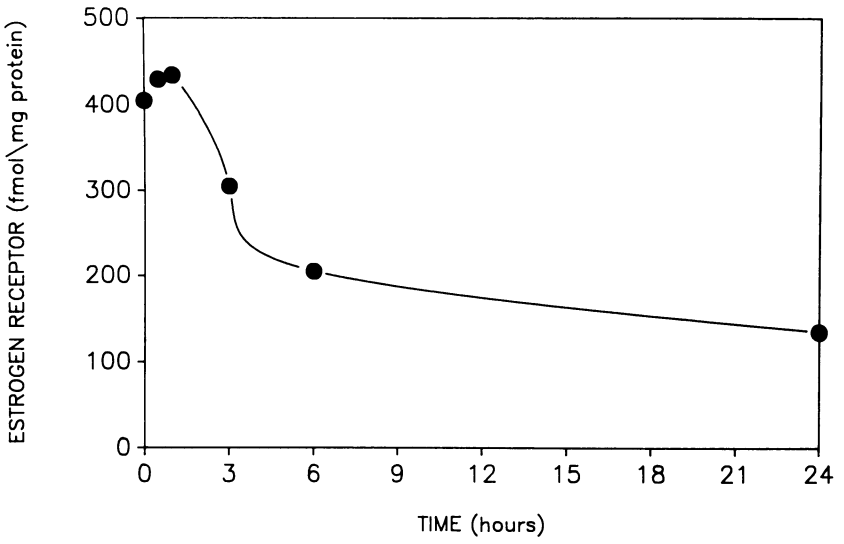

Figure 1. Effect of estrogen on the steady-state level of ER protein. MCF-7 cells were grown in IMEM medium supplemented with 5% charcoal-treated calf serum. At approximately 80% confluence, the medium was replaced with phenol red-free IMEM containing 5% charcoal-treated calf serum. After 2 days, cells were treated with estradiol, 10^{-9} M, or ethanol for various times. Cells were washed, harvested, and homogenized by sonication. Total estrogen receptor was determined with an EIA kit from Abbott Laboratories, using D547 and H222 monoclonal antibodies. Results are presented as femtomoles of ER per mg protein. Each point is the mean of several experiments.

However, the mechanisms of regulation are not understood. Recent cloning of steroid receptors has made it possible to investigate the molecular mechanism underlying regulation. Previously it had been shown that treatment of MCF–7 breast cancer cells with estrogen results in a down regulation of estrogen-binding sites [56–59]. Our studies indicate that transcriptional as well as posttranscriptional events contribute to the suppression of receptor expression by estrogen [60]. However, posttranscriptional regulation appears to be the predominant mechanism suppressing receptor expression.

To define the molecular mechanism of regulation of ER expression, we have measured simultaneously the effects of estradiol on the relationship between ER protein concentration and binding capacity, the steady-state levels of receptor mRNA, and the level of ER gene transcription [60]. Treatment of MCF–7 cells with estrogen resulted in the suppression of the ER (figure 1). The level of binding also decreased in a manner similar to the decline in receptor protein [data not shown]. The decline in receptor protein to a new steady-state level accompanied a parallel decrease in the level of receptor mRNA (figure 2). This result has been verified in several laboratories [61–63]. In contrast to the effect on protein and mRNA, estrogen treatment resulted in a transient decrease in ER gene transcription followed by an enhanced level of expression (figure 3). Although estrogen treatment resulted in a transient decrease in ER gene transcription, it is improbable that this decrease is responsible for the suppression of receptor mRNA. It suggests

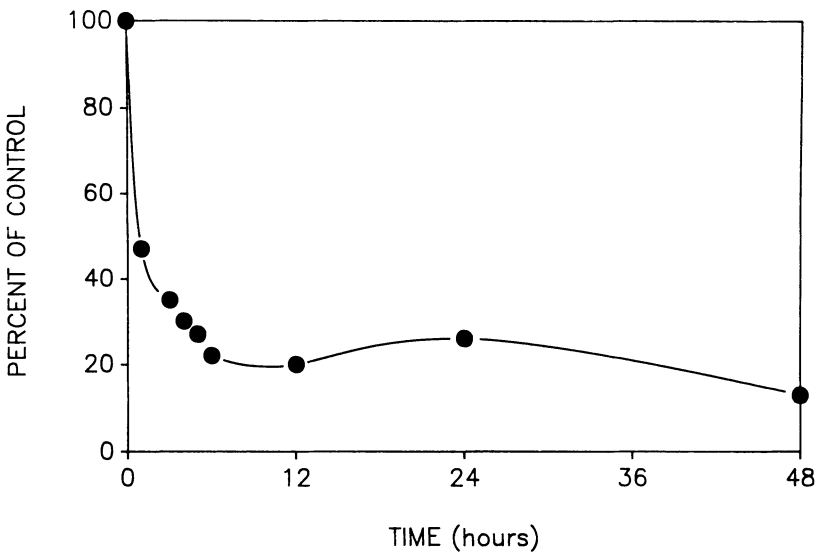

Figure 2. Effect of estrogen on the steady-state level of ER mRNA. Autoradiographs from the RNase protection assay were quantified by scanning densitometry, and the values were expressed as the ratio of the integrated ER signal divided by the integrated 36B4 signal. The results are presented as percent of control. The points represent the average of a minimum of three values and in some cases as many as ten values.

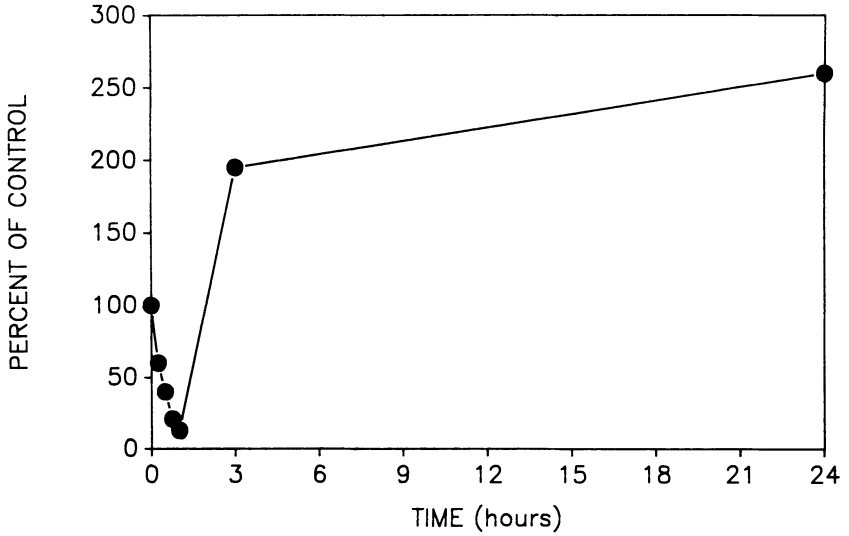

Figure 3. Effect of estrogen on ER gene transcription. MCF–7 cells were treated as described in the legend to figure 1. Nuclei were isolated at the indicated time points by homogenization in 1.5 M sucrose buffer containing 0.1% Brij 58; elongation of nascent transcripts was performed in a reaction buffer containing 32p–UTP. For detection of specific transcripts, the newly synthesized transcripts were isolated and hybridized to filters containing an excess of plasmid DNA. The level of transcription was determined by autoradiography and quantified by scanning densitometry. The level of transcription was expressed as the ratio of the integrated ER signal divided by the integrated 36B4 signal. The results are presented as percent of control.

that the predominant mechanism regulating ER expression is a posttranscriptional event. The mechanism of autoregulation of the estrogen receptor by estrogen appears to be different than the mechanism of autoregulation of the glucocorticoid receptor by dexamethasone. Suppression of the glucocorticoid receptor expression is determined by both transcriptional and posttranscriptional processes [64].

It previously had been proposed that nuclear processing was responsible for the new steady-state level of ER seen in MCF–7 cellls after estrogen administration. Processing of ER complexes was measured as a decrease in competible ^{3}H–estradiol binding in 0.6 M KCl nuclear extracts [56]. The new steady-state level of ER may be due to one of several factors, including a decreased half life of receptor or a decreased synthesis of receptor protein. However, previous studies report a rapid turnover of ER with a half life of 2.5 to 4.5 hours in the presence or absence of ligand [65, 66]. In addition, the more recent data that show that estrogen results in the suppression of ER mRNA suggest that decreased synthesis of receptor protein plays an important role in the loss of estrogen receptors. Although the more recent data do not discount nuclear processing as an early event in ER regulation, they do suggest that the new steady-state level of receptor is largely determined by suppression of ER mRNA.

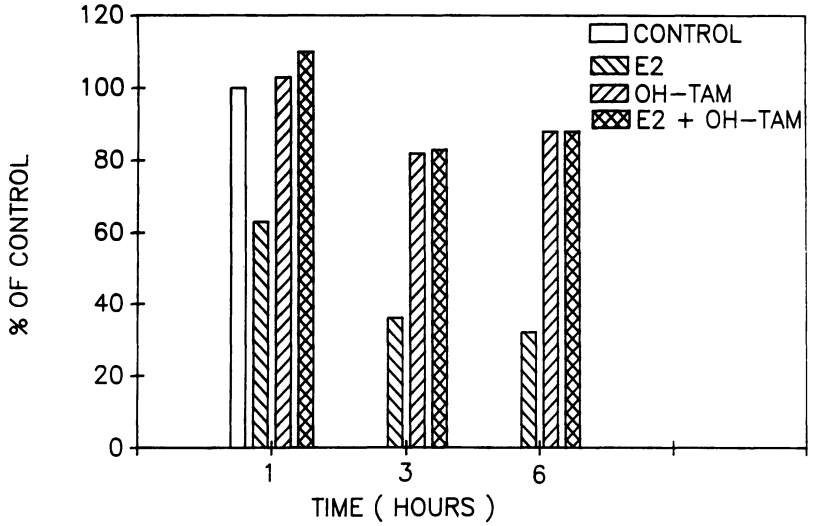

Figure 4. Effect of estradiol and 4–hydroxytamoxifen on the level of ER mRNA. Autoradiographs from the RNase protection assay were quantified by scanning densitometry and the values were expressed as the ratio of the integrated estrogen receptor signal divided by the integrated 36B4 signal. The results are presented as percent of control. Values are the mean of at least four experiments.

Although steroid hormone action has been shown to involve posttranscriptional regulation of mRNA stability, the nature of the posttranscriptional event and the role of steroid receptors are not well understood. To determine whether posttranscriptional regulation of ER gene expression is mediated by a receptor-dependent mechanism independent of protein synthesis, the competitive estrogen antagonist 4–hydoxytamoxifen and the inhibitor of protein synthesis cycloheximide were employed to study the posttranscriptional regulation of ER mRNA by estradiol [67]. Alone 4–hydroxytamoxifen had no effect on the level of ER mRNA in MCF–7 cells but effectively blocked the suppression of ER mRNA by estradiol (figure 4). Because estradiol treatment results in a maximum suppression of ER mRNA by six hours, the time of onset of maximum suppression suggests that estradiol may induce a new gene product that mediates the posttranscriptional regualtion of receptor mRNA. However, cycloheximide does not block the effect of estradiol on ER mRNA, suggesting that the effect of estradiol on ER mRNA level is independent of portein synthesis (figure 5). These data suggest that the effect of estradiol on receptor mRNA is a primary effect of the ER; however, other possibilities cannot be ruled out, such as that the estrogen receptor-mediated effect is through the induction of an RNA species. Studies are in progress to determine if the posttranscriptional suppression of ER mRNA is mediated through the induction of specific RNAs.

To identify the site of posttranscriptional regulation of ER mRNA, nuclear and cytoplasmic RNA from MCF–7 cells was isolated, and the level of ER

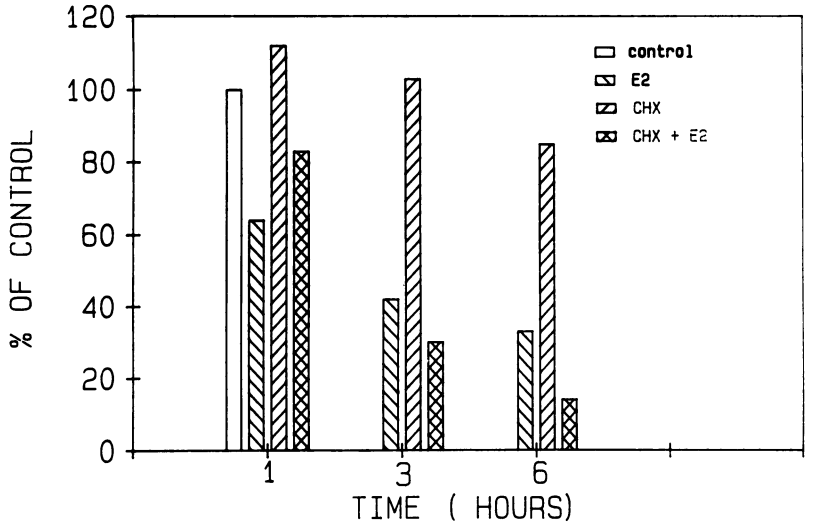

Figure 5. Effect of estradiol and cycloheximide on the level of ER mRNA. Autoradiographs from the RNase protection assay were quantified as in figure 2. Values are the mean of at least four experiments.

mRNA was measured. Treatment of cells with estradiol results in suppression of both nuclear (figure 6) and cytoplasmic (figure 7) levels of ER mRNA. Although the decrease in the cytoplasmic level of ER mRNA was greater than the decrease in the nuclear level of ER mRNA, the data suggest that the posttranscriptional suppression of ER mRNA is a nuclear event. It is possible that estradiol alters the manner in which nascent ER transcripts are processed or transported from the nucleus. In addition to processing of nuclear RNA and export from the nucleus, mRNA degradation may also be a site of differential hormonal regulation. Stability of mRNA is recognized as an important posttranscriptional mechanism for altering cytoplasmic mRNA levels. Although the data suggest that suppression of ER mRNA by estrogen is a nuclear event, cytoplasmic stability cannot be ruled out. Steroid hormone receptors have been reported to form complexes with RNA [68–70]. Although the data provide evidence that the posttranscriptional regulation of ER mRNA by estradiol is mediated by the classical estrogen receptor, independent of protein synthesis, the exact role of ER in posttranscriptional regulation remains to be defined. The effect of estradiol on ER mRNA stability is currently under investigation in this laboratory.

In addition to estrogen and 4–hydroxytamoxifen, we have investigated the role of the antiestrogen, N–butyl–N–methyl (10–trien–7–yl0) undecanamide (ICI 164,384) in the regulation of the ER in MCF–7 cells. ICI 164,384 is a new steroidal antiestrogen, which exhibits no estrogenic effects on uterine growth in immature rats and mice and reverses estrogen-induced proliferation of cultured MCF–7 cells [71–73]. Treatment of cells with ICI 164,384, 10^{-7}

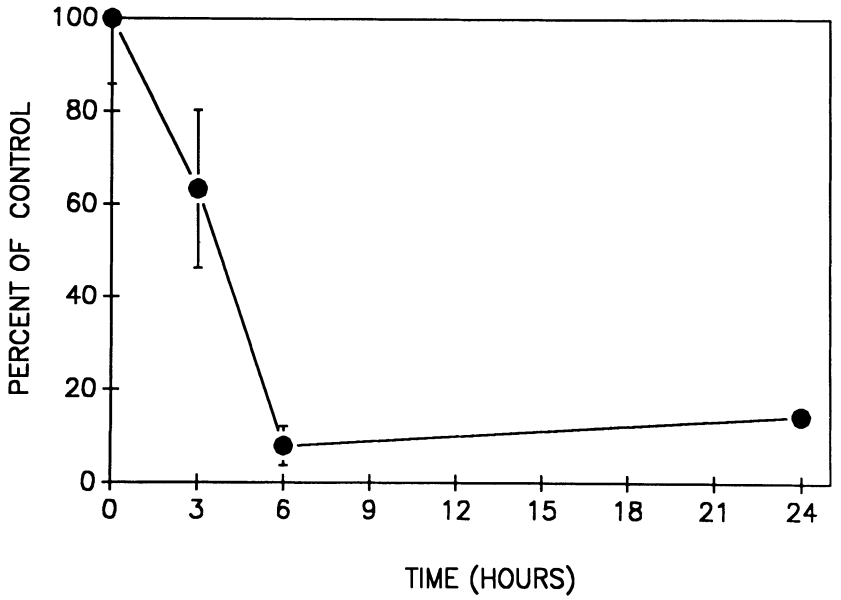

Figure 6. Effect of estradiol on the levels of nuclear ER mRNA. Autoradiographs from the RNase protection assay were quantified as in figure 2. Values are the mean of at least three experiments.

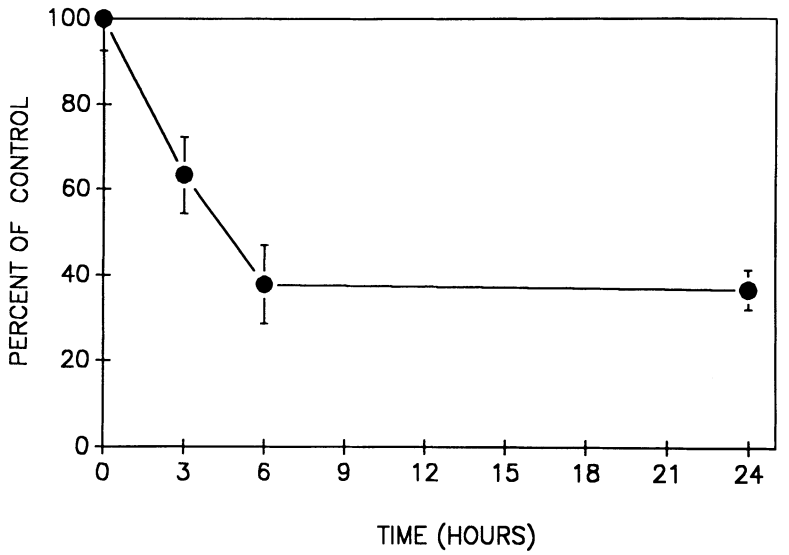

Figure 7. Effect of estradiol on the level of cytoplasmic ER mRNA. Autoradiographs were quantified as described in figure 2. Values are the mean of at least three experiments.

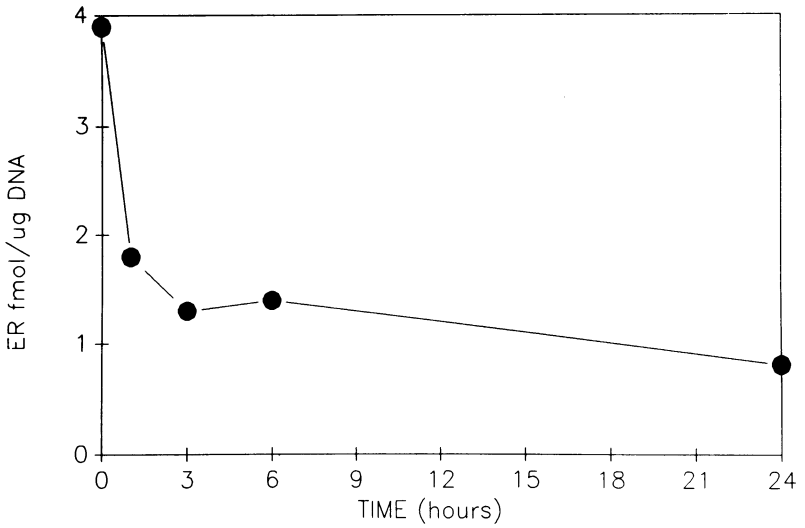

Figure 8. Effect of ICI 164,384 on the steady-state level of ER protein. MCF–7 cells were grown and total estrogen receptor was determined as described in figure 1. Cells were treated with ICI 164,384, 10^{-7} M, or ethanol for various times. Results are presented as femtomoles of ER per μg DNA. Each point is the mean of several experiments.

M, resulted in a decline in total receptor protein by about 60%. The level of receptor decreased as early as 1 hour and remained depressed for up to 48 hours (figure 8). These data are in agreement with the effects of estrogen, except that the effect of ICI 164,384 was much more rapid. To confirm that the rapid decrease in the level of ER protein was not an artifact of the enzymeimmunoassay [Abbott Laboratories], the effect of ICI 164,384 on the assay was tested. When ICI 164,384 was added to the assay, the level of receptor was measured as 608.8 fmol/mg protein compared to 640 fmol/mg protein in controls, suggesting that the compound had no significant effect on the sensitivity or accuracy of the assay [data not shown]. Although ICI 164,384 treatment resulted in the suppression of ER protein, the steady-state level of ER mRNA remained constant under these conditions [data not shown]. In contrast to the effects of estradiol, these data suggest that the ICI compound suppresses receptor gene expression by a translational or posttranscriptional mechanism. Previous studies with estrogens and other antiestrogens suggest that the bound ligand does not significantly effect the stability of the estrogen receptor [65, 66]. In these studies, the half life of the estrogen receptor was determined as approximately 2.5 to 4.5 hours. Based on these studies, ICI 164,384 is not expected to significantly effect the stability of the estrogen receptor. At present the nature of the translational/posttranslational regulation of estrogen receptor expression is under investigation.

Although estrogen appears to suppress ER expression in MCF–7 cells, this

does not appear to true under all culture conditions of MCF–7 cells [74, 75] or for all breast cancer cell lines [61, 62]. In the breast cancer cell line T47D, estrogen treatment has been shown either to have no effect on ER [61] or to result in a 2.5-fold increase in ER mRNA [62]. Interestingly, in the rat, estrogen differentially regulates ER expression in a tissue-specific manner [76]. In the rat uterus following ovariectomy, there is a threefold to sixfold increase in ER mRNA; subsequent administration of estradiol results in the return of ER mRNA to intact levels, suggesting that estrogen negatively regulated ER expression in the uterus. Ovariectomy has the opposite effect on ER expression in the liver and pituitary; there is a 1.5- to 3-fold decrease in liver ER mRNA and a 3-fold decrease in pituitary ER mRNA. The level of ER mRNA returns to the intact level following administration of estradiol, suggesting that estrogen positively regulates ER expression in liver and pituitary. In contrast to the effect on rat liver ER mRNA, ovariectomy has the opposite effect on steroid-binding capacity in the liver. Following ovariectomy, there is at least a 150% increase in the level of steroid binding [77, 78]. It is not clear why there is an increase in estrogen-specific binding sites and a decrease in ER mRNA in the liver; however, these findings suggest that estrogen may modulate translational or posttranscriptional processing of the estrogen receptor in the liver. The mechanisms for tissue-specific regulation of ER expression are not understood.

In addition to estrogen receptor, autoregulation of progesterone receptor (PR) by progestins has been demonstrated in the breast cancer cell lines MCF–7 [51] and T47D [22, 51, 79]. Treatment of breast cancer cells with progestins results in a suppression of PR expression. The decline in receptor protein accompanies a parallel decrease in the steady-state level of progesterone receptor mRNA and a decrease in the level of PR gene transcription. In contrast to the effects of estrogen on ER expression, progestins have no effect on PR mRNA half life but mediate a decrease in progesterone receptor protein half life [79]. It appears that the predominant mechanisms regulating PR expression are transcriptional and posttranslational events.

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14. Growth factors as mediators of estrogen/antiestrogen action in human breast cancer cells

Carlos L. Arteaga and C. Kent Osborne

It is generally accepted that the induction and maintenance of breast cancer is, at some point and to a variable degree, under control by estrogen. However, the mechanisms by which estrogen regulates the proliferation of human breast cancer cells remain speculative. Recently it has been reported that breast cancer cells can synthesize and secrete a number of polypeptide growth factors or growth factor-like activities, including transforming growth factors alpha and beta (TGF–alpha and TGF–beta), the insulin-like growth factors I and II (IGF–I and IGF–II), platelet derived growth factor (PDGF), and the protease cathepsin D. For some of these growth factors, specific cell surface receptors have been identified in breast cancer cells. Furthermore, addition of exogenous growth factors to these cells under serum-free conditions induces growth stimulation (TGF–alpha, IGF–I, IGF–II, and cathepsin D) or growth inhibition (TGF–beta). Interestingly, the synthesis and secretion of these growth factors are regulated by estrogens and antiestrogens in some estrogen receptor (ER)-positive hormone-dependent cells, whereas higher levels are expressed constitutively in some ER-negative hormone-independent cells. These data have led to the hypothesis that estrogens and antiestrogens regulate breast cancer progression and tumorigenicity indirectly by inducing the expression and secretion of polypeptide growth stimulators or inhibitors, which then bind and act on breast cancer cells in an autocrine fashion [1]. On the other hand, the growth advantage associated with hormone-independent breast cancer cells may be due to the high constitutive expression of autocrine growth factors.

Despite a number of published studies, confirmation of the autocrine hypothesis in human breast cancer cell systems is still elusive. In the following section we will summarize data from our laboratories that examine the interaction of some of these polypeptide growth factors with hormone-dependent and -independent human breast cancer cells. We will also present and discuss studies that suggest a potential diagnostic or prognostic utility for some of these secreted growth factor activities in clinical breast cancer.

M. Lippman and R. Dickson (eds.), REGULATORY MECHANISMS IN BREAST CANCER.

Transforming growth factor–alpha as a mediator of estrogen-induced growth

TGF–alpha is a 6 Kd secreted polypeptide originally discovered in medium of certain retrovirus-transformed fibroblasts [2]. It binds to the epidermal growth factor (EGF) receptor and derives its name from the observation that it reversibly transforms immortalized normal rat kidney (NRK) fibroblasts in vitro [3]. Induction of a high level of expression of TGF–alpha in immortalized Rat–1 and NRK fibroblasts [4, 5] confers tumorigenicity in vivo in an autocrine fashion.

Using specific cDNA probes, mRNA transcripts for TGF–alpha have been identified in extracts of breast cancer cell lines as well as in breast tumor tissue [6, 7]. Biologically active or immunoreactive TGF–alpha activity with a molecular weight of 30 Kd has been found in conditioned medium from these cells [8–10]. Physiological doses of estradiol increase TGF–alpha mRNA expression [7] and secretion [9, 10] in ER-positive hormone-responsive breast cancer cells whereas antiestrogens inhibit TGF–alpha expression. Secretion of TGF–alpha is higher, and it is independent of hormonal control in the ER-negative MDA–231 cell line [9, 10], but it is unknown if TGF–alpha high constitutive secretion is a hallmark of ER-negative human breast cancer, in general.

Evidence that TGF–alpha-like peptides may mediate estrogen-induced tumorigenicity of breast cancer cells was reported in 1986 by Dickson et al. [11], who utilized the ER-positive hormone-dependent MCF–7 cell line as a model. In these experiments, serum-free conditioned medium from estrogen-primed MCF–7 cells was able to partially and transiently support the growth of these cells in ovariectomized nude mice in the absence of estrogen supplementation. Infusion of EGF also stimulated MCF–7 tumor formation in vivo in the absence of exogenous estradiol [11]. This same MCF–7 cell line, when transfected with the v–Ha–*ras* oncogene, secretes fourfold higher levels of TGF–alpha and becomes tumorigenic in vivo in the absence of estrogen supplementation [12, 13]. These data suggest the following: (1) that secreted TGF–alpha may be a mediator of the mitogenic effect of estrogen in hormone-dependent breast cancer cells and (2) that an increase in TGF–alpha secretion may contribute to the escape of these cells from estrogen dependence.

We have examined whether secreted TGF–alpha is an important estrogen-regulated autocrine growth factor for hormone-responsive human breast cancer cells in vitro, by utilizing antibodies that block the EGF/TGF–alpha receptor. We hypothesized that if secreted TGF–alpha mediates estrogen-induced growth, then blockade of the EGF receptor should antagonize estrogenic stimulation. We utilized the anti-EGF receptor mouse monoclonal antibodies 528 and 225 [14] and the rabbit polyclonal antibody 451 [15]. All of these inhibit EGF receptor binding and antagonize the EGF-stimulated tyrosine kinase activity associated with the EGF/TGF–alpha receptor [14, 15]. Although antibody-induced blockade of the EGF receptor (with each of

Table 1. Effect of EGF receptor blockade on TGF–alpha-induced and estrogen-induced anchorage-independent growth of MCF–7 cells.

	Colonies		Colonies
Control	25 ± 4	Control	45 ± 6
225ab (100 nM)	21 ± 6	451ab (1:500)	42 ± 8
TGF–alpha (2 nM)	93 ± 6	TGF–alpha (2 nM)	136 ± 15
TGF–alpha + 225ab	31 ± 6	TGF–alpha + 451ab	65 ± 11
Estradiol (1 nM)	288 ± 19	Estradiol (1 nM)	341 ± 29
Estradiol + 225ab	322 ± 13	Estradiol + 451ab	346 ± 27

MCF–7 cells (5×10^3 cells per Petri dish) were cloned in 0.8% agarose, phenol red-free IMEM with 5% CSCS, 10 nM insulin, 10 mM Hepes supplemented with the hormones and/or antibody mentioned in the table. After 10 to 14 days, colonies measuring ≥50 μ were counted using a Bausch and Laumb Omnicon Feature Analysis Stem Model II image analyzer. Values represent mean number of colonies ± SE of triplicate determinations.

the three antibodies) inhibited TGF–alpha-induced growth of MCF–7 cells, it had no effect on growth stimulation by estradiol, suggesting that secreted TGF–alpha is not a major mediator of the mitogenic effect of estrogen in these cells [16].

Table 1 shows the effect of EGF receptor blockade on estrogen-stimulated growth of MCF–7 cells. The concentrations of antibody utilized inhibit more than 95% of the binding of 60 pM 125–I–TGF–alpha to these cells [not shown]. To avoid the presence of estrogen or related substances, these experiments were done in phenol red-free medium supplemented with charcoal-stripped calf serum (CSCS). TGF–alpha and estradiol induced a marked increase in colony formation. Both antireceptor antibodies blocked TGF–alpha-induced but not estrogen-induced clonogenicity.

A similar experiment was done with the ER-positive ZR–75–1 and T47D human breast cancer cells but now using [^{3}H]thymidine incorporation 24 hours after the addition of either hormone as an indicator of growth. As illustrated in figure 1, similar results were observed. Thus, not only late estrogen-induced changes (clonogenicity) but also an early mitogenic response (DNA synthesis) in response to exogenous estradiol is unaffected by EGF/TGF–alpha receptor blockade. Taken together these data support the conclusion that secreted TGF–alpha is not a primary mediator of the growth effects of estrogen in hormone-dependent breast cancer cells. One cannot conclude from these data, however, that TGF–alpha has absolutely no role on estrogen-regulated growth of these cells. It is conceivable that breast cancer cell growth regulation by hormones involves a very complex interaction of several stimulatory and inhibitory secreted factors. Inhibition of only one of these autocrine loops might be inadequate to significantly alter estrogen-induced growth. However, in preliminary experiments we have been unable to block estrogen-stimulated growth of MCF–7 cells in vitro using the EGF receptor antibody 225 simultaneously with a monoclonal antibody against the IGF–I receptor. This receptor mediates the mitogenic effects of IGF–I and IGF–II, another potential estrogen-regulated autocrine

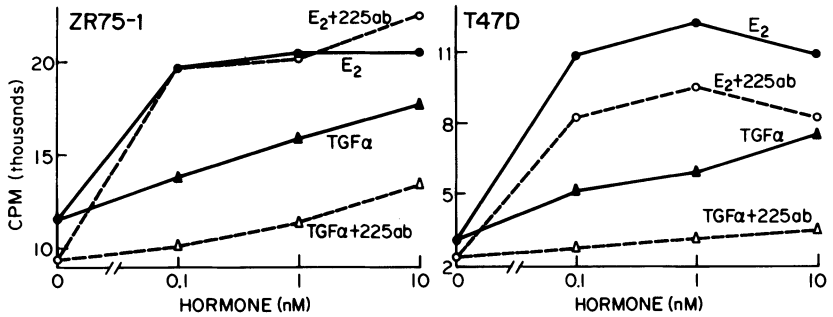

Figure 1. Effect of EGF receptor blockade on TGF–alpha-induced and estrogen-induced DNA synthesis in ZR75–1 and T47D cells. Cells were plated in 24-well plates in their regular growth medium (3×10^4 cells per well). Twenty-four hours later the medium was changed to phenol red-free IMEM with 5% CSCS. After 24 hours, 17–beta–estradiol or TGF–alpha with (○, △) or without (●, ▲) the anti-EGF receptor antibody 225 (100 nM) were added to the wells. Incorporation of [^{3}H]thymidine into acid-precipitable material was estimated 18 hours after the addition of either hormone with or without antibody as described [16]. Data shown represent means of triplicate determinations. SE were less than 10%.

growth factor in these cells [CL Arteaga and CK Osborne, unpublished data].

The previous conclusion is consistent with a recent study reported by Clarke et al. [17] in which MCF–7 cells were transfected with plasmids containing a TGF–alpha cDNA fragment coding for the entire TGF–alpha precursor peptide. The transfected cells secreted very high levels of biologically active TGF–alpha, but they still required estrogen supplementation in order to form tumors in ovariectomized nude mice. Thus the induction of high levels of secreted TGF–alpha cannot replace the in vivo effects of estrogen.

The question of whether estrogen-regulated secreted growth factors, such as TGF–alpha, can mediate estrogen-induced growth was addressed in a different way by Osborne et al. [18]. The ER-negative MDA–231 cell line was used as a source of growth factors in an attempt to stimulate in vivo growth of MCF–7 cells in an estrogen-depleted nude mouse. The MDA–231 cells secrete high constitutive levels of TGF–alpha and IGF–I-like activities into conditioned medium [9, 19]. Results from this experiment are summarized in table 2. When inoculated into athymic nude mice, the MDA–231 cells rapidly formed tumors with or without estrogen supplementation. In contrast, control mice injected with only MCF–7 cells did not form tumors unless supplemented with an estradiol pellet. When MCF–7 cells were inoculated on the flank opposite to the MDA–231 cells, either simultaneously or after large MDA–231 tumors had formed, they did not form tumors unless the mice were supplemented with estrogen. Thus growth factors secreted by the MDA–231 tumors were unable to replace estrogen for growth of MCF–7 cells inoculated at a distant site.

To determine if growth factors from MDA–231 cells could support MCF–7

Table 2. MCF–7 tumor growth stimulation by MDA–231 cells inoculated at a distant site.

Group	MDA–231	MCF–7
Control, estrogen –	7/7[a]	0/7
Control, estrogen +	7/7	7/7
Simultaneous bilateral inoculation		
estrogen –	7/7	0/7
estrogen +	7/7	7/7
Sequential bilateral inoculation		
estrogen –	10/10	0/10
estrogen +	10/10	10/10

[a]Tumors formed/total mice inoculated.
MDA–231 cells (5×10^6 cells per mouse) were inoculated subcutaneously into the flank of 4-week-old castrated female nude mice. A similar size inoculum of MCF–7 cells was injected into the opposite flank either simultaneously or 2 weeks after (sequential) MDA–231 tumor formation (approximately 1 cm in maximum diameter). Some mice were supplemented with a 0.25 mg subcutaneous estradiol pellet. Control mice were inoculated with either MDA–231 or MCF–7 cells only. Adapted from Osborne et al. [18].

tumor growth by paracrine mechanisms, the two lines were mixed together and injected into a single site in estrogen-supplemented or castrated mice. Analysis of estrogen receptor status and DNA content by flow cytometry of the resulting xenografts in mice supplemented with estradiol revealed that both cell types, MDA–231 and MCF–7, were present. On the contrary, the resulting tumors in castrated mice contained predominantly MDA–231 cells, indicating that TGF–alpha and other growth factor activities released by these hormone-independent cells were unable to support growth of adjacent MCF–7 cells in the absence of systemic estrogens.

The cumulative data from experimental models summarized above fail to support an essential role of TGF–alpha in estrogen-regulated breast cancer cell proliferation or tumorigenicity. However, they do not exclude the possibility that TGF–alpha could modulate tumor growth or progression in clinical breast cancer in an autocrine/paracrine fashion. Furthermore, since this protein is known to have several other effects in vivo in addition to its effects on cell proliferation [20], it is likely that future experiments will define additional biological roles for TGF–alpha in breast cancer.

Transforming growth factor–alpha as a potential tumor marker

If breast cancer cells are capable of secreting growth factors in vivo, it is plausible that detectable growth factor activities in body fluids could be used as tumor markers. Moreover, if these putative autocrine loops are operative in vivo, one would expect those tumors secreting higher levels of growth factors to have a proliferative advantage and thus a worse prognosis.

Supporting this possibility is the finding by Stromberg et al. [21] of

immunoreactive TGF–alpha in pooled urine from three patients with extensive metastatic breast cancer. Pooled urine from control women did not contain measurable quantities of this growth factor. In another study, urinary levels of immunoreactive TGF–alpha were significantly higher in patients with hepatocellular carcinoma than in urine from sex-matched healthy controls [22].

We have measured immunoreactive TGF–alpha in 130 effusions, pleural or ascitic, from patients with different types of cancer. Fifty-five of 130 effusions (42%) contained a detectable level of TGF–alpha, whereas only 3 of 17 (18%) control effusions from noncancer patients had a low level of growth factor activity present ($p = .015$) [23]. In this study the presence of immunoreactive TGF–alpha in the effusions correlated with tumor cell clonogenicity in soft agar, with high tumor burden, and with poor patient outcome. Of 34 specimens from breast cancer patients, 13 (38%) contained TGF–alpha. When we correlated these findings with several established clinical parameters of breast cancer aggressiveness, we found that a significantly larger percentage of TGF–alpha-positive samples were from patients with a poor performance status, with a high tumor burden as estimated by the number of metastatic sites, with premenopausal status, and with hormone receptor-negative tumors (figure 2). Not unexpectedly, the presence of

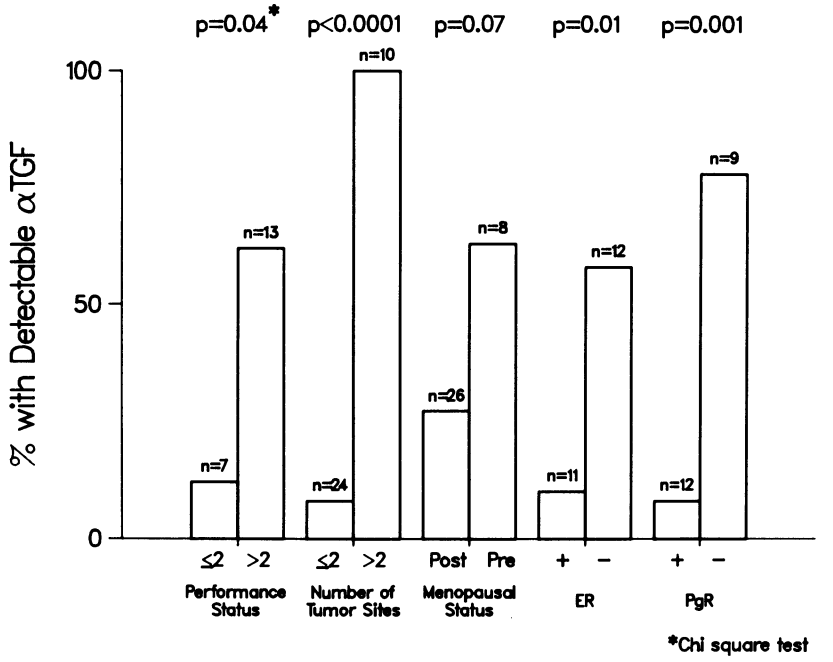

Figure 2. Percentage of breast cancer patients with detectable TGF–alpha immunoreactivity in either pleural or ascitic effusions, as a function of several clinical and biochemical prognostic parameters. (Reproduced with permission [23].)

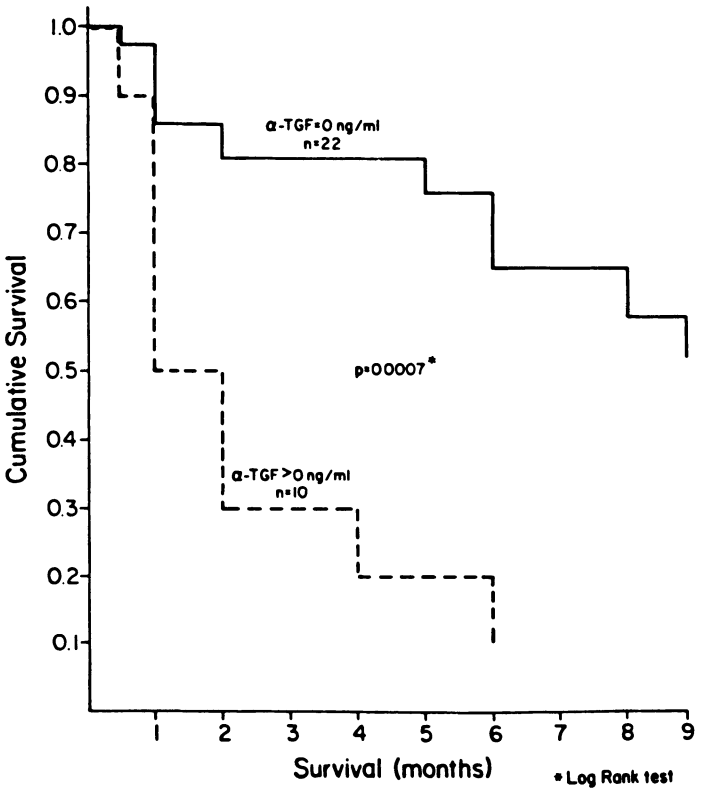

Figure 3. Kaplan–Meier survival curves according to the presence or absence of immunoreactive TGF–alpha activity in breast cancer patients' effusions (n = 32 patients). Survival was calculated from the time of collection of the specimen.

TGF–alpha in the effusions also predicted for a shorter survival, as shown in figure 3. Those patients with TGF–alpha-positive samples had a median survival of one month, whereas those without a detectable level reached their median survival at nine months ($p = 0.0007$).

These data demonstrate that TGF–alpha activity can be measured in a body fluid in patients with advanced breast cancer and that it correlates with other clinical markers of biologic aggressiveness. Future studies in serum or urine are needed in order to establish the potential value of TGF–alpha as a tumor marker or as an indicator of a poor prognosis.

Obviously these studies are subject to the criticism that they cannot discern if the growth factor measured is coming from the cancer cells or from normal tissues elsewhere in the body, perhaps as a response to the malignant process. Since other nontransformed cells [24–26], including normal breast epithelial cells maintained in tissue culture [27, 28], also synthesize TGF–alpha, future studies should carefully address the cellular source of this potentially important marker.

Other possible roles for TGF–alpha in breast epithelial cell biology

Normal human breast epithelial cells also express TGF–alpha mRNA and secrete immunoreactive TGF–alpha into conditioned medium [27, 28], raising the possibility that TGF–alpha may play a role in normal breast epithelium proliferation. In a study by Valverius et al., TGF–alpha mRNA levels in these cells were comparable to those seen in breast cancer cell lines, but the levels of secreted immunoreactive TGF–alpha were 10- to 100-fold less than that in conditioned medium from the transformed cells [28]. Since these benign cells are grown in the presence of EGF [29], it is unclear to what extent the expression of TGF–alpha message reflects autoinduction in vitro in response to exogenous growth factors in the medium. These results suggest that the expression of TGF–alpha is not restricted to malignant mammary epithelium. More comparative studies like that of Valverius et al. are needed in order to address possible quantitative and qualitative biological differences between the expression of TGF–alpha in normal as well as transformed breast epithelial cells.

In certain in vivo models, TGF–alpha has been associated with certain (physiological) processes that could be relevant to breast cancer cell behavior. TGF–alpha is a potent inducer of angiogenesis and of DNA synthesis in endothelial cells in vivo [30]. In a dog artery model, exogenous TGF–alpha increases blood flow and antagonizes vasoconstricting substances much more effectively than EGF [31]. These observations support a possible role for TGF–alpha in the processes of invasion and metastases. TGF–alpha is also a potent inducer of calcium release from bones in culture [32] and has been proposed as a mediator of the bone resorption and subsequent hypercalcemia seen in some epithelial malignancies. Interestingly, the three patients from whom Stromberg et al. [21] isolated urine immunoreactive TGF–alpha had extensive osteolytic metastases.

Several studies have suggested a possible link between the EGF/TGF–alpha receptor in breast cancer cells and increased tumorigenicity. Sainsbury et al. [33] reported that breast cancer biopsies expressing a larger number of EGF-binding sites tended to lack hormone receptors and to behave clinically more aggressively. An additional study utilizing breast cancer tissue showed preferential binding of 125–I–EGF to highly proliferating mammary cancer cells [34]. Furthermore, hormone-independent cell lines consistently express higher levels of functional EGF receptors and respond less to exogenous EGF or TGF–alpha [35] [CL Arteaga, unpublished data]. One possibility is that in these highly tumorigenic cells EGF receptors are already under maximal stimulation by endogenous TGF–alpha. Taken together these data suggest that EGF/TGF–alpha receptors may also have a role in breast cancer cell proliferation and tumorigenicity. One could speculate that in those breast cancer cells expressing TGF–alpha and high levels of activated EGF receptors, this putative autocrine stimulatory pathway is operative. Confirmation of this hypothesis may identify new therapeutic targets in some breast cancer cells.

Transforming growth factor–beta: A potential autocrine growth inhibitor of human breast cancer cells

TGF–beta is a multifunctional family of polypeptides [36] first identified for the ability to induce anchorage-independent growth of nontransformed fibroblasts [37, 38]. It is stimulatory to mesenchymal cells, but in epithelial cell systems it is primarily a growth inhibitor (39, 40). To date, no epithelial cell, benign or malignant, has been reported to be stimulated by TGF–beta, and in fact, a large number of carcinoma cell lines in tissue culture are inhibited by exogenous TGF–beta [39, 40]. The widespread distribution of cellular receptors for TGF–beta [41] and the discovery of TGF–beta mRNA in a number of normal and malignant epithelial cell lines and tissues [42, 43] have raised the possibility that this molecule may be a functional autocrine growth inhibitor of epithelial cells. Consequently, it has been proposed that in some malignant epithelial cells, a loss of the inhibitory response to endogenous TGF–beta may account for a proliferative advantage and thereby contribute to the neoplastic phenotype [44].

Previous studies have reported that human breast cancer cells express and secrete TGF–beta activity and are inhibited by exogenous TGF–beta, suggesting the possibility of a functional autocrine growth inhibitory pathway [9, 40, 45]. Dickson et al. reported that TGF–beta activity in conditioned medium from the hormone-dependent MCF–7 and T47D cell lines was decreased in the presence of 17–beta–estradiol [9]. In this same study, high constitutive levels of bioactive TGF–beta were detected in media from the hormone-independent MDA–231 and HS578T cell lines. Knabbe et al. reported that early passage MCF–7 cells, when exposed to inhibitory concentrations of antiestrogens, secreted 8- to 27-fold higher levels of TGF–beta activity [45]. With these findings it was then proposed that TGF–beta is a hormonally regulated negative growth factor that could partially mediate the inhibitory effects of antiestrogens in ER-positive breast cancer cells.

We have examined the interaction of TGF–beta with a panel of ER-positive and ER-negative human breast cancer cell lines. Four of four ER-negative cell lines were inhibited by picomolar concentrations of TGF–beta, whereas all three ER-positive cells examined were unaffected by concentrations of TGF–beta as high as 1 nM [46]. The disparate effects of TGF–beta on ER-positive and ER-negative cells could be explained by the observation that typical TGF–beta receptors were identified only in the estrogen-independent cells. Furthermore, higher levels of TGF–beta activity were detected in media from the ER-negative cells, supporting a potential autocrine growth inhibitory role for TGF–beta in these estrogen-autonomous breast cancer cells.

Our results with the three ER-positive cell lines that are not inhibited by TGF–beta argue against the possibility that increased TGF–beta secretion in response to tamoxifen mediates the growth inhibitory effects of the antiestrogen. As shown in table 3, experiments with these cell lines showed a dissociation between TGF–beta sensitivity and tamoxifen inhibition. These

Table 3. Effect of tamoxifen and of TGF–beta on DNA synthesis in ER-positive human breast cancer cells.

	MCF–7	ZR75–1	T–47D
Control	15958 ± 450	10504 ± 1030	2281 ± 246
Tamoxifen (1 μM)	4804 ± 175	5484 ± 307	1401 ± 104
TGF–beta (1 nM)	15133 ± 284	11317 ± 790	2049 ± 94

Cells were plated in 24-well plates in their regular growth medium (3–4 × 10^4 cells/well). Twenty-four hours later the medium was changed to phenol red-free IMEM supplemented with 5% CSCS plus tamoxifen or TGF–beta at the concentrations shown in the table. DNA synthesis was estimated at 48 hours as the amount of tritiated thymidine incorporated after a 1 hour pulse of 0.25 uCi/well of [^{3}H]thymidine. Values represent mean cpm ± SE of triplicate wells.

results are in disagreement with the results from Knabbe et al. [45] and may reflect differences among the clones utilized in both studies or even differences in the culture conditions. Alternatively, with serial passages some breast cancer cells may lose their sensitivity to TGF–beta while retaining their response to estrogen and antiestrogens. Another study has recently reported the failure of TGF–beta to inhibit growth of MCF–7 and T47D cells [47]. Furthermore, it has been reported that antiestrogen-induced growth inhibition of T47D cells decreased the level of TGF–beta mRNA, arguing against a pivotal role of TGF–beta on growth inhibition in these hormone-dependent breast cancer cells in vitro [48].

It is paradoxical that the highly tumorigenic ER-negative cell lines utilized in this study secrete higher levels of TGF–beta activity and are the most sensitive to TGF–beta-induced growth inhibition [46]. Furthermore, contrary to other cells that secrete TGF–beta in an inactive form [49, 50], some of the TGF–beta secreted by these cells is bioactive in the absence of acidification [46]. In preliminary experiments, anti-TGF–beta antibodies induced faster cell proliferation of hormone-independent cell lines [71], suggesting that the TGF–beta-negative autocrine loop is indeed operative in these cells. The possibility that some breast cancer cells are regulated in vivo by endogenous inhibitory factors (i.e., TGF–beta) could lead to new treatment strategies aimed at increasing the synthesis of the inhibitor and thereby arresting tumor growth.

Despite a large body of in vitro data showing an inhibitory effect of TGF–beta against transformed epithelial cells, the role of exogenous or endogenous TGF–beta on in vivo cancer cell proliferation is unknown. Although TGF–beta inhibits some breast cancer cells in vitro, several recent publications suggest that endogenously produced TGF–beta by cancer cells may have certain in vivo effects that could potentially maintain the neoplastic phenotype. These reported TGF–beta effects include: the potent induction of angiogenesis [51]; the inhibition of immune surveillance mechanisms involving T and B lymphocytes [52] and natural killer cell activity [53] as well as the deactivation of macrophages [54]; and the stimulation of cancer cell-

associated proteolytic activity [55]. Accordingly, MCF–7 cells transfected with the v–Ha–*ras* oncogene secrete higher levels of TGF–beta and acquire increased tumorigenicity in athymic mice [12].

Insulin-like growth factors as potential targets for inhibition of human breast cancer cells

The insulin-like growth factors are mitogenic for cultured human breast cancer cells [19, 47, 56–58]. Several of these cell lines secrete IGF activities into their culture medium [19, 58, 59]. In ER-positive cells the expression and secretion of both IGF–I and IGF–II-like proteins are increased by physiological concentrations of estradiol [58, 60]. One study has shown that either exogenous IGF–I or serum-free conditioned medium from MCF–7 cells containing IGF–I activity was able to transiently support the growth of these hormone-dependent cells in castrated nude mice in the absence of estrogen supplementation [11]. IGF–I receptors, also known as type I somatomedin receptors, have been reported in breast cancer cell lines [56, 61] and in breast cancer biopsies [62, 63]. Furthermore, breast cancer tissue from patients exhibits higher IGF–I binding than adjacent normal tissue [62], suggesting a link between this receptor and breast cancer cell proliferation. These cumulative data suggest that IGFs are important endocrine or autocrine/paracrine growth regulators of human breast cancer cells.

Utilizing a mouse monoclonal antibody, alpha–IR3, that blocks the IGF–I receptor-binding domain [64], we have found that this receptor mediates the proliferative effects of IGF–I and IGF–II in breast cancer cells [58, 65]. This observation has potentially important clinical implications since blockade of this receptor might inhibit the growth stimulatory effect of several related hormones (IGF–I, IGF–II, and insulin) and perhaps offer a new therapeutic strategy. However, alpha–IR3 did not inhibit growth of any of seven breast cancer cell lines in serum-free medium [70]. Furthermore, in three ER-positive, estrogen-responsive cell lines (MCF–7, ZR-75–1, and T47D), IGF–I receptor blockade with the antibody did not block estrogen-induced cell proliferation [70]. Taken together these results indicate that secreted IGF activity may not be a critical autocrine growth mechanism in cultured breast cancer cells.

In contrast, the growth of six of seven breast cancer lines in serum-supplemented medium was inhibited by the receptor-blocking antibody [70], suggesting that serum IGFs may be important growth regulators of breast cancer growth in vivo. This inhibitory effect, which has already been reported with MCF–7 cells under similar culture conditions [66], was reversible with the addition of excess IGF–I, indicating that it was not due to antibody-mediated nonspecific cytotoxicity. These results are consistent with recent results using human breast cancer tissue that demonstrated preferential expression of IGFs by stromal cells with negligible expression by the

malignant cells, suggesting that IGFs might be growth stimulatory to breast cancer cells in an endocrine/paracrine fashion [67, 68].

In an in vivo model using athymic mice, alpha–IR3-induced blockade of the IGF–I receptor inhibited growth of the hormone-independent MDA–231 breast cancer cells in a dose-dependent fashion [65]. A control antibody had no effect on MDA–231 tumor growth, and a mononuclear cell infiltrate was not observed in the tumor sections, arguing against the possibility that host immune mechanisms were responsible for tumor growth inhibition. These results suggest that IGFs may be important in vivo growth regulators for some human breast cancers and could serve as potential targets for treatment manipulation. Since IGFs may mediate several normal physiological functions [69], future studies need to address the potential toxicity of this treatment approach. Nevertheless, just as blockade of estrogen-mediated pathways by antiestrogens has had enormous clinical utility, antagonism of these polypeptide growth factor pathways could provide another form of 'endocrine therapy' for breast cancer.

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15. Steroids, growth factors, and cell cycle controls in breast cancer

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Introduction

Since the initial availability of hormone-responsive human breast cancer cell lines in the early 1970s, many studies have shown that the control of proliferation and differentiation in these cells involves complex interactions between steroid hormones, peptide hormones, and growth factors [1]. By contrast, the vast clinical literature provides evidence that the major stimulus for growth of breast cancer is estrogen. The low incidence of breast cancer in females without functional ovaries and in males [2], the importance of menstrual and reproductive history as risk factors [3], the marked arrest of tumor growth following ovariectomy in some patients [4], and reinitiation of tumor growth upon administration of estrogen [5] all support a fundamental role for estrogen in breast cancer progression and growth. Furthermore, the fact that treatment with compounds that act via antagonism of estrogen action, i.e., synthetic antiestrogens and perhaps progestins, leads to remission in a high proportion of patients with receptor-positive tumors adds additional weight to this view [6–8].

Although antiestrogens are thought to act predominantly via the estrogen receptor (ER) [9, 10] and progestins via the progesterone receptor (PR) [7, 11], the molecular mechanisms by which these agents inhibit human breast cancer cell proliferation remain unknown despite active and fruitful research in this area from a number of laboratories. Paradoxically, not all ER-positive and PR-positive tumors respond to these treatments, while a small but significant number of tumors in which ER and PR are undetectable do show responsiveness [12]. Furthermore, most tumors that are initially controlled, later develop resistance to the growth inhibitory effects of steroids and their antagonists. These observations provide impetus for the development of a more complete understanding of the mechanisms underlying hormone-dependent proliferation in human breast cancer. It is generally agreed that more detailed information in this area will pave the way for the more rational use of existing hormonal or antihormonal agents, the development of more potent and more specific agents, a more accurate discrimination between sensitive and insensitive tumors, and the identification of biochemical

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markers of poor prognosis, i.e., indicators of tumor progression and hormone resistance.

Several models of the mechanism of estrogen stimulation of proliferation have been proposed, primarily based on experimental data obtained using breast cancer cells in culture. These models postulate that estrogens may act either directly to modulate the transcription of one or several of the limited number of genes controlling progression through the cell cycle, or secondarily, by promoting the production of autocrine or paracrine peptide growth factors that in turn act upon cell cycle progression genes [1, 2, 13]. A third possibility is that estrogen may stimulate proliferation by negating the growth inhibition resulting from 'negative growth factors' naturally present, but as yet unidentified, in blood and extracellular fluid [14]. Data that allow unequivocal determination of which, if any, of these models is correct are not yet available. However, the in vitro effects of steroid inhibitors, particularly antiestrogens, have been well studied, providing some insight into the interrelationships of the steroid hormones and growth factors that govern breast cancer cell cycle progression. This chapter summarizes data that document the effects of steroids (progestins and 1,25–dihydroxyvitamin D_3) and steroid antagonists (antiestrogens) on breast cancer cell proliferation and cell cycle kinetics, and the modulation of these effects by peptide growth factors. These data are interpreted in the light of known effects of growth factors on cell cycle progression and the potential modulation of steroid hormone sensitivity by growth factor-induced changes in cellular steroid hormone receptor levels. The recent deeper understanding of cell cycle control mechanisms, particularly the cloning and functional analysis of a number of mammalian cell cycle progression genes, provides new insights that should aid in the design of experiments aimed at enhancing our understanding of hormonal control of breast cancer cell cycle progression.

Growth regulatory and cell cycle effects of steroids

Estrogens

Estrogens have well-documented mitogenic effects predominantly in female reproductive tissues [15], and indeed the first bioassays for estrogenic compounds followed from such observations. Typically the response is characterized by a bell-shaped dose-response curve, i.e., by stimulation of growth at low, physiological, estrogen concentrations but inhibition at high concentrations [15]. Inhibitory effects of high (10–100 μM) concentrations of estrogen are observed in breast cancer cell lines and include both cytotoxicity and changes in the cell cycle phase distribution, i.e., increases in the percentages of S and G_2 + M cells, in both ER-positive and ER-negative cell lines [16]. These changes in cell cycle distribution are seen in exponentially growing, but not plateau-phase, cultures of T–47D cells [16] and are

therefore likely to represent a cell cycle-specific mechanism of action, distinct from cell-cycle independent cytotoxicity.

Lippman and his colleagues were the first to demonstrate direct effects of stimulatory concentrations of estrogens on the proliferation of cultured breast cancer cells. Using MCF–7 cells in low serum or serum-free medium, they demonstrated that addition of estrogens increased both the rate of tritiated thymidine incorporation and the cell numbers following treatment, indicating an increase in the growth fraction [9, 17]. The thymidine incorporation was stimulated in parallel with an increase in the activity of a number of the enzymes involved in DNA synthesis, including those catalyzing nucleotide salvage and de novo synthesis [18–22]. Some reports suggest that an increase in cell numbers is dependent on the continuous presence of estrogen: ZR–75–1 cells grown in defined medium and then deprived of estradiol fail to further increase their cell numbers, despite the continued incorporation of tritiated thymidine [23]. This appears to be due to increased cell death in the absence of estrogen, since in cell populations prelabelled with thymidine, considerably more label is lost into the medium of estrogen-deprived than estrogen-treated cells [23]. Finally, MCF–7 cells growth-arrested by tamoxifen are 'rescued,' i.e., thymidine incorporation is stimulated, by the addition of estradiol [9, 17, 24]; although this stimulation is also observed with cells merely changed to fresh, serum-containing medium, it is less pronounced than in the presence of estradiol [24]. These limited cell cycle kinetic studies on the effects of estrogens on breast cancer cells in vitro support the data that are available from in vivo studies using the rodent reproductive tract as a target tissue [reviewed in 15], leading to the following conclusions: estrogens increase cell viability, recruit non-cycling or quiescent cells into the cell cycle, and reduce the duration of cell cycle phases, primarily G_1 phase.

Antiestrogens

Inhibition of breast cancer cell proliferation in vitro by synthetic antiestrogens is thought to be mediated by antiestrogen binding to the ER, since the effect is confined to ER-positive cell lines; and in studies with series of structurally related triphenylethylene antiestrogens, potency of growth inhibition is positively correlated with affinity of the antagonist for the ER [10, 25–29]. At antiestrogen concentrations of 2–5 μM or below, the decrease in cell proliferation rate can be negated by the simultaneous or subsequent addition of estradiol [10, 19, 24–33]; but at higher antiestrogen concentrations, estradiol no longer completely restores growth rates to control levels [25–33]. There is thus strong evidence for estrogen-irreversible as well as estrogen-reversible mechanisms of growth inhibition by synthetic nonsteroidal antiestrogens in vitro. These two effects can be most clearly distinguished in the biphasic dose-response curves generated when MCF–7 cells are treated with a series of hydroxylated antiestrogens with high affinity for the ER, e.g., hydroxyclomiphene. Under these circumstances the estrogen-reversible

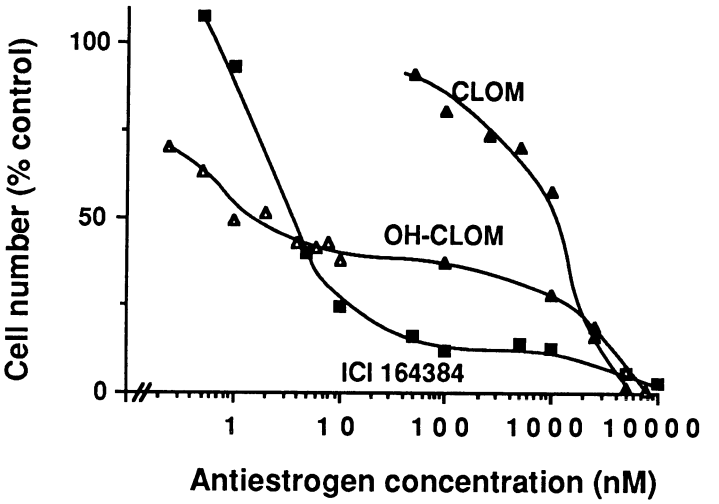

Figure 1. Cell growth inhibition by antiestrogens. The effects of the nonsteroidal antiestrogen clomiphene (CLOM, ▲), hydroxyclomiphene (OH–CLOM, △), a structurally related compound with high affinity for the ER, or the steroidal antiestrogen ICI 164,384 (■) on MCF–7 cell growth are expressed as cell numbers relative to control, after approximately four cell doublings of control vehicle-treated cultures [From references 29, 31, 42].

action reaches its maximal effect at a concentration of ~10 nM [29, 32, 33], at least two orders of magnitude lower than the 2 μM concentration at which the estrogen-irreversible actions are seen (figure 1). The dose-dependence of the effects of structurally related compounds with lower affinity for the ER, e.g., clomiphene, differs from that of their hydroxylated derivatives over the estrogen-reversible but not estrogen-irreversible concentration range.

Nonsteroidal antiestrogens can display partial agonist activity in addition to their predominant antagonist activity; this is most easily demonstrated by their stimulation of uterine growth in immature or ovariectomized rats [6] and their stimulation of the growth of endometrial tumors transplanted in nude mice [34], but also observed in breast cancer in vivo [6, 8] and in vitro under some culture conditions [35–38]. However, the recently described steroidal antiestrogen ICI 164384 [39] has displayed only antagonist activity when tested in the same experimental systems [38–41] and has therefore been termed a 'pure' antiestrogen. This compound inhibits the growth of breast cancer cells in culture over a concentration range that overlaps the estrogen-reversible range of hydroxylated nonsteroidal antiestrogens (figure 1), but is more effective [41, 42]. One explanation for this difference in efficacy is that the nonsteroidal antiestrogens behave as agonists as well as antagonists in these circumstances, with a resultant decrease in maximal growth inhibition.

The observation that in cells treated with an antiestrogen then 'rescued' with estrogen, thymidine incorporation reached levels greater than those in control cells led Lippman to postulate as early as 1975 that antiestrogens

arrested cells in a specific phase of the cell cycle from which they could be rescued in a synchronous manner by estrogen [17]. It was later demonstrated, first in this laboratory [25–33] and subsequently by others [24, 43–45], that antiestrogen treatment of breast cancer cells in culture is associated with the arrest of cells in the G_1 phase of the cell cycle and a resulting decrease in the relative proportion of cells in S phase. These observations have recently been extended to include the steroidal 'pure' antiestrogen ICI 164384 as well as nonsteroidal antiestrogens [42, 46]. Cells synchronized by mitotic selection and then treated with antiestrogens have been used to further delineate the cell cycle kinetic changes. Early experiments clearly demonstrated that only those cells in early-to-mid-G_1 phase were susceptible to growth arrest by the antiestrogen tamoxifen [47], and they were corroborated by the observation that cells in plateau phase, where a high proportion of cells might be expected to be in G_0 rather than G_1 phase, are relatively insensitive to tamoxifen [26, 31]. The possibility that ER-mediated effects might not have been predominant at the concentration of antiestrogen used in the studies with synchronous cells (7.5 μM), together with our demonstration of the biphasic mechanisms of growth inhibition of hydroxylated nonsteroidal antiestrogens and the availability of the 'pure' antiestrogen ICI 164384, prompted a reexamination of the timing of the multiple modes of antiestrogen actions. The steroidal pure antiestrogen was demonstrated to have an estrogen-reversible action in mid-G_1 phase coincident with the sensitivity previously found with the nonsteroidal compound tamoxifen [42]. Furthermore, the estrogen-reversible and estrogen-irreversible actions of hydroxyclomiphene were also confined to the same portion of G_1 phase [42]. This latter compound is structurally related to tamoxifen but has a much higher affinity for the ER [29, 33]. These results confirm that the specific, cell cycle-related growth arrest is characteristic of antiestrogens as a class rather than peculiar to compounds of one particular structure. The timing of the sensitivity is compatible with that expected for cell cycle regulatory events, which are believed to take place predominantly in G_1 phase.

The binding of tamoxifen and other nonsteroidal antiestrogens to a number of intracellular binding sites that might mediate estrogen-irreversible actions has been described. Of these, a specific high-affinity antiestrogen-binding site (AEBS) [48–50] and calmodulin [51] have been studied in some detail. The available data argue against the AEBS functioning directly in this role, for two reasons: potency of a series of antiestrogens in the estrogen-irreversible range is not highly correlated with relative affinity for the AEBS [29, 33], and secondly, there is an approximately thousand fold difference between affinity for the AEBS and the concentrations of antiestrogen required for estrogen-irreversible growth inhibition. In contrast, an antiestrogen action mediated by calmodulin antagonism is supported by a correlation between estrogen-irreversible growth inhibitory potency and inhibition of calmodulin-dependent phosphodiesterase activity [52], both apparent within the same micromolar concentration range of a group of compounds

containing both antiestrogens and calmodulin antagonists. Further support is provided by qualitative similarities in the changes in cell cycle kinetic parameters in breast cancer cells treated with high concentrations of antiestrogen or with calmodulin antagonists [33, 42]; the calmodulin antagonist R 24571 has actions within the same portion of G_1 phase as have hydroxyclomiphene and ICI 164384 [42]. These data are compatible with high concentrations of antiestrogen acting, at least in part, via inhibition of calmodulin-dependent pathways.

Progestins

Synthetic long-acting progestins are effective agents in the treatment of some breast cancers [7]. There is at present considerable debate as to their molecular mechanisms of action as antitumor agents, since these compounds have both glucocorticoid and progestational activity that could be responsible for central actions via the hypothalamo-pituitary axis and/or direct effects on tumor cells. A number of studies have demonstrated direct inhibition of human breast cancer cell proliferation by progestins in cell culture [53–56], and we have recently extended these studies with a broader spectrum of cell lines and detailed cell cycle kinetic analysis [11].

Progestin inhibition of breast cancer cell growth is accompanied by cell cycle kinetic changes qualitatively similar to those that occur in the presence of antiestrogens. There are, however, important differences between the

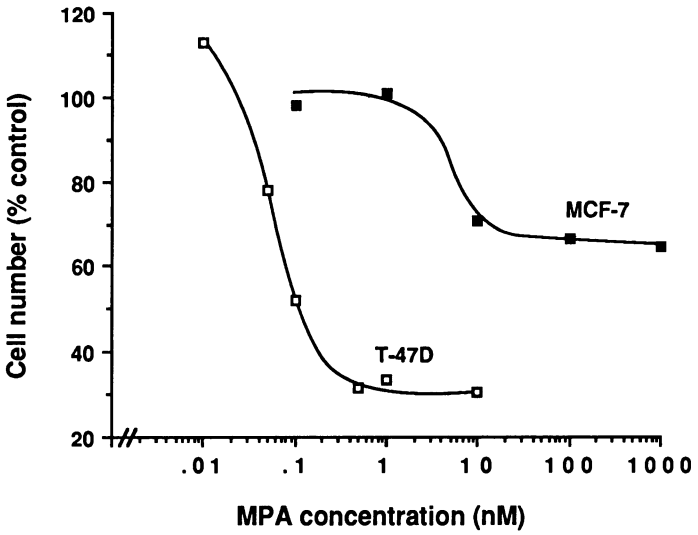

Figure 2. Cell growth inhibition by progestins. The differential sensitivity of MCF–7 (■) and T–47D (□) cells to the growth inhibitory effects of the progestin medroxyprogesterone acetate is shown. Cell numbers are expressed as a percentage of the number of cells in vehicle-treated control flasks after approximately four cell doublings of control cultures. [From reference 11.]

effects of the two classes of compounds. Although sensitivity to progestins is correlated with PR status (in that ER-negative, PR-negative cell lines are insensitive, while ER-positive, PR-positive cell lines are growth inhibited), in a series of five receptor-positive cell lines treated with the progestin medroxyprogesterone acetate (MPA), there was little correlation between sensitivity to progestin-induced growth inhibition and PR concentration [11]. The maximum growth inhibitory effect after four population doubling times of control cultures varied from 15% to less than 70% and was not related to the progestin sensitivity, which differs by approximately a thousandfold (figure 2) [11].

The cell cycle kinetic effects associated with the growth inhibition are consistent with an action predominantly in G_1 phase. However, since the changes in cell cycle phase distribution only become apparent after treatment times longer than the duration of G_1 phase (estimated from the population doubling time and cell cycle phase distribution), it is necessary to postulate a 4 to 5 hour delay between the addition of the drug and evidence of its action. In the most sensitive cell line, T–47D, within one cell cycle of treatment with 10 nM MPA (i.e., ~24 hours), greater than 90% of cells are in G_1 phase, with a concomitant fall in the percent of S phase cells (figure 3). The increase in the percent of G_1 cells reflects an almost total inhibition of efflux from that phase following progestin treatment [11]. Although profound, the arrest in G_1 phase is transient and is followed by a resumption of cell cycle progression and consequent efflux from G_1, albeit at lower than control rates [11]. In contrast, under similar experimental conditions the effects of antiestrogens are cumulative and can result in complete arrest of cell cycle progression without evidence for cytotoxicity [25–27, 31].

Although some studies have suggested that only estrogen-dependent growth is responsive to progestins [53], progestin-mediated growth inhibition is not reversed to any major degree by the addition of exogenous estrogen [11] and can be demonstrated in cultures depleted of estrogens [56, EA Musgrove and RL Sutherland, unpublished]. Furthermore, in the same cell line the magnitude of growth inhibition by antiestrogens and progestins can differ markedly; for example, MCF–7 cells treated with progestins are growth inhibited to a maximum of ~30%, while they are inhibited by antiestrogens to a maximum of more than 70% under identical experimental conditions (compare data in figures 1 and 2). It is apparent that ER- and PR-mediated effects on proliferation are distinct and that even in hormone-responsive cells, growth control does not entirely depend on either pathway.

1,25–dihydroxyvitamin D_3

1,25–dihydroxyvitamin D_3 (1,25–$(OH)_2$ D_3), the active hormonal form of vitamin D_3, is a seco-steroid that exerts many of its effects at the cellular level by interacting with a specific high-affinity receptor that is a member of the thyroid hormone/steroid hormone receptor superfamily of nuclear transcrip-

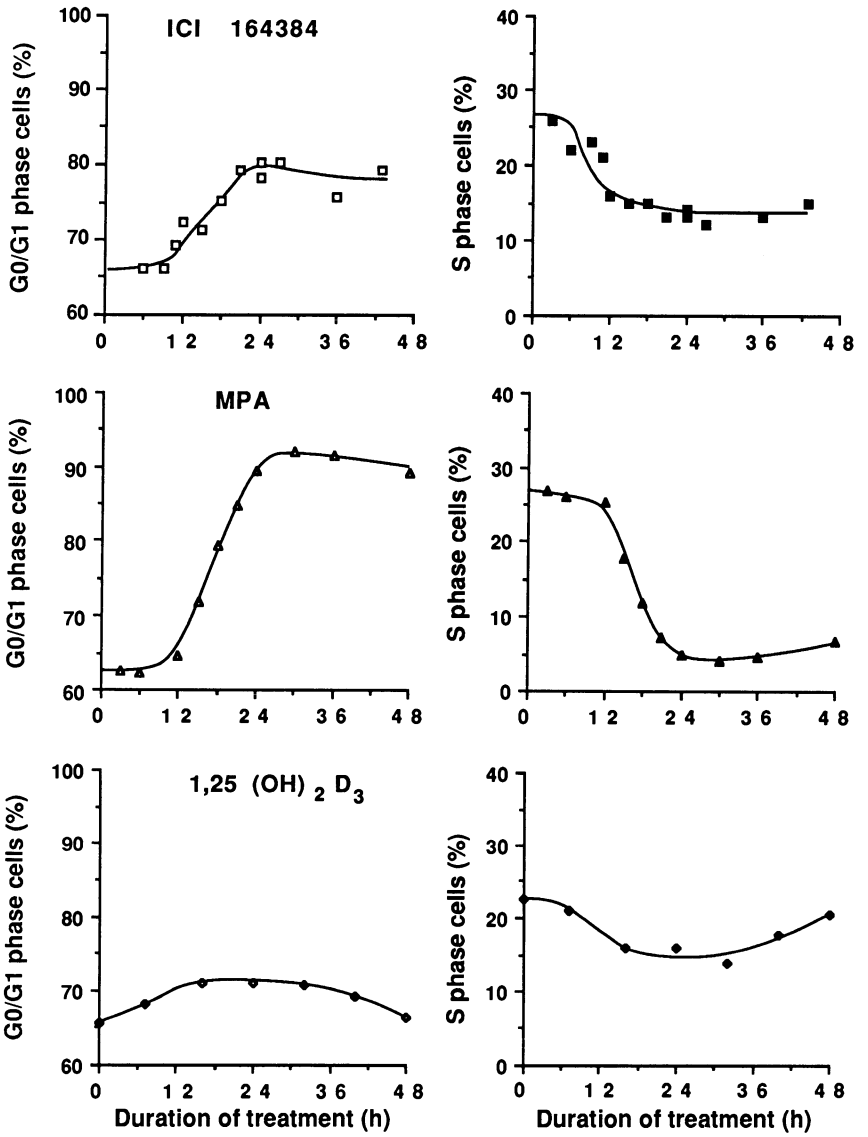

Figure 3. Time-course of cell cycle kinetic changes of cells treated with antiestrogen, progestin, or 1,25-dihydroxyvitamin D_3. Cell cycle phase distributions, measured by analytical flow cytometry, of T-47D cells treated with the antiestrogen ICI 164,384, 100 nM (□, ■); the progestin medroxyprogesterone acetate, 10 nM (△, ▲); or 1,25-dihydroxyvitamin D_3, 100 nM (◇, ◆). The distributions of vehicle-treated control cells were essentially unchanged during the period shown. [Data for ICI 164,384 are from our own unpublished work, the remainder are from references 11, 60.]

tion factors. This agent has well-documented effects on cellular proliferation and differentiation, which have been studied in most detail in lymphoid cells but have also been investigated in breast cancer cell lines.

At concentrations of more than 1 nM, 1,25-$(OH)_2$ D_3 inhibits cellular replication in several types of cancer cell lines, including breast cancer cell lines [57–60]. In T-47D cells, a complex series of cell cycle kinetic changes accompanies the growth inhibition. The effects observed after two to six days of treatment are an increase in G_2 + M phase cells of ~2-fold compared with control cultures, despite a fall in the proportion of cells in S phase. Following an initial rise, the proportion of cells in G_1 phase returns to control levels despite a sustained ~7-fold decrease in the G_1 exit rate [60]. The simplest explanation of these changes is that there are delays in transit through both G_1 and G_2 + M phases, and there is previous evidence for such transition delays in other cell types. In T-47D cells, although the G_2 + M phase accumulation predominates after prolonged exposure to 1,25-$(OH)_2$ D_3, the effects of the G_1 phase arrest become apparent first, and cells accumulate in G_1 phase during the first 24 hours of treatment (figure 3). This part of the response is maximal at 16–24 hours, i.e., up to 6 hours before the maximal G_1 phase increase in response to the progestin MPA, and in agreement with the time-course of G_1 phase increase in response to antiestrogens under the same experimental conditions, providing circumstantial evidence for an early-to-mid G_1 phase effect of 1,25-$(OH)_2$ D_3.

Response to 1,25-$(OH)_2$ D_3 in some breast cancer cell lines has parallels with the effects of estrogens in that both steroids are stimulatory at low concentrations but become inhibitory at high concentrations [9, 15, 16, 61], and that inhibition is characterized by accumulation of G_2 + M phase cells, in marked contrast with the effects observed after treatment of receptor-positive cell lines with antiestrogens or progestins. At high concentrations both antiestrogens [27] and 1,25-$(OH)_2$ D_3 [59] have at least additive effects in combination with micromolar concentrations of estrogens. It is therefore likely that, in this concentration range at least, the mechanisms of inhibition are different for the three compounds.

Other steroids

A number of reports suggest that other steroid hormones, including glucocorticoids [62, 63] and androgens [64, 65], inhibit the proliferation of some human breast cancer cells in culture. Little information is available on differential sensitivity of different cell lines, particulary with respect to receptor status and receptor concentrations; nor is there, to our knowledge, detailed data on the cell cycle correlates of growth inhibition.

The morphogen retinoic acid, which acts through a receptor of the steroid hormone family, also inhibits the proliferation of some breast cancer cell lines in vitro [66–73] and in vivo in athymic mice [71, 74]. Retinoic acid appears to be cytostatic rather than cytotoxic, since its growth inhibitory effects are

reversible and since proliferation, although markedly slowed, is not wholly abolished. This characteristic is shared with both antiestrogens and progestins. Furthermore, antiestrogens (tamoxifen and hydroxytamoxifen) and retinoic acid are additive in inhibiting breast cancer cell proliferation [72, 73], an effect not mediated by binding of retinoic acid to the ER [72]. The limited cell cycle kinetic data on the effect of retinoic acid on human breast cancer cells are contradictory. Ueda et al. [68] reported decreases in tritiated thymidine uptake in parallel with decreases in proliferation rate, while Marth et al. [69, 70] reported an increased uptake of thymidine and an increased percent of S phase, again accompanying decreased proliferation. In other cellular systems in which retinoic acid causes growth inhibition, e.g., HeLa cells, the predominant point of action has been demonstrated to be in G_1 phase [75].

Summary of steroidal effects

The data outlined above demonstrate cell cycle-specific actions in G_1 phase for estrogens, antiestrogens, progestins, and 1,25–$(OH)_2$ D_3. For antiestrogens, at least, this action can be further ascribed to a restricted portion of G_1 phase, supporting the hypothesis that the estrogen/estrogen-receptor complex is intimately involved in the completion of a biochemical event crucial to cell cycle progression. Each inhibitor has distinct cell cycle kinetic effects, e.g., the accumulation of cells in G_2 + M phases upon 1,25–$(OH)_2$ D_3 treatment; and the delay before MPA treatment, compared with antiestrogen or 1,25–$(OH)_2$ D_3 treatment, induces accumulation of cells in G_1. However, it is not clear from the available data whether each has a distinct cell cycle-related target or whether the differences in the time at which their effects become apparent (figure 3) reflect different pathways that ultimately converge at or before a cell cycle control event.

Growth factor modulation of steroid effects

Growth factors and cell cycle progression

The factors that govern cell cycle progression in mammalian cells have been extensively explored using rodent fibroblast cell lines. Once rendered quiescent by density inhibition, these cells can be induced to reenter the cell cycle and progress into S phase by combinations of polypeptide mitogens. Two classes of growth factors, apparently acting sequentially, have been defined: competence factors, e.g., platelet-derived growth factor, which initiate cell cycle traverse; and progression factors, e.g., epidermal growth factor (EGF) and insulin/insulin-like growth factor I, which provide the stimuli required to continue through the cell cycle into S phase [76, 77]. The pathway of cells entering G_1 phase from mitosis converges with this tightly controlled sequ-

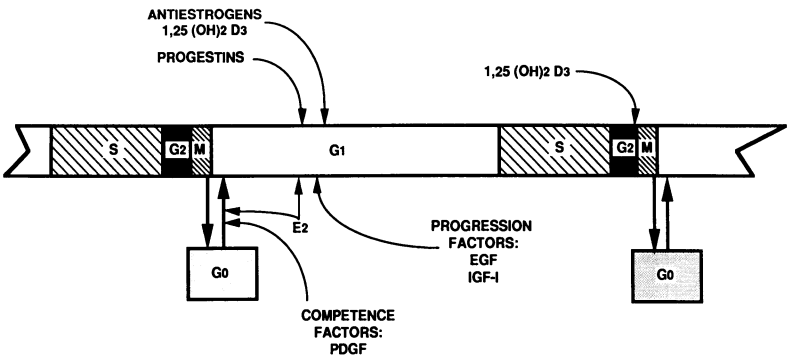

Figure 4. Cell cycle actions of steroids, steroid antagonists, and growth factors.

ence of events at a point where transcription and protein synthesis in the presence of progression factors are required for DNA synthesis to commence [78]. Although there may not be exact parallels between this series of cell cycle control mechanisms and those that operate within epithelial, cells and in breast cancer cells in particular, several lines of evidence suggest a link between the actions of polypeptide growth factors and the regulation of breast cancer cell proliferation.

Breast cancer cell lines possess receptors for EGF, insulin, and insulin-like growth factor–I (IGF–I) [79–83], and proliferation is often but not always stimulated by ligands that bind to these receptors [81, 84–90]. Furthermore, the evidence outlined above suggests that sensitivity to growth inhibition, including estrogen antagonism, can be ascribed to discrete points within the cell cycle and, in particular, within G_1, similar to the positions at which control of proliferation by progression factors occurs in fibroblasts (figure 4). Finally, breast cancer cells produce peptide growth factors, including EGF [91] and transforming growth factor α (TGF–α) [92–95], both of which exert their biological actions via binding to the EGF receptor. Autocrine production of such factors is an attractive hypothesis to explain the escape of cancer cells from normal growth regulation; and a number of reports have suggested that the production and/or action of these factors are regulated by steroid hormones and are linked to the proliferative status of the cell [1, 2, 13, 93–99]. This line of reasoning prompted a series of experiments, described below, in which EGF-and/or insulin-mediated antagonism of growth inhibition by antiestrogens [100] and progestins [101] were demonstrated. Similar results have also been obtained in other laboratories [36, 38, 56, 102, 103].

Effects of EGF and insulin on steroid-induced growth inhibition

Recently published data from this laboratory have shown a concentration-dependent reversal of the growth inhibition of T–47D cells by either antiestrogens [100] or progestins [101] with EGF. The effect was apparent in

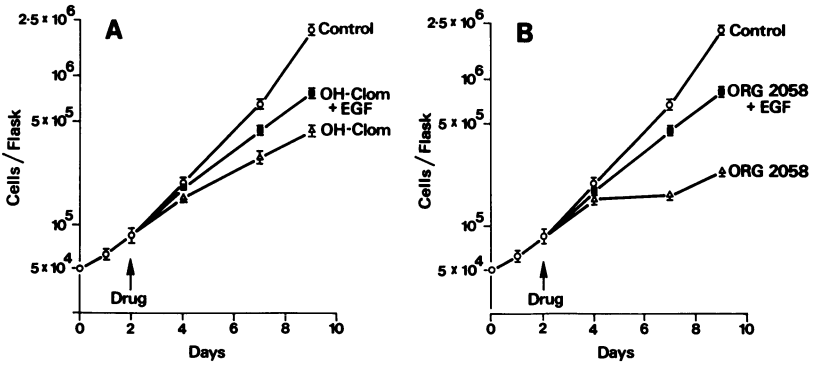

Figure 5. Effect of EGF on growth inhibition by antiestrogen or progestin. Replicate cultures of T-47D cells were treated with (A) 10 nM hydroxyclomiphene (OH-Clom) alone (△) or 10 nM hydroxyclomiphene with 10 ng/ml EGF (■); and (B) 10 nM ORG 2058 alone (△) or 10 nM ORG 2058 with 10 ng/ml EGF (■). Treatments were added simultaneously 2 days after plating; control cultures (○) received vehicle alone. [From reference 101.]

the concentration range 0.01–10 ng/ml (1.6 pM–1.6 nM), with maximum reversal at EGF concentrations greater than 0.5 ng/ml. When compared with cultures treated with either inhibitor alone, this represents an increase in cell numbers of ~50% over times equivalent to four doublings of untreated cultures, but not a restoration of growth to control rates (figure 5). Others, using different experimental conditions, have observed complete negation of antiproliferative effects of antiestrogens and progestins with EGF [36, 38, 56]. These effects are likely to be EGF receptor mediated, since they occur at concentrations similar to the Kd of the EGF receptor and since TGF-α, acting by the same receptor, stimulates thymidine incorporation in cells pretreated with tamoxifen [104].

In contrast to EGF, which has similar effects on both progestin- and antiestrogen-treated cultures, insulin is much more effective in increasing the growth rates of antiestrogen-treated cultures than the growth rates of progestin-treated cultures (figure 6). Cell numbers in cultures passaged for three to eight weeks in the absence of insulin and then treated with 10 nM of the progestin ORG 2058 were increased only slightly, if at all, by insulin concentrations in the range 5 ng/ml to 5 μg/ml (0.87 nM–870 nM). Insulin concentrations of 50 ng/ml or greater did, however, markedly increase cell numbers in cultures treated with the antiestrogen hydroxyclomiphene. At the highest dose tested, 5 μg/ml, this represented a doubling of cell numbers. Insulin and EGF added together exerted approximately additive effects to antagonize antiestrogen-mediated growth inhibition and exerted more than additive effects in antagonizing progestin inhibition (figure 6). This suggests a divergence of the pathways by which these growth factors increase cell numbers in this experimental design, since a response to insulin can occur in the presence of a maximally effective concentration of EGF. In addition, the contrast between the responses of progestin- or antiestrogen-treated cells further defines differences in their mechanism of growth inhibition.

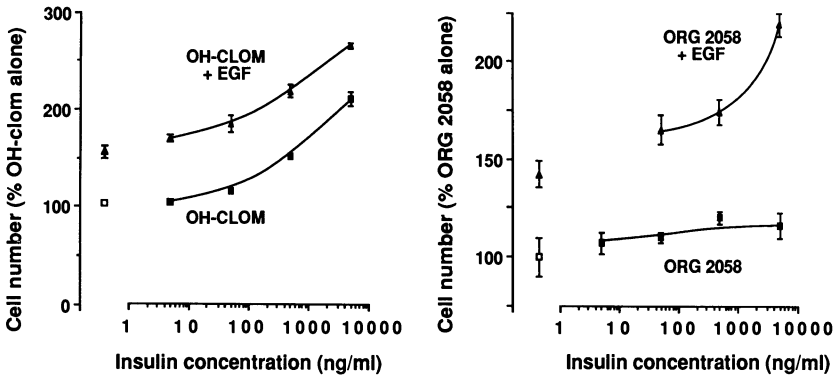

Figure 6. Dose dependence of EGF and/or insulin effects on antiestrogen- or progestin-treated cells. Cells cultured in the absence of insulin for at least 3 weeks were plated into replicate flasks and treated 2 days later in either the presence (closed symbols) or absence (open symbols) of insulin. Treatments were (A) 10 nM hydroxyclomiphene (OH–CLOM) alone (□, ■) or 10 nM hydroxyclomiphene with 10 ng/ml EGF (△, ▲); and (B) 10 nM ORG 2058 alone (□, ■) or 10 nM ORG 2058 with 10 ng/ml EGF (△, ▲). Cell numbers are recorded as a percentage of those in flasks treated with inhibitor alone. [From reference 101.]

Steroidal regulation autocrine growth factor production

One hypothesis compatible with the data presented above is that the action of antiproliferative agents (antiestrogens and progestins) is mediated by inhibition of production and secretion of autocrine growth factors and can therefore be mitigated by the exogenous addition of such growth factors. Supportive evidence for this view is provided by reports that estrogens increase and antiestrogens decrease the production of EGF and TGF-α-like peptides [93–95]. However, the regulation of expression of these growth factor genes is not directly linked with proliferation, since progestins also increase both EGF and TGF-α production [91, 103, 105; CSL Lee, PRJ Healey, and RL Sutherland, upublished]. Furthermore, rescue of tamoxifen-arrested cells by estrogen is not inhibited by the presence of an antibody to the EGF receptor, whereas rescue by TGF-α is inhibited [104], also arguing against this mechanism.

Potential mechanisms for growth factor modulation of steroid effects

The differences in the response of progestin-and antiestrogen-treated cells to EGF and insulin, either alone or in combination, suggest that although both progestins and antiestrogens act in G_1 phase, there is a qualitative difference in their mechanisms of growth inhibition. One possible explanation is that the proliferative response of breast cancer cells may be triggered by any of a number of agents, and the pathways for these responses may be redundant rather than dependent. Treatment with antiestrogen or progestin may therefore be specific to only one or several, but not all, of the pathways that culminate in the signal to proliferate; and EGF and insulin would be able to

stimulate proliferation by routes independent of the growth inhibition. The data outlined above, particularly evidence that EGF-stimulated growth is not inhibited by either progestins or antiestrogens in some systems [38, 56], and the fact that the effects of EGF and insulin are at least additive in antagonizing growth inhibition are compatible with this hypothesis.

An alternative possibility is that steroid receptor concentrations could be modulated by growth factors, which might be expected to regulate cellular sensitivity to steroids and their antagonists and in turn regulate proliferation. There is also evidence for the converse, i.e., that growth factor receptor numbers and therefore sensitivity to growth factors are modulated by steroids. Some laboratories that report a decrease in growth inhibition by progestins or antiestrogens in the presence of EGF have presented evidence that EGF causes a decrease in ER and PR [38, 56, 106], although increases in ER and PR upon EGF treatment have also been reported [107]. Although EGF may therefore act in this situation by reducing the cellular sensitivity to growth inhibition by progestins or antiestrogens, this potential mechanism requires further substantiation and is apparently at variance with the observed decrease in growth inhibition by antiestrogens, but not progestins, in the presence of insulin, since insulin has been reported to decrease the number of ER sites in MCF–7 cells [102] and to also decrease the number of PR sites in both MCF–7 and T–47D cells [56].

Progestins do not appear to induce insulin receptors in the T–47D cells used for these studies [97], although in a variant subline, growth inhibition by progestins is accompanied by an increase in insulin receptor levels [55]. The EGF receptor is induced by progestin treatment of T–47D cells [56, 97, 98], but the effect is not sustained [56]. In contrast, antiestrogen treatment does not alter EGF receptors over short time courses, but reduces EGF binding to 25% of control after five days [100, 107]. These data do not readily provide an explanation of similarities between EGF action on progestin- and antiestrogen-treated cells. The relationship between sensitivity and receptor modulation is clearly not simple and is only likely to be clarified by experiments in which receptor and growth modulation are tested in parallel under conditions where the hormonal and growth factor milieu are defined, to minimize the effects of reciprocal regulation of receptors. However it still appears likely that receptor regulation is involved in some growth factor modulation of steroid effects.

Cell cycle progression genes and their regulation

The agents necessary for cell proliferation have been classified by Baserga [108] into those that are necessary but not specific, e.g., ATP, Mg^{2+} and Ca^{2+}, and those that are both necessary and specific. The latter are further subdivided into regulatory components (e.g., DNA-synthesizing enzymes) and controlling components, which are responsive to environmental

signals and do not depend on the previous expression of other cell cycle genes. Cell cycle control genes are thus defined as those that initiate the events leading to DNA synthesis and mitosis. It is as yet unclear which genes actually perform this role in mammalian cells, although a number of candidate genes, some of which are described below, are the subject of intensive investigation.

Genes for DNA-synthesizing enzymes

The activities of a number of DNA-synthesizing enzymes, including enzymes of DNA metabolism (e.g., thymidine kinase, dihydrofolate reductase, and ribonucleotide reductase as well as DNA polymerases), are cell cycle dependent in that they increase coordinately at the G_1/S boundary, although they are detectable in G_1 and G_0 cells [108]. The expression of mRNA encoding some but not all of these enzymes also increases specifically and abruptly at the beginning of S phase [108]. The proliferating cell nuclear antigen (PCNA/cyclin) has been grouped together with these enzymes, since it shares their pattern of cell cycle regulation at the level of both mRNA and protein and has recently been shown to be a cofactor for DNA polymerase δ [109, 110]. It has been suggested that DNA polymerase δ is the leading strand replicase, whilst DNA polymerase α is the lagging strand replicase [for review see 111]. Publications that document inhibition of DNA synthesis in cells exposed to antibodies to PCNA/cyclin [112] or antisense oligodeoxynucleotides to PCNA/cyclin mRNA [113, 114] imply that PCNA/cyclin expression is a prerequisite for DNA replication. Although the function of the DNA synthetic enzymes suggests that their regulation is a consequence of the stimulus for DNA synthesis rather than being an initiating event, the available data for PCNA/cyclin are consistent with the possibility that its synthesis triggers DNA replication, and considerable debate exists over its precise role.

A number of publications, primarily the work of Aitken and Lippman, have addressed the regulation of DNA synthetic enzymes in human breast cancer cells. These studies have shown that, in general, antiestrogen treatment decreases and estrogen treatment increases the activities of enzymes including de novo pyrimidine synthetic enzymes [20], thymidine kinase [21, 22], and DNA polymerase [18]. This regulation of enzyme activity correlates with changes in tritiated thymidine incorporation and the rate of cell growth [21, 22]; and in the case of thymidine kinase, at least, is mediated by transcriptional control of mRNA levels [115]. The lag of more than 12 hours after estrogen exposure before increases in thymidine incorporation or thymidine kinase mRNA are apparent and the dependence of increases in thymidine incorporation on both protein and new mRNA synthesis suggest that these responses are not primary responses to estrogen [22]. This observation parallels the results obtained by Jaskulski et al., who described the modulation of both thymidine kinase and PCNA/cyclin mRNA in fibroblastic cells stimulated with serum [116], and supports their suggestion

that the regulation of these genes is dependent upon the products of separate growth factor-regulated genes.

Calmodulin

Calmodulin, which mediates many calcium-dependent processes, regulates a number of intracellular events and has been implicated in the regulation of cell proliferation. Its concentration increases sharply as cells enter S phase [117], and calmodulin antagonists inhibit the proliferation of a variety of cell types [117–120], including breast cancer cells [42, 52, 121]. The growth inhibition has been ascribed to cell cycle arrest both within G_1 and during mitosis [42, 117, 119, 122, 123]. More direct evidence that the rate of cell cycle progression is sensitive to the intracellular calmodulin concentration is provided by the inverse relationship between the length of G_1 and the intracellular calmodulin concentration in cells transfected with a calmodulin minigene [123, 124].

Some convergence of the mechanisms of calmodulin- and estrogen-dependent proliferation is implied by the temporal coincidence of the action of their antagonists (see the section on antiestrogens) and by the ability of EGF to diminish the effects of both antiestrogens [100] and some calmodulin antagonists [119]. One possibility, as yet untested experimentally, is that calmodulin gene expression is hormonally regulated in breast cancer cells. Alternately, there is evidence for a more direct interaction, since tyrosine phosphorylation of the ER can be stimulated by both estrogen and calmodulin and inhibited by antiestrogen [125]. This kinase activity, and hence conversion of the ER from a non-binding to a hormone-binding state, may be critical to estrogen action [125].

Oncogenes

The expression of a family of 'early-response' or 'competence' genes, first identified as markers of the competence state discussed above, is markedly enhanced within one hour of treatment of cells with platelet-derived growth factor (PDGF) [126]. This induction is not cell cycle phase specific and is characterized by a rapid increase in mRNA followed quickly by an equally rapid decline. In many cases treatment with protein synthesis inhibitors leads to 'super-induction,' suggesting that preexisting nuclear factors activate transcription and that repression of transcription requires the synthesis of a protein product. The proto-oncogenes c–*fos* [127–130] and c–*myc* [130, 131] belong to this family of genes and are induced in a range of cell types by a wide variety of mitogens, including serum, polypeptide growth factors, and hormones. Although some debate exists as to their role in continually cycling cells, c–*fos* and c–*myc* appear to be necessary for proliferation, since expression of antisense RNA, incubation with antisense oligodeoxynucleotides, or microinjection of antibodies directed against their protein products decreases

the transition of fibroblasts from G_0 to G_1 and also retards the entry of asynchronously cycling cells into S phase [132–136]. However, there are also reports describing uncoupling of proliferation from c–*fos* and c–*myc* induction [137], suggesting that, although necessary, their induction is not sufficient for proliferation. The c–*fos* protein and the product of another early-response oncogene c–*jun*, together for m the transcription factor AP–1 [138, 139]. This protein complex binds to specific DNA sequences thereby activating transcription from heterologous promoters [140–143]. Thus evidence to date suggests that these oncogenes, and c–*fos* in particular, are generalized modulators of transcription that are involved in coupling signals from cell-surface receptors to nuclear responses that in turn, enable proliferation to proceed. This is not incompatible with a controlling function in cell cycle progression.

It has been demonstrated that regulation of *fos* and *myc* occurs as a result of estrogen treatment both of target tissues in vivo [144–147] and of breast cancer cells in vitro [148–151], in the same manner as growth factor regulation of their expression in nonhormone-responsive cells. C–*fos* and c–*myc* mRNA are transiently increased by up to 20-fold, frequently concomitant with an increase in transcription rate, one to two hours after estrogen treatment. This induction follows closely after the appearance of occupied ER [146] and does not depend on protein synthesis, nor is it observed after treatment with nonmitogenic steroids [147]. In some reports a later, broader peak of mRNA induction is also observed. Furthermore, some hormonal (progesterone) or antihormonal (tamoxifen) inhibitors of proliferation decrease c–*myc* mRNA [148, 150, 152] in responsive cells, while c–*myc* mRNA levels are stable in ER-negative, antiestrogen-resistant cell lines treated with either estradiol or tamoxifen [148, 150, 151]. However, as observed in other systems, induction of c–*fos* and c–*myc* mRNA can be dissociated from growth stimulation (e.g., the MDA MB–468 cell line is growth inhibited by EGF but demonstrates a 15-fold to 20-fold increase in c–*myc* mRNA one to two hours after EGF treatment [153]), while TPA, a known inhibitor of MCF–7 cell proliferation, induces c–*fos* and c–*myc* [149].

These data provide evidence for some of the earliest effects of hormonal agents on the transcription of genes thought to be necessary for cellular replication; clearly, steroids can regulate such genes. Indeed, it has been suggested that the effects of estradiol on c–*fos* transcription may be mediated by the direct interaction of ER with the *fos* promoter [146], which contains sequences similar to the 13 base-pair palindromic estrogen-response element [147].

Cell division cycle (cdc) genes

Genes with the potential for cell cycle control have been extensively studied in yeasts, where the ease of genetic manipulation provides an advantage over other eukaryotic systems. Significant progress has recently been made

towards an understanding of the regulation of these 'cell division cycle' (cdc) genes and their products. The product of the fission yeast gene *cdc2* is required for both the transition from G_1 into S phase and the initiation of mitosis [154]; the two functions appear to be regulated independently at a posttranscriptional level and can be genetically dissociated [155]. The protein encoded by *cdc2* ($p34^{cdc2}$) is a protein kinase [156, 157], and homologues have been identified in every eukaryotic species so far examined, including man [158, 159]. They have been best characterized as part of a high-molecular-weight complex, termed maturation promoting factor (MPF), closely related to histone H1 kinase and transiently activated at mitosis [160–164]. In human cells both the phosphorylation of $p34^{cdc2}$ and its association with other proteins contribute to the control of kinase activity [164, 165]. However, the protein kinases responsible for the phosphorylation of $p34^{cdc2}$ have not been identified. There is evidence to support the existence of a second group of regulatory genes that act upon $p34^{cdc2}$ in G_1, where both its substrate specificity and kinase activity may be different to that present at mitosis; and certainly such a role is not necessarily excluded by the data outlined above. This and several additional lines of evidence have been used to argue for a cell cycle model in which an interaction between cyclins, another highly conserved family of cell cycle-dependent proteins, and $p34^{cdc2}$ has a key role in the G_1 commitment of somatic cells to DNA synthesis [166]. This possibility has yet to be investigated. Whether or not this kinase is involved in cell cycle control only at mitosis or in G_1 as well, it will be interesting to determine whether the cell cycle-specific effects of hormones and/or their antagonists are paralleled by, and thus possibly mediated by, changes in the phosphorylation or kinase activity of $p34^{cdc2}$, particularly in view of the coincidence of their actions within the cell cycle.

Conclusions and prospects

The concept that estrogen, growth factors, and other steroids exert their effects on cell cycle progression by actions at major control points for cell division underlies much of the experimental data discussed here. The evidence that most, if not all, of these agents act within G_1 phase supports this idea but does not allow distinction between events that control cell replication and those that accompany it. Furthermore, it is still unclear whether estrogens induce mitogenesis directly or as a consequence of a stimulation of growth factor production. These questions may be addressed by the examination of direct interactions of estrogen, other steroids, and/or growth factors on genes or gene products with established roles in cell cycle progression. A necessary step before such experiments can be undertaken is the delineation of the actions of growth regulatory agents, alone and in combination, in hormonally defined model systems. Reports of defined or steroid and growth factor-depleted media that support the growth of breast

cancer cell lines have long been available [85, 86], but limitations, including low plating efficiency [85] and the recent identification of the estrogenic activity of preparations of the medium component phenol red [167], necessitate further development of suitable systems. Several recent publications have addressed this question [89, 90] and, with our own unpublished data, give confidence that such experiments are now feasible.

The refinement of such experimental systems in conjunction with the recent advances in knowledge of the control of cell cycle progression suggests that further advances in our understanding of the steroidal control of breast cell cycle progression are imminent. Some questions that urgently require answers include:

1. Does estrogen act directly to initiate cell cycle progression, or alternately, is its action mediated by growth factors?
2. Is the estrogen-responsive mitogenic response supplemented by equally effective responses to any single peptide growth factor or combination of growth factors?
3. What are the molecular mechanisms by which these responses are inhibited by antiproliferative steroids?
4. Is growth inhibition by each steroid or steroid antagonist mediated by a different pathway, or does each converge on a common event?
5. Is modulation of steroid- or steroid antagonist-induced growth inhibition by growth factors due to effects on cell cycle control mechanisms or to effects on the concentration or phosphorylation of the steroid receptors?
6. Do steroids and their antagonists, through interactions with their receptors, directly control the transcription of cell cycle progression genes, particularly proto-oncogenes?
7. What is the role of phosphorylation in controlling steroid hormone actions on proliferation, i.e., the interaction between steroid hormone receptor-mediated pathways and phosphorylation of cellular proteins critical in cell cycle progression by growth factor receptor tyrosine kinases, calmodulin, and the $p34^{cdc2}$ kinase?

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IV.

Stromal-Epithelial Interactions and Metastases

16. Stromal regulation of epithelial function

Annemarie A. Donjacour and Gerald R. Cunha

Abstract

Stromal influences upon epithelia are part of a continuum of cellular interactions that begins at fertilization and extends into adulthood. In parenchymal organs, the most thoroughly characterized interactions have been those that occur during development between mesenchyme, embryonic stroma, and epithelium. Mesenchyme is essential for epithelial proliferation, morphogenesis, and differentiation. Hormones affect stromal-epithelial interactions, and in some cases, steroid hormones may produce their effects on the epithelium indirectly, acting via the mesenchyme. In many adult organs the epithelia continually proliferate and differentiate and consequently may be considered developing systems within the mature organism. This is especially true in organs with a rapidly renewing epithelium, such as the intestine, and in organs that have cycles of functional activity, such as those of the female reproductive system. The mechanisms by which stroma affects epithelial structure and function are not well understood. Current models of how signaling may be accomplished include transmission via diffusible substances, via the extracellular matrix (ECM), and via direct cell–cell contact. Growth factors and organ-specific paracrine factors are candidates for stromal cues that affect the epithelium in some systems. Components of the ECM appear to play a role in permissive interactions and may affect epithelial function by changing cell shape or by binding ECM to the cell surface integrin receptors. Signaling via direct stromal-epithelial contact may be accomplished via interactions between complimentary cell surface adhesion molecules. The importance of stromal-epithelial interactions is reemphasized by several models of carcinogenesis that suggest that perturbations in these interactions may be involved in tumor progression.

Introduction

All parenchymal organs are composed of a stroma, or connective tissue, which is derived from embryonic mesenchyme, and an epithelium, which may

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be derived from any of the three embryonic germ layers. Although it s usually the epithelium that is directly responsible for the major functional activity of an organ at maturity, the stroma and epithelium work as an integrated unit. The stroma influences the biology of the epithelium in many ways, especially during the development of the organ, but this influence also continues into adulthood. In fact, stroma may play a role in epithelial carcinogenesis. Several excellent reviews are available that discuss in detail earlier work on mesenchymal-epithelial interactions [1–4]. Specific features of the mammary gland are discussed in the chapter by Haslam [this volume]. This review will describe the general characteristics of stromal-epithelial interactions during development and in adulthood, consider the possible mechanisms that may underlie such interactions, and finally will discuss the ways in which abnormal stromal influences may affect the development of cancer.

The nature of stromal influences on epithelium

Developmental events

The effects of mesenchyme (embryonic or fetal stroma) on epithelium during organogenesis have been studied in mammals for many years, beginning with the work of Grobstein [1, 3, 5, 6]. To examine these interactions, several experimental approaches were used and are still used today [2]. Embryonic organ rudiments have been grown in organ culture either as whole organs, as separated tissue components, or as experimental tissue recombinations of different epithelia and mesenchymes. Grafting of organ rudiments or tissues into host animals is also used for growth of tissue recombinants. It was clear from early experiments that the survival, proliferation, morphogenesis, and differentiation of the epithelia from many organs depend upon the presence of mesenchyme [3].

When mesenchyme and epithelium from two different organs are recombined (heterotypic recombinants), the resulting tissue recombination generally develops in one of two ways: the epithelium may continue to develop as it would have in vivo (a permissive interaction or induction), or its fate may be changed often to coincide with the original fate of the mesenchyme (an instructive interaction or induction) [7]. These two common classifications of embryonic interactions should be recognized as operational definitions, though, in general, they probably describe in vivo processes. During an instructive interaction, the responding epithelial cell is being prompted by the mesenchyme to choose between two or more developmental options. Once this choice is made, the epithelium is said to be 'determined,' thus allowing the epithelium to stably express its final phenotype. Full expression of this phenotype may not occur for some time, however, and it is during this time that permissive interactions become important [3]. The stable, continued expression of a specific epithelial phenotype in adulthood appears to be

predicated upon continued contact between epithelium and stroma (see below).

To assess whether or not the fate of the epithelium is induced to change in heterotypic recombinants, morphological criteria, such as epithelial branching pattern or histological organization, were initially used. In more recent experiments, however, the production of specific epithelial products has been examined. In some cases mesenchyme induces changes in epithelial branching pattern, cytodifferentiation, and function in accord with the origin of the mesenchyme. However, in other heterotypic recombinants, morphological changes occur but biochemical parameters of the epithelium remain unaltered. For example, mammary gland epithelium recombined with salivary gland mesenchyme produces and organ whose epithelium has a salivary gland-like branching pattern, but which produces α–lactalbumen, a milk protein [8]. Similar mixed inductions have been observed with digestive tract tissues [9]. The distinction between an instructive and a permissive induction becomes unclear in these cases, but these experiments may reflect underlying properties of the signals required for morphogenesis versus those required for secretory differentiation.

An important aspect of mesenchymal-epithelial interactions during organogenesis is that they are part of a continuum of cellular interactions that begins with fertilization. These interactions are sequential and the consequences of each set of interactions influences cellular activity in successive interactions [7, 10]. Both the epithelium and stroma of each organ rudiment already have a developmental 'history.' Therefore, one might expect that not all mesenchymes could support the differentiation of a given epithelium and that the possibilities for instructive interactions between heterotypic mesenchyme and epithelium would be relatively limited. A successful instructive interaction suggests that a similarity in inductive mechanisms exist between the two organs used to construct the tissue recombinant. The outcome of heterotypic tissue recombinations varies and is not readily predictable [2, 11–13], although tissues that have a common developmental history tend to interact more successfully, e.g., ectodermally-derived epidermis is capable of being induced to form a variety of ectodermal derivatives [4].

The initial step in an organogenetic tissue interaction is an instructive or determinative event in which the epithelium becomes committed to a particular prospective fate. It is at this stage that the developmental fate of the epithelium becomes fixed. Subsequently permissive signals from the mesenchyme come into play, and the epithelium differentiates and in some cases becomes more independent of mesenchymal cues. For example, during pancreatic development in the mouse, the prepancreatic endoderm from the 7 to 9 somite stages will differentiate into pancreatic tissue in vitro only when it is in close contact with its own mesenchyme, but not with other mesenchymes. After the 15 somite stage (9 days of gestation), development of pancreatic epithelium can be supported by heterologous mesenchyme [15]. During this time the epithelium begins to bud into the mesenchyme. At the 30

somite stage specific pancreatic products can be detected in the epithelium [16–18]. Embryonic pancreatic epithelium is unusual in that after the 30 somite stage it can continue to develop in vitro without mesenchyme, provided embryo extracts or conditioned medium from mesenchyme are added [15, 19].

Temporal factors play an important role in interactions between mesenchyme and epithelium, especially in instructive interactions. In organ systems that must function at birth, mesenchymal-epithelial interactions elicit complete morphological and biochemical differentiation during embryonic and fetal stages. In other organs, especially those of the reproductive system, development occurs to a large extent postnatally and is not completed until after puberty. This means that organogenetic tissue interactions may be telescoped into brief periods prenatally or spread over considerable periods postnatally. Organogenetic tissue interactions are dependent upon both inductive and responsive tissues, which in parenchymal organs are mesenchyme and epithelium, respectively. The appropriate developmental outcome is abortive if either the inductor fails to produce its inductive message or the responsive tissue loses its competence or responsiveness. Both inductive activities and competence may be influenced by the age of the tissue. Early embryonic inductions, such as induction of mesoderm or neural tube, studied in amphibia appear to be limited in time mainly by the period of competence of the responding tissue [20]. In the case of the female urogenital sinus (UGS) of the mouse, it appears that the age-dependent failure of this rudiment to form prostate in response to exogenous androgens is due to a loss of the inductive signal from the mesenchyme [21]. Both the male and female UGS are capable of forming prostate under the influence of androgens [21–23]. However, as the intact female urogenital sinus undergoes vaginal and urethral development in the absence of androgens, it gradually loses its ability to form prostate in response to androgens. To examine whether alterations in the inductive activity of the mesenchyme or the responsiveness of the epithelium are responsible for this change, urogenital sinus epithelium (UGE) or vaginal epithelium (VE) from mice of different ages (16 days embryonic to 20 days postnatal) was recombined with UGM (16 days embryonic) and grafted into male hosts. Prostatic differentiation resulted in the majority of these tissue recombinants, which indicates that epithelial competence to prostatic induction is maintained until at least 20 days post partum. However, when mesenchyme from vaginal rudiments (VM) of mice at increasing ages (0 days to 20 days postnatal) was recombined with UGE (16 days embryonic), the percentage of VM + UGE tissue recombinants that produced prostate decreased with the age of the mesenchyme and was zero by 20 days of age [21]. Thus the loss in the ability of the female UGS to form prostate correlates with a loss in prostatic inductive activity of the vaginal mesenchyme. The converse situation exists in instructive inductions using vaginal stroma and uterine epithelium. In this case the uterine epithelium is induced to express vaginal differentiation provided it is obtained from mice that are less than

9 days of age. However, uterine epithelium from mice older than 9 days of age does not respond to the inductive influence of vaginal stroma but instead continues to express uterine differentiation. Even adult (150-day-old) vaginal stroma is capable of inducing competent neonatal uterine epithelium to undergo vaginal differentiation [24]. Therefore, the loss of epithelial competence is responsible for the failure of an instructive induction to occur in this tissue recombination. In contrast to instructive interactions, permissive interactions appear to be less restricted in time and may continue throughout adulthood to maintain epithelial function (see below).

It is unclear in most systems whether epithelial competence is due to the ability of each individual cell to change its phenotype in response to the mesenchyme, or whether epithelia are heterogeneous and respond to the mesenchyme by a process of cell selection. Since epithelial proliferation accompanies determination, it is difficult to distinguish between these two possibilities. However, the induction of mesoderm in animal pole cells by vegital pole cells in Xenopus does not require cell division and appears to be a case of a direct change in cellular phenotype rather than of cell selection [20, 25]. The relevance of these findings to stromal-epithelial interactions in high vertebrates is unclear.

Spatial factors also play an important role in stromal-epithelial interactions. During early embryonic inductions, such as that of mesoderm and neural tube, more epithelial cells can respond to a given mesenchymal signal than actually do [20], suggesting that the transmission of the inductive signal within the tissue determines the spatial distribution of the response. This also appears to be true during organogenesis. For example, the optic cup induces lens formation where it makes close contact with head ectoderm [26]. The precise patterning of feathers in the chick [14] and the vibrissae follicles in the mouse [27–29] are presumably due to the earlier patterning of the dermal papillae in feather germs and hair follicles, as in both cases it is known that the dermal papillae are the inductors of these structures. The branching pattern of lung [30, 31] and the specification of structures within the urogenital sinus [32] are also determined by the mesenchyme. The precise ordering observed in the structures suggest that the inductive signal from the mesenchyme acts only over a relatively short range.

Systemic hormones impinge upon stromal-epithelial interactions during organogenesis in a number of ways. For example, male secondary sex organs, which develop as a result of epithelial-mesenchyme interactions, [33] have an absolute requirement for androgens for their development [34–36]. In other organs, hormones affect only a subset of developmental events; for example, glucocorticoids stimulate the synthesis of specific products in the differentiating lung but do not affect lung growth [37, 38]. Finally, the development of another group of organs, such as the uterus or vagina, do not require hormonal stimulation [39] but are responsive to the administration of exogenous hormones, e.g., estrogens, which may elicit teratogenic or carcinogenic changes [40–45].

In the developing reproductive system, the receptors for the steroid hormones relevant to development, androgens in the male and estrogens in the female, first appear in the mesenchyme (mammary gland [46, 47]; prostate [48]; seminal vesicle [49, 50]; bulbourethral bland, ductus deferens, and epididymis [49]; uterus and vagina [45, 51]). In the mouse, androgen receptors are present at embryonic day 16 in the UGM but do not appear in the epithelium until approximately four days after birth [48]. During this time, such androgen-dependent processes as branching morphogenesis of the epithelium are initiated [23, 52, 53], and the prostate increases in size [54]. A similar situation exists in the uterus of the Balb/c mouse. Stromal estrogen receptors are present at birth, but epithelial estrogen receptors do not appear until 5 days postnatal or later in Balb/c mice [45, 51, 55], after 8 days in NMRI mice [56], or 3 days in D–1 mice [57–59], yet these epithelial cells clearly proliferate in response to estrogen [45, 51]. In the rodent mammary gland, fetal androgens cause the destruction of the epithelial mammary gland rudiment in the male [60, 61]. Mammary epithelial cells do not have androgen receptors at the time when cell death is elicited by androgens, but the mesenchymal cells do have these receptors [46, 47]. In this case a steroid hormone is acting indirectly to cause epithelial cell death [62, 63].

Experiments using tissue recombinants composed of epithelium or mesenchyme from mice with the testicular feminization mutation (Tfm) also strongly support the model of an indirect mechanism of steroid hormone action. Tfm mice lack functional androgen receptors [64–67], and although they have testes and produce nearly normal levels of androgens as neonates [68], their secondary sex organs and external genitalia are feminized [69]. If recombinants are made with UGM from Tfm mice plus epithelium from normal, androgen receptor-positive mice and grown in the presence of androgens, the resulting tissue recombinants will fail to become prostate. Instead, these recombinants form vaginal tissue. If a tissue recombant is made with wild type UGM plus epithelium from a Tfm mouse, normal prostatic development occurs in the presence of androgens [22, 70, 71]. The prostatic ductal epithelium in these tissue recombinants never expresses androgen receptors [72, 73]. Thus prostatic development occurs in the complete absence of epithelial androgen receptors. Similar tissue recombination experiments have been done with mammary gland epithelium from Tfm mice, and similar conclusions have been drawn. In tissue recombinant composed of wild type mammary mesenchyme plus Tfm epithelium, regression of the epithelium in response to androgens occurs in the genetic absence of androgen receptors in the epithelium [62, 63].

At later stages of development in the reproductive system, an indirect action of androgens is suspected as well. In the testes of pubertal rats, in which the Sertoli cell are committed but still differentiating, the Sertoli cells in culture are stimulated to secrete transferrin and androgen-binding protein (ABP) by a factor (P–mod–S) produced by the surrounding peritubular (stromal) cells [74]. Secretion of P–mod–S is stimulated by androgens [74], and the peritubular cells possess a significantly higher concentration of 5

α–reductase and androgen receptors than do Sertoli cells [75, 76]. In very pure Sertoli cell cultures, (i.e., few myoid cells), testosterone produces only a slight increase in ABP secretion by Sertoli cells [77]; this response to testosterone is augmented by the presence of peritubular cells in coculture with the Sertoli cells [77]. Although androgens may be acting directly on Sertoli cells as well, it is quite possible that the peritubular cell factor is important in mediating the effect of androgens in the developing testes.

An indirect mechanism of steroid action is not restricted to the reproductive system. During normal development of the lung, glucorcorticoids act via the mesenchyme to stimulate the synthesis of surfactant by alveolar type II cells. Lung fibroblasts secrete a soluble factor, termed fibroblast pneumonocyte factor (FPF), in response to glucocorticoids [78, 79] during a specific time of development [80]. FPF then acts on the type II cells to make surfactant phospholipids [38]. In males (presence of androgens), lung development and surfactant synthesis are retarded [81]; this response also appears to be mediated by the stroma. Dihydrotestosterone decreases the stimulatory effect of cortisol on stromal FPF synthesis [82, 83] but does not decrease the epithelial response to FPF [84]. During lung development, two different steroid hormones may affect lung maturation via the stroma.

Certainly not all hormonal effects on developing epithelia are indirect. The direct effects of hormones can modulate and are often required for normal stromal-epithelial interactions. In the case of the lung, thyroid hormone works together with FPF on type II alveolar cells to increase surfactant production. Without FPF, thyroid hormone has no effect [38]. In pure uterine epithelial cell cultures, estrogen stimulation increases the number of progesterone receptors [85]. Mammary epithelial cells in vitro require prolactin, insulin, and cortisone, which act directly on the epithelium to stimulate milk protein production. The extracellular matrix (ECM) on which the cells are cultured affects the degree to which prolactin is effective [86].

Although this chapter focuses on stromal influences, it should be noted that in many systems the epithelium has a clear reciprocal effect upon the stroma. The epithelia of the developing intestine and uterus play an important role in organizing the smooth muscle that surrounds them [87, 88]. During embryonic development in the mammary gland, the epithelium induces androgen receptors in the adjacent mesenchyme [46]. Other carefully studied examples of reciprocal tissue interactions include the effect of ureteric bud epithelium on the metanephric mesenchyme of the kidney [10], the influence of ameloblast basal lamina on odontoblast differentiation in the tooth [89], and the influence of the apical ectodermal ridge on limb mesenchyme [90].

Interactions in adulthood

Processes resembling embryonic development continue into adulthood, particularly in the mammary gland and urogenital tract. Some organs, such as those of the reproductive system, undergo extensive growth and differentia-

tion postnatally, especially at the time of puberty. In females and in seasonally breeding males, dramatic tissue remodelling and development occurs cyclically during adulthood. More subtly, most epithelia undergo renewal throughout life, and individual cells must continually divide and differentiate within the epithelium. This process is especially prominent in rapidly renewing epithelia, such as those of the gastrointestinal tract, female genital tract, or the skin. These continuous processes of development are likely to proceed via mechanisms similar to those operating in the embryo and fetus. Several lines of evidence indicate that stroma continues to exert important influences on the epithelium in the adult.

Fibroblasts from some neonatal tissues retain the ability to change the phenotype of, that is induce, heterotypic adult epithelium. In the intestine throughout life, epithelial cells proliferate in the crypts and differentiate as they move up toward the villi. The pericryptal fibroblasts also proliferate deep in the crypts and move toward the villi in concert with the differentiating epithelium [88, 91, 92]. The intestinal epithelium and adjacent stroma can therefore be considered as a continually differentiating system within the adult organism. This view is consistent with the ability of the neonatal intestinal stroma to instructively induce gizzard epithelium to form intestine [93]. The uterus and vagina are not fully differentiated at birth but will only achieve that status at puberty. Stroma from the neonatal uterus can instructively induce neonatal vaginal epithelium to undergo uterine differentiation. Conversely, vaginal stroma from mice as old as 150 days of age can induce the simple, columnar uterine epithelium from a neonate to become a stratified cycling vaginal epithelium [24, 33]. The inductive abilities of the neonatal vaginal and uterine stromas are retained even if the stromal cells are grown in culture for as long as four weeks [94]. Throughout adulthood both uterus and vagina change their morphology throughout each estrous/menstrual cycle, and therefore these organs can also be viewed as continually developing systems.

Grafting experiments with adult skin suggest that the dermis maintains its ability to induce regional differentiation and, to a limited extent, the epidermis remains competent to respond. These experiments were done with animals of different pigmentation so that contamination with host epidermis could be excluded. In the guinea pig, epidermis from the sole, ear, or trunk changes its histology in accord with source of the dermis when grafted to either sole, ear or trunk sites. In contrast, lingual and esophageal epithelium retain their specificity when grafted onto the dermis of the sole or ear [95]. In a somewhat different type of experiment, dissociated epithelial cells from the trunk, esophagus, or cornea were injected into trunk dermis in situ. The epithelium of esophagus and cornea were not instructively induced but formed cysts whose morphology resembled that of the original epithelium [96]. Instructive inductions were more successful with closely related epithelia (e.g., ear dermis with various other skin surface epithelia) than with more distantly related epithelia (e.g., ear dermis with mucosal epithelia).

In a limited way, differentiated avian scale dermis is also capable of instructively inducing heterotypic epithelium. At embryonic day 16, the scutate and reticulate scales of the chick have assumed their adult forms and are no longer capable of inducing scale morphogenesis in chorioallantoic epithelium [97]. These dermises, however, remain capable of directing the epithelium of the chorioallantoic membrane to produce keratins that are specific to the type of scale produced [98].

In many in vitro systems, maintenance of adult epithelial differentiation and physiological responsiveness can be achieved in organ culture but not in pure epithelial cell cultures (intestine [88], liver [99], bladder [100]), suggesting that stromal factors do play an important role. This appears to be especially true for the reproductive tract organs where steroid hormone responsiveness is lost when the epithelial cells are grown in culture [85, 101–105]. In some cases inproved epithelial differentiation in vitro has been achieved by partially reconstructing elements of the stroma by seeding the epithelium in or on ECM [86, 100, 106], associating them with fibroblasts, or by using fibroblast-conditioned medium [107].

As is the case during fetal development of the reproductive tract, in adulthood steroid receptors are sometimes present exclusively in the stroma during stages of the female reproductive cycle [108]. For example, in the uterus of the macaque during the transition between the luteal and follicular phases, estradiol receptors reappear in the stroma just prior to the estradiol-stimulated increase in epithelial proliferation [109]. Estradiol receptors return to the uterine epithelial cells as epithelial proliferation increases, but the distribution of receptors is patchy [109]. By simultaneous estrogen receptor immunocytochemistry and thymidine autoradiography, it has been shown that epithelial cells that do not have estrogen receptors can proliferate in response to estradiol [110]. In the oviduct, estradiol stimulates ciliogenesis, yet the ciliated cells never possess estrogen receptors [108]. Electron microscopic observations provide additional evidence for stromal-epithelial interactions in the adult reproductive tract. In the human uterus during the late proliferative and early luteal phases, there is an increase in the number of interruptions in the basal lamina. Epithelial processes extend through these holes and make contact, sometimes apparent junctional contact, with the underlying stromal cells [111, 112]. These data suggest that in the adult reproductive tract, steroid hormones can act on the epithelium via the stroma.

Epithelia from various adult organs retain their ability to proliferate and differentiate in response to fetal stroma [113]. Embryonic mammary mesenchyme or salivary gland mesenchyme, when grafted into the mammary gland of adult virgin mice, induces the otherwise quiescent epithelial ducts to proliferate and form new ductal structures [8, 114]. The ability of the mammary epithelium to respond is somewhat restricted, however, as proliferation cannot be elicited with other mesenchymes, nor can salivary gland mesenchyme stimulate proliferation by lactating mammary epithelium [114].

The epithelia of the adult prostate, ductus deferens, and seminal vesicle also remain able to respond to mesenchyme [115–117]. In tissue recombinants of small segments of adult prostatic duct plus UGM grown as renal subcapsular graft, the adult epithelium proliferates tremendously and forms mature prostate; prostatic ducts grafted alone do not grow [115].

A particulary dramatic example of the plasticity of an adult epithelium is the response of adult bladder epithelium (BLE) to UGM. After four weeks of growth in a male host, the epithelium of UGM + BLE recombinants not only proliferates a great deal but also differentiates into prostate [118, 119]. This instructive interaction is not limited to morphological changes but also includes the induction of androgen receptors in the epithelium [120], a shift in the profile of newly synthesized proteins [120], and the secretion of prostatic proteins by the induced bladder epithelium [121]. Bladder epithelium can also be instructively induced by neonatal seminal vesicle mesenchyme; in this case, however, the recombinant forms prostate, a tissue to which neither epithelium nor mesenchyme would ordinarily give rise [122]. Thus bladder epithelium appears to be especially competent to respond to mesenchymal signals. Since bladder epithelium has a very low proliferative rate in vivo [123], it appears that the normal rate of epithelial renewal is not correlated with its ability to respond to exogenous inductive signals.

The nature of stromal signals in adulthood is not clear. Stromal influences on adult epithelia in vivo are most likely to be permissive rather than instructive, since their function is the maintenance rather than the genesis of the differentiated state. However, it is important to recognize that each epithelium is constantly renewing itself, and therefore many parenchymal organs are, to a certain extent, developing systems.

Mechanisms of stromal-epithelial interactions

Stromal-epithelial interactions mediate a variety of developmental processes that comprise organogenesis, and consequently a variety of mechanisms are likely to be involved [124]. In no system has the mechanism of the entire developmental process been completely characterized. In studying the mechanism of these interactions, it may be useful to distinguish the signal molecule(s) itself from its mode of transmission, since these aspects might be experimentally separable. The three most likely modes of transmission are free diffusion, cell–ECM contact, and direct cellular contact either via cell surface molecules or intercellular junctions. Stromal signals must act over a relatively short distance and must be able to affect changes in the genome of the responding cell. The differing natures of instructive and permissive interactions indicate that they might be accomplished by different types of mechanisms. The specificity of instructive inductions suggests a similarity with receptor-mediated responses [20], whereas permissive interactions may be mediated by relatively nonspecific factors, such as the availability of particular amino acids or lipids.

Systems for which a soluble inductive agent have been isolated are few. One of the most well-characterized instructive inductions, and the only one for which the putative regulatory factors are well characterized, is the induction of mesoderm in Xenopus. In vivo cells of the marginal zone are induced by vegital hemisphere cells to form mesoderm. In this system it appears that at least two diffusible substances are involved; one is similar to mammalian fibroblast growth factor (FGF) and the other is simliar to transforming growth factor–β (TBF–β). Purified basic FGF [125–127] or TGF–β_2 [128] causes Xenopus animal pole cells to differentiate in culture into several types of mesodermal tissue. TGF–β_1 can work synergistically with basic FGF to induce mesodermal tissue as well [126]. Animal pole cells, which normally give rise only to epidermis, are clearly sensitive to these exogenous growth factors, which elicit mesodermal differentiation. An mRNA with significant homology to TGF–β (Veg 1) is localized in the vegital hemisphere of Xenopus oocytes [129], and FGF is also present within the egg [126]. Therefore, it is highly likely that the Xenopus blastula uses homologues of these two growth factors in the induction of mesodermal tissues.

Growth factors have also been implicated as mediators of local growth and proliferation during organogenesis in a few systems. For example, supernumery bud formation can be induced in the fetal lung by grafting onto the trachea a piece of agar that had been soaked in epidermal growth factor (EGF) [130]. In the mammary gland, implanted pellets of EGF stimulate proliferation and branching morphogenesis locally, even in ovariectomized mice [131]. In addition, EGF receptors are found in the highest concentrations in the specialized epithelial cap cells, the myoepithelial cells, and in the stromal cells adjacent to the growing end buds of mammary ducts [131]. Though it is far from clear, EGF may also be involved in autocrine or paracrine control of uterine growth. EGF can stimulate the proliferation of uterine epithelial cells in culture [132, 133], while estradiol cannot [105]. In vivo estradiol administration increases the concentration of EGF receptors in the uterus [134]. All cell types in the immature and adult uterus have EGF receptors [135, 136], but it is not known whether estradiol elicits a selective increase in these receptors in any one cell type. In the immature and adult uterus, EGF is localized in the epithelium and myometrium, not in the stroma. The majority of the EGF is, however, in the membrane-bound precursor form, and its levels are stimulated only weakly or not at all by estradiol [137].

TGF–β influences the proliferation and differentiation of many types of cells, with the specific effect being highly dependent on the cell type. In general, however, TGF–β increases the proliferation of mesenchymal cells, inhibits the proliferation of epithelial cells, and stimulates epithelial differentiation [138, 139].

For several stromal-epithelial interactions during organogenesis, candi-

dates for a diffusible regulatory substance are being characterized but have not yet been purified. As mentioned previously, fibroblast pneumonocyte factor is secreted by embryonic lung fibroblasts in response to cortisol [78, 79]. It appears to act specifically to stimulate surfactant production and has no effect on proliferation in the lung [37, 78]. FPF has been partially purified and appears to be a 5 KD polypeptide [78]. Another soluble factor that very specifically affects the differentiation of a committed cell is P–mod–S, which is secreted by testicular peritubular cells and which stimulates the synthesis of a subset of Sertoli cell secretory products [74]. Like FPF, this factor also has no effect on proliferation [74]. P–mod–S activity has been purified and consists of two polypeptides of 56 and 59 KD; both proteins bind to heparin [140]. A third putative modulator of epithelial behavior is 'scatter factor,' a 50 KD protein that has been isolated from a variety of embryonic fibroblasts. Its primary effect, as observed in culture, is to prompt the dissolution of epithelial sheets and stimulate the migration of individual cells [141]. This factor seems to be nonspecific, as it works on several types of adult, neonatal, and fetal epithelia. A role for scatter factor in any developmental system has yet to be determined.

In the mammary gland, the epithelial cells proliferate and differentiate within an adipose stroma, and stromally derived lipids have been implicated in stromal-epithelial interactions [142]. Certain fatty acids, such as phophatidic acid, appear to be mitogenic for mammary epithelial cells in culture, even in the absence of mammogenic hormones [143, 144]. Similar phospholipids can also increase the proliferation of certain cultured fibroblasts. These lipids appear to act via multiple intracellular signaling pathways [144]. Linoleic acid can potentiate the effect of growth factors, such as EGF, on mammary epithelial cells, but it is not mitogenic alone [145, 146]. In addition, the differentiation of these epithelial cells in culture can be modulated by linoleic acid [147].

Most known growth factors act via plasma membrane receptors to stimulate one or several of a variety of second messenger systems [139, 148–150]. It is as yet unknown precisely how proliferation or changes in transcription of specific genes is achieved, though certain proto-oncogenes and DNA-binding proteins, such as NF–1, have been implicated [139]. The induction of mesoderm appears to involve a similar intracellular signaling pathway, as does malignant transformation with polyoma virus. If mRNA for the polyoma middle T antigen is micoinjected into frog oocytes, isolated animal cap cells from the resulting embryos will differentiate into mesoderm [151]. Microinjection of middle T antigen mRNA from a transformation-defective mutant fails to cause mesoderm formation [151]. Middle T antigen is found in the plasma membrane and associates with and activates $pp60^{c-src}$, a cellular typrosine kinase [152, 153].

Some growth factors have a significant effect on ECM deposition and degradation. TGF–β is especially potent in this regard. Its effect on most cells is to increase the accumulation of several ECM constituents, e.g., type I

collagen and fibronectin, by either increasing their deposition, reducing their degradation, or both [138, 139].

Some nonproteinaceous signaling molecules also act via specific cellular receptors. For example, retinoic acid appears to be the endogenous morphogen that confers anterior-posterior polarity in the chick limb [154]. This small molecule acts via an intranuclear receptor that is in the same family as the steroid receptors [155]. Its mechanism of action is likely to be similar to that of the steroid receptors and involve binding to specific sites on the DNA to modulate transcription [156, 157].

Extracellular matrix

Based on his experiments on salivary gland morphogenesis, Grobstein [6] first postulated that the interaction between epithelium and mesenchyme was mediated by ECM during organogenesis. Subsequent experiments with other systems, both fetal and adult, have emphasized the importance of the ECM in many permissive interactions [1, 158, 159].

The most comprehensive model for how mesenchyme affects epithelial morphogenesis has been developed by Bernfield and coworkers through studies on the salivary gland [160]. Epithelial branching morphogenesis in this gland requries a temporally and spatially coordinated synthesis, deposition, and degradation of basal laminar glycosaminoglycans in the lobules and intervening clefts of the salivary gland. Proliferation of the epithelium is highest at the ductal tips of the branching gland [161]. As a cleft deepens between adjacent lobules, collagen produced by mesenchymal cells stabilizes the branch point and proximal duct. The process is dependent upon an epithelial-mesenchymal interaction since in the absence of mesenchyme, proliferation is no longer localized and cleft formation fails to continue [161].

During tooth development, a reciprocal interaction of epithelium with mesenchyme, the ameloblast epithelium stimulates differentiation of the mesenchymal odontoblasts. In this process mesenchymal cell processes must make contact with the intact basal lamina of the ameloblast epithelium in order to trigger odontoblast differentiation [89, 162]. In the cornea, contact with killed lens capsule stimulates differentiation of the corneal epithelium, implicating ECM, and not the cells of the lens, as the inductive signal [163]. In fact, pure collagen can substitute for the lens in eliciting corneal epithelial differentiation from determined corneal epithelium. This lead Meier and Hay [163] to question the use of the term 'permissive induction' and suggested that in some systems all that was needed for differentiation was the proper reconstitution of the ECM. This idea is supported by a number of epithelial cell culture systems in which many aspects of epithelial functional differentiation can be maintained by an acellular ECM of the proper composition. On the other hand, adult bladder epithelium will not maintain its phenotype when grown alone on plastic or on a collagen-coated surface but requires a three-dimensional substrate, such as a collagen gel, in order for fibroblast-

conditioned medium to have a beneficial effect. In this system at least two mechanisms, ECM and a diffusible factor, are involved in maintaining adult urothelial differentiation [100]. Cultured hepatocytes also maintain many aspects of their differentiated state when grown in a three-dimensional collagenous substrate [164]. Extensive work on mammary epithelium in culture also shows that ECM greatly improves the degree of differentiation of these cells [86; Bissell, this volume]. Specific ECM components, such as laminin, type IV collagen, and tenacin, which appear at sites of mesenchymalepithelial interactions and of mesenchymal condensation, have also been suggested as important regulators of epithelial differentiation in the mammary gland in vivo [165, 166; Sakakura, this volume; Haslam, this volume].

Though the distributions of certain ECM components show interesting correlations with morphogenesis, a causal role for these molecules is in doubt in some cases. For example, stabilization of the basal lamina by collagen type I [167] is thought to be an important step in branching morphogenesis [160]. However, branching morphogenesis is apparently normal in embryos that are genetically deficient in collagen type I [168]. Other types of fibrillar collagens may substitute for type I collagen [168]. During kidney tubule formation, the spatial distribution of fibronectin changes in a specific way, implicating it as a developmentally important signaling molecule. Fibronectin is present in abundance in uninduced metanephric mesenchyme and disappears from areas where kidney tubules form. It remains associated with the branching ureter and the stroma close to the developing kidney tubule and is later located in the developing glomerular basement membrane [169]. Nevertheless, in culture antibodies to fibronectin and peptides that bind to the fibronectin receptor fail to block the branching of the ureteric bud or the development of kidney tubules [170].

One of the few systems in which an instructive induction may be mediated by ECM is the differentiation of axolotl neural crest cells into either melanocytes or neurons. When undifferentiated neural crest cells migrate via the subepidermal pathway, they become melanocytes, whereas if they migrate via the medioventral route, they differentiate into peripheral nervous system structures [171]. Perris and coworkers [172] implanted a nitrocellulose membrane along each of the two neural crest pathways. ECM from these two microenvironments was deposited on the filters, which were then placed in culture. Undifferentiated neural crest cells grown on either of these two filters differentiated into the cell type that corresponded to the site from which the filters had been removed, i.e., cells grown on filters from subdermal sites became melanocytes, and those grown on filters from the presumptive region of the dorsal root ganglia became neurons. Two-dimensional gel analysis of proteins from the two types of filters revealed several differences [172]. Interpretation of these experiments is complicated, since some growth factors bind to ECM [139, 148, 173], and therefore growth factors may have actually been responsible for this induction.

As in the case of soluble factors, the precise mechanism by which the ECM affects the target epithelium is not fully understood. In some case ECM might be the mode of transmission of growth factors and other paracrine regulatory molecules [139, 148, 173]. FGF binds avidly to the heparan in glycosaminoglycans and may be bound to heparan even before it is secreted [148]. This binding would serve to concentrate FGF activity in a localized area; the mechanism by which the FGF subsequently becomes available to act on neighboring cells is not yet clear [148]. The fact that several types of epithelia in culture require ECM in a three-dimensional array [100, 164] suggests that ECM may be modulating cell function by changing cell shape. This mechanism has been addressed in detail by Bissel [this volume].

Many ECM components, such as fibronectin, laminin, vitronectin, fibrinogen, thrombospondin, and collagen types I, IV, and VI, bind to epithelia via a family of cell surface receptors called integrins [174–178]. The density and the type of integrin receptor on a cell's surface can modulate its adhesive properties and may also stimulate an intracellular signal. Antibodies to the fibronectin receptor induce an increase in the level of mRNA and protein of two metalloproteases, collagenase and stromelysin, in synovial fibroblasts in culture [179]. In contrast with other agents that induce metalloproteases, binding of this antibody does not induce a concurrent change in fibroblast shape. This suggests that these antibodies to the fibronectin receptor, and possibly fibronectin itself, may not be affecting transcription via the cytoskeleton but via another transmembrane signal [179].

Cell–cell contact

Direct cell to cell contact has been implicated as a mechanism for stromal-epithelial interactions in situations where there are significant breaks in the basal lamina that would allow for association between epithelial and mesenchymal cell processes [3]. For example, when odontoblasts stimulate ameloblast differentiation in the developing tooth, epithelial protrusions cross the basal lamina and make close contact with the underlying odontoblast cells [89]. The same phenomenon is observed in the rodent gut three to four days before birth [180] as well as in the embryonic lung [181]. In the adult uterus, not only do processes traverse the basal lamina, but junctional associations may be present between stromal and epithelial cells [111, 112].

The developing kidney has been the most thoroughly studied experimental system in which cell contact has been implicated as the mechanism by which induction occurs. In transfilter experiments, the degree to which the spinal cord induces metanephric mesenchyme to form tubules is in direct proportion to the degree of cell–cell contact allowed by the filter [182, 183]. No organized junctional contacts were seen between the interacting tissues.

In a few cases, intracellular junctions between epithelial and stromal cells have been reported [111, 112]. However, this does not appear to represent a major mechanism of stromal-epithelial interaction in organogenesis.

If direct cell to cell contact is necessary for stromal-epithelial interactions, the actual signaling may be accomplished by the interaction of complimentary surface molecules on each cell. There is little data suggesting direct membrane–membrane contact. Circumstantial evidence points to a growing class of cell surface glycoproteins, the cell adhesion molecules (CAMs) as possible mediators of induction [184–186]. CAMs may bind to other identical or different CAMs and are certainly able to effect the selective adhesion of cells [185]. The pattern of expression of these molecules marks the boundaries of morphogenetically important tissue compartments [184, 185]. In some cases, especially in the adult, CAMs are associated with zonula adherens junctions and may be linked to the actin cytoskeleton [186]. Antibodies to the adhesion molecule L–CAM can disrupt feather formation in vitro [187]. L–CAM is found solely on the epithelial cells [187]; however, interference by the antibody alters the pattern of cell condensation within the mesenchyme, indicating a possible role for L–CAM in reciprocal epithelial-mesenchymal interactions. Cell contact-mediated stimulation of the enzyme choline acetyltransferase in sympathic neurons is inhibited by antibodies to N–CAM [188]. The role of N–CAM in this system does not appear to be that of a direct signaling molecule. Rather, N–CAM seems to be important in bringing the cell membranes into close apposition so that an independent signal can act. The sialidation state of the N–CAM molecule is important in this interaction as well, since N–CAM isoforms with a high sialic acid content appear to block this putative independent signaling mechanism [188].

A cell surface glycolipid, ganglioside G_{D3} has been implicated in the control of branching morphogenesis in the ureteric bud as well as in kidney tubule formation in the metanephric mesenchyme [170]. Antibodies to G_{D3} block both facets of kidney morphogenesis as well as inhibit kidney growth when applied to 12-day embryonic kidneys in vitro. The ganglioside is localized to mesenchymal cells close to the ureter and is not found in the epithelial cells or in induced mesenchyme, which would later form tubular epithelium [170]. Conversely, antibodies to the cell adhesion molecules L–CAM and N–CAM had no effect on kidney development in this system [170]. The mechanism of action of G_{D3} is not known, but alterations in various glycolipids are consistantly linked to oncogenic transformation [189, 190]. Changes in cell surface glycolipids are associated with the entry of cultured cells into the cell cycle and with contact inhibition cell growth [189, 190]. Glycolipids may be involved in certain cases of selective cellular adhesion and recognition and may interact allosterically with growth factor receptors as well [189, 191]. Ganglioside G_{D3} is also expressed consistantly in melanoma cells, in normal adult kidney, and in isolated stromal cells in the normal dermis, intestine, prostate, and testis [192], suggesting an ongoing role for this molecule in some mature organs.

These three mechanisms of transmission of stromal signals to epithelia (diffusion, ECM, and cell–cell contact) are certainly not mutually exclusive. In fact, there is likely to be significant interactions among them. Growth

factors may be diffusible or bound to ECM, and they may in turn affect the overall accumulation of various ECM components or change the pattern of expression of CAMs [193] or the integrins [194]. Some interactions of ECM components (e.g., laminin) with their cellular receptors may trigger intracellular changes in the pathways used by growth factors [195]. Binding of cells to ECM or their release from the matrix may stimulate the expression of cell adhesion molecules, or vice versa. The possible mechanisms for stromal-epithelial interactions have a great potential for 'cross-talk,' and within one organ, several stromal signals may be acting sequentially or in concert to affect the growth, differentiation, and maintenance of functional activity of the epithelium.

Abnormal stromal-epithelial interactions

Stromal-epithelial interactions clearly play a key role in the normal development and maintenance of epithelial phenotype; therefore, a disruption or perturbation of this interaction might lead to abnormal epithelial growth and development. There are several examples of abnormality in stromal-epithelial interactions associated with cancer; however, there is no unifying model for how aberrant stromal influences might participate in carcinogenesis. Since the precise role of stroma in development seems to differ from organ to organ, stromal involvement in the induction and growth of neoplasia is likely to be varied.

As discussed above, the ECM, and in particular the basal lamina, is likely to be essential for the maintenance of epithelial differentiation [158]. The abnormal morphology of the basal lamina in some tumors is one indication that stromal-epithelial interactions are perturbed [196]. For example, in the relatively well-differentiated Dunning tumor [197], the basal lamina is highly redundant [198]. Some individual epithelial cells protrude through this basal lamina and eventually escape and enter the stromal compartment in a type of micrometastasis [198]. Certain basal cell carcinomas have continuous basal laminae that are atypical in composition [199]. In addition, during tumor metastatsis, interruptions are commonly observed in the basal lamina [196, 199, 200]. Although these data are suggestive, they do not establish a causal role of stromal-epithelial interactions in carcinogenesis.

Several experimental paradigms for studying carcinogenesis indicate that both the epithelium and stroma are involved in tumorigenesis, even if the vast majority of the tissue within a tumor is epithelial. An example of cell–cell interactions in tumor formation can be found in polyma virus-induced carcinogenesis of the salivary gland in mice [201]. If salivary gland epithelium and mesenchyme are infected separately in vitro, then grafted together into a host mouse, tumors will form. In contrast, tumors do not form if infected epithelium or mesenchyme are grafted separately [202]. These experiments suggest that the formation of these tumors consists of at least three interac-

tions: of polyoma virus with the epithelium, of polyoma virus with the mesenchyme, and the mesenchyme with epithelium.

Bladder carcinogenesis induced with the chemical carcinogen NMU (N–methyl–nitrosourea) have also been studied from the perspective of epithelial-stromal interactions. Stroma treated with NMU can induce a change in cell surface architecture in normal epithelium, but normal stroma cannot rescue NMU-treated epithelium [203]. These data indicate that in this system, once cancerous changes occur in the epithelium, they need not be maintained by the mesenchyme, and that carcinogen-treated stroma can alter the surface morphology of normal epithelial cells. A similar conclusion appears to hold true for the the origin and maintenance of an abnormal growth of the vaginal epithelium known as ovary-independent persistant vaginal cornification, which frequently progresses, after approximately a year, to epithelial hyperplasia, then to precancerous lesions, and finally to frank tumors [42, 204]. Treatment of the neonatal mouse with relatively high doses of exogenous estrogen causes the vaginal epithelium to subsequently escape from the normal hormonal control of its proliferation and to continually express a cornified phenotpye even in the absence of estrogen [42]. During the neonatal period, at which time estrogen is effective in causing this condition, the mesenchyme contains a substantial level of estrogen receptors [51], but the levels of these receptors in the vaginal epithelium are undetectable [51, 55]. If the vaginal epithelium from a neonatally estrogenized mouse is recombined with normal vaginal mesenchyme, the epithelium continues to express ovary-independent cornification [205]. When the mesenchyme from an estrogen-treated mouse is recombined with normal vaginal epithelium, this epithelium is stimulated to express ovary-independent cornification [205]. These experiments also suggest that estrogen can act indirectly on the epithelium to stimulate ovary-independent vaginal cornification, but that neonatally estrogenized vaginal epithelium undergoes changes that are not reversible by normal mesenchyme.

Normal mesenchyme can modulate the phenotype of various tumors [203]. The epithelium from a transitional cell bladder carcinoma, when recombined with UGM and growth in a male host, changes to a glandular morphology but appears to continue to express a variety of atypias, suggesting that the transformed phenotype is maintained [206]. The phenotype of the Dunning tumor can also be changed when it is recombined and grown with a variety of genital tract mesenchymes. The epithelial cells of this slow-growing glandular tumor are squamous-to-cuboidal and nonsecretory [207], but when combined with UGM they become columnar and secrete a proteinaceous fluid [208]. In this case the UGM appears to 'normalize' the neoplastic epithelium, though no conclusion can yet be drawn concerning whether or not the induced, highly differentiated, columnar epithelial cells are still capable of tumorigenesis. Some degree of change toward normal phenotypes has also been observed with other types of carcinoma combined with normal mesenchyme (mammary [209, 210], basal cell carcinoma [211], colon [212, 213], oral [214]). In some

instances normal stroma can stimulate the growth of certain carcinomas [215–221]. The fact that the morphology of a number of tumors can still be modulated by normal mesenchyme indicates that some of the neoplastic changes may not be totally irreversible.

Though the mechanism of abnormal stromal-epithelial interactions may be even more varied than those controlling normal development, certain possibilities seem more likely than others. Faulty regulation of stromal influences on epithelium would be expected to affect tumor progression rather than tumor initiation. In all of the above examples, carcinogenesis required an outside agent (e.g., NMU, exogenous estrogen, polyoma virus) in whose absence the tumors would not have formed. In some cases of benign tissue growth, the primary defect may be in the stroma. Abnormal stromal signals have been hypothesized to be the causative agent in benign prostatic growth (BPH). In the human prostate, pure stromal nodules tend to form first, followed by epithelial proliferation into these nodules. This pattern of growth lead MacNeal [222, 223] to postulate that the formation of new ductal-tumor tissue in BPH may result from a 'reawakening' of embryonic inductive activity in the stroma. In experimental situations, embryonic stroma by itself, even when implanted in or recombined with adult tissues, will not cause tumor formation [114, 115, 224] unless some other agent, such as a virus, is present [224].

Several of the mechanisms by which stromal-epithelial interactions might go awry may relate to the inappropriate expression of various fetal properties in the stroma. These activities may include the secretion of growth stimulators, the loss of growth inhibitors, the stimulation of epithelial mobility (e.g., increased secretion of scatter factor [114]), the alteration of cell surface molecules (such as CAMs or gangliosides [192]), or the modulation of the ECM. Loss of fibronectin or collagen from the matrix [225] or loss of certain fibronectin receptors [226] is associated with malignant transformation in many tumors. As in the study of viral and cellular oncogenes, the elucidation of the defects in stromal-epithelial interactions during carcinogenesis may lead to the understanding of new mechanisms that control normal development.

Conclusions

Although the details of the mechanism of interactions between stroma and epithelium remain unclear, several general conclusions can still be drawn. Mesenchymal influences on epithelium are part of a continuum of tissue interactions that occur within an organism from fertilization until adulthood. During normal organogenesis, the mesenchyme supports and sometimes directs epithelial proliferation and differentiation. In some cases, especially within the reproductive tract, the mesenchyme or stroma appears to be the mediator of steroid hormone action upon the epithelium. In the adult the stroma contributes to the maintenance of epithelial phenotype, notably in

organs in which epithelial cell renewal is rapid. There is great variability in the degree of influence that the stroma has over the epithelium both during development and in adulthood; in addition, the mechanism by which the stroma exerts its influence is likely to vary from organ to organ. Under certain circumstances this finely tuned interaction of stroma and epithelium may malfunction and possibly contribute to the progression of epithelial tumors.

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17. Mammary epithelial cells, extracellular matrix, and gene expression

Charles H. Streuli and Mina J. Bissell

The mammary gland is an appropriate system with which to study mechanisms that control gene expression during both growth and differentiation. It is one of the few tissues with a developmental potential after birth, since following the onset of pregnancy, epithelial cells in the mammary gland proliferate and differentiate into milk-secreting cells. A schematic cross-section of one of the ten mammary glands of the mouse shows the gross changes in structure that occur during progression of the gland in pregnancy (figure 1, upper panel), from the virgin state (V) to lactation (L). During pregnancy (P), certain epithelial cells lining the ducts (which themselves are embedded in stromal tissue) proliferate and form alveolar buds. By the time of parturition, cells in the alveoli become secretory and occupy the entire gland. This structural development is accompanied by the onset of lactogenic function, in which expression of milk proteins begins during pregnancy and culminates with the secretion of milk after parturition [1]. The steady-state levels of the mRNA for β–casein (figure 1, lower panel) illustrate this type of progression with no expression in the virgin state, some during pregnancy, and the highest levels at lactation. After weaning when the gland involutes, β–casein expression is down regulated (figure 1, lower panel, lane I).

In order to explore the molecular mechanisms that regulate development of mammary epithelial cells, we asked initially whether a system could be provided for cells from a partially differentiated gland at mid-pregnancy (days 10–14) to become 'lactating' in culture (figure 2). In conditions under which the cells spread into sheets and lose their polarized morphology, such as on a conventional plastic culture dish, they also lose most of their functional characteristics, even in the presence of lactogenic hormones [for review see 2]. However, in vivo mammary epithelia develop in a stromal environment, is largely composed of fibroblastic and adipose tissues. The classic experiments of Emerman and Pitelka (1977), where they plated cells onto a stromal type I collagen gel that was subsequently released to allow the cells to contract the gel [3], demonstrated that in addition to lactogenic hormones, substrata, as well as cell shape changes and cell-cell interactions, play important roles in morphological and functional differentiation [4, 5, 6,

M. Lippman and R. Dickson (eds.), REGULATORY MECHANISMS IN BREAST CANCER.

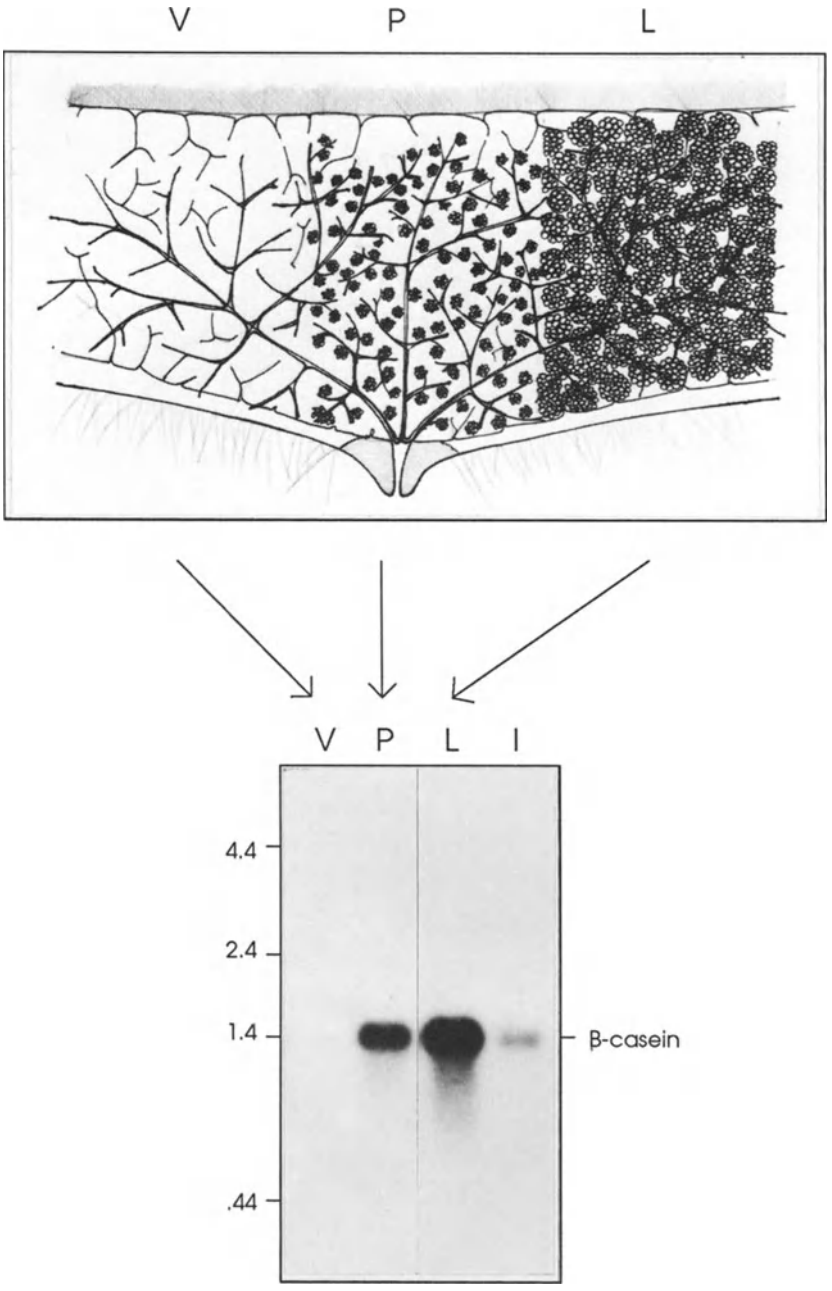

Figure 1. Developmental progression of the mouse mammary gland from virgin (V), through pregnancy (P), to lactation (L) and involution (I) in schematic form (upper panel) and at the functional level (lower panel) by Northern blot analysis of β-casein mRNA. Ducts infiltrate the fatty stroma in the virgin tissue, and certain epithelial cells lining the ducts proliferate at pregnancy to form alveolar buds, visible as clusters of cells.

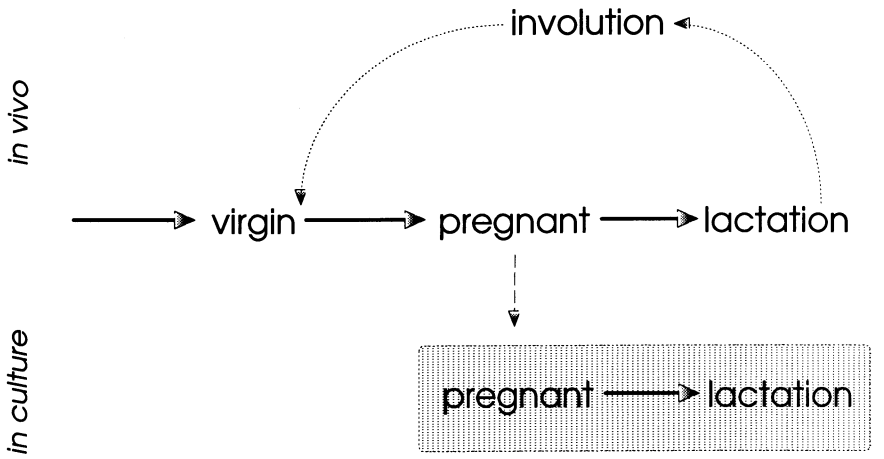

Figure 2. Scheme of the mammary developmental cycle in vivo and of the stages that our systems mimic in culture.

7, 8]. It is evident from morphological studies that during pregnancy and lactation, secretory epithelial cells additionally have a multicellular structure in which groups of many cells are arranged as spherical arrays to form alveoli. This is essential for apical secretion of milk proteins into alveolar lumina. To mimic this in culture, we put cells onto a matrix of basement membrane (BM) components [9, 10] instead of stromal type I collagen gels. In this brief review, some recent work from our laboratory that addresses the question of structure and function of mammary epithelial cells in culture is discussed, with specific emphasis on the role of ECM.

By transmission electron microscopy, the cells cultured on plastic are squamous; but on released collagen gels that are floating in the medium, they are polarized and columnar [4]. When cultured on a BM derived from Engelberth–Holm–Swarm (EHS) tumor, the cells additionally form spherical structures (figure 3) that have similarites to in vivo alveoli. A model for their formation can be constructed from transmission electron micrographs, in which clusters of cells reorganize to give rise to alveolar structures that enclose a lumen [22] (figure 4). Complex dynamic events between individual spheres are involved in this progression, since aggregates in early cultures often amalgamate (figure 5).

A cross-section of an alveolus from a lactating mammary gland (figure 6a) shows a layer of secretory cells surrounding the lumen, which is filled with milk proteins and fat globules. On approximately the same scale is an electron micrograph through the center of a cultured alveolar structure (figure 6b). The cells are highly polarized, with basal nuclei, apical microvilli, and a prominant secretory apparatus. They are joined by tight junctions that seal the lumen and thus form a distinct compartment from the extracellular

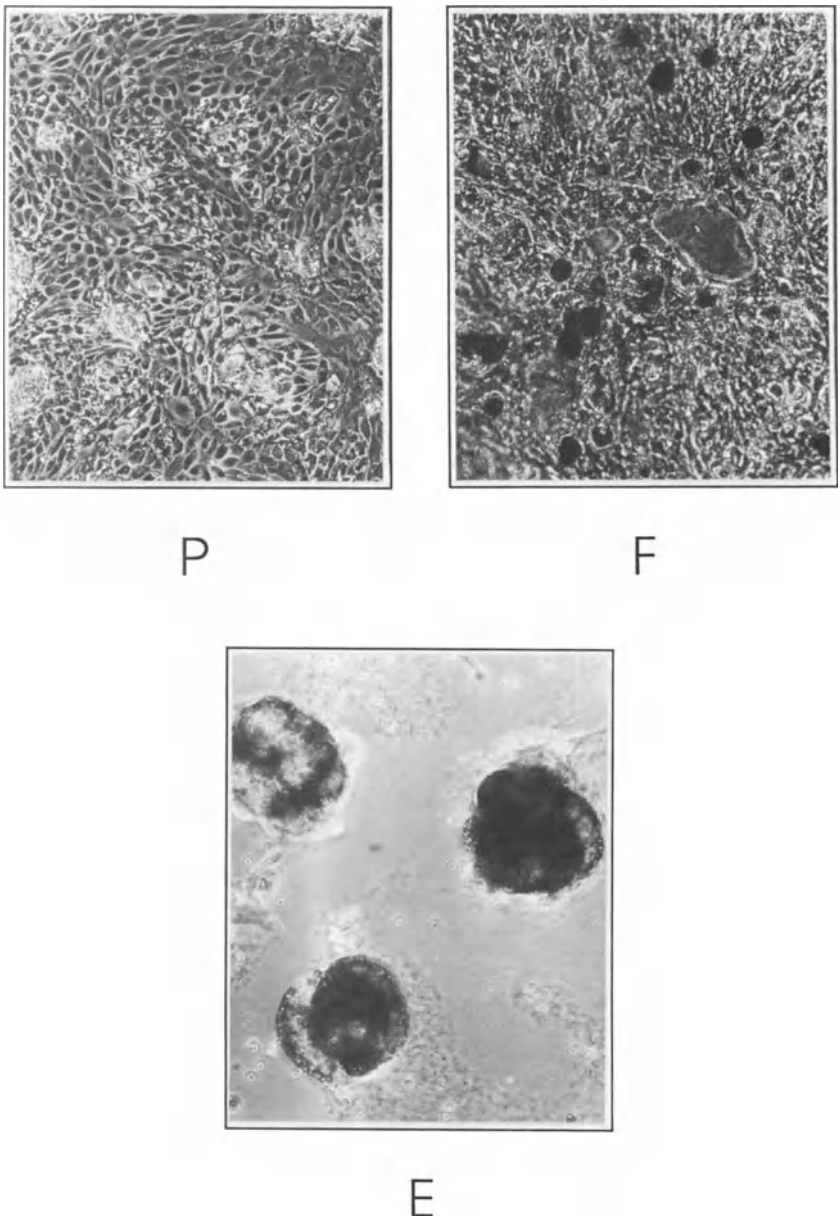

Figure 3. Phase contrast views of primary mammary cells cultured on plastic substrata (P), on floating collagen gels (F), and on the EHS-derived BM matrix (E): the lactogenic hormones hydrocortisone, insulin, and prolactin are present in all three culture systems.

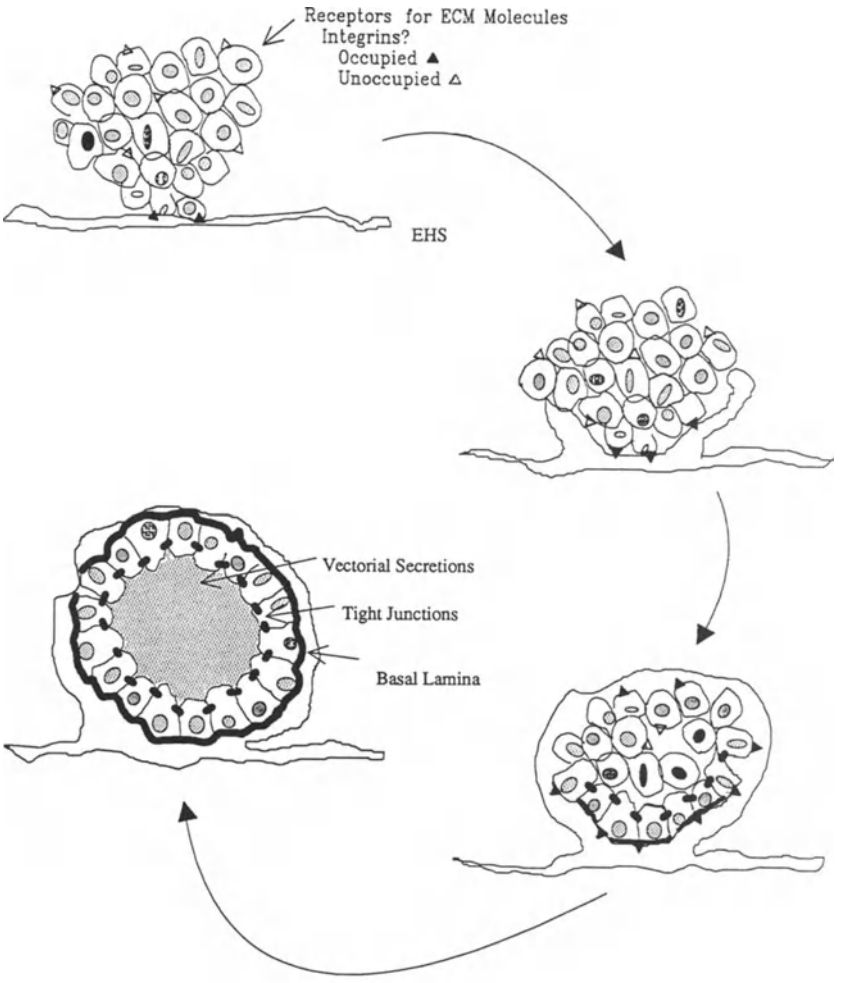

Figure 4. In a model for the generation of alveolar structures on the EHS-derived BM matrix, cell aggregates become enveloped in the matrix and subsequently polarize with apical surfaces facing inwards. [From Barcellos-Hoff and Bissell, 1989; reproduced with permission.]

medium [10]. The cells secrete milk proteins apically into this central lumen, demonstrated both by immunofluorescence with β–casein-specific antibodies [not shown] and by using an antibody with broad-spectrum specificity for milk proteins [8] to precipitate newly made proteins secreted either into the lumen or basally into the medium outside the spheres. A full spectrum of milk proteins are secreted apically, while transferrin and lactoferrin are secreted both ways (figure 7) [see also 10].

Thus primary epithelial cells from mid-pregnant mammary gland differentiate if plated onto a BM matrix or, as has been shown previously [8], onto

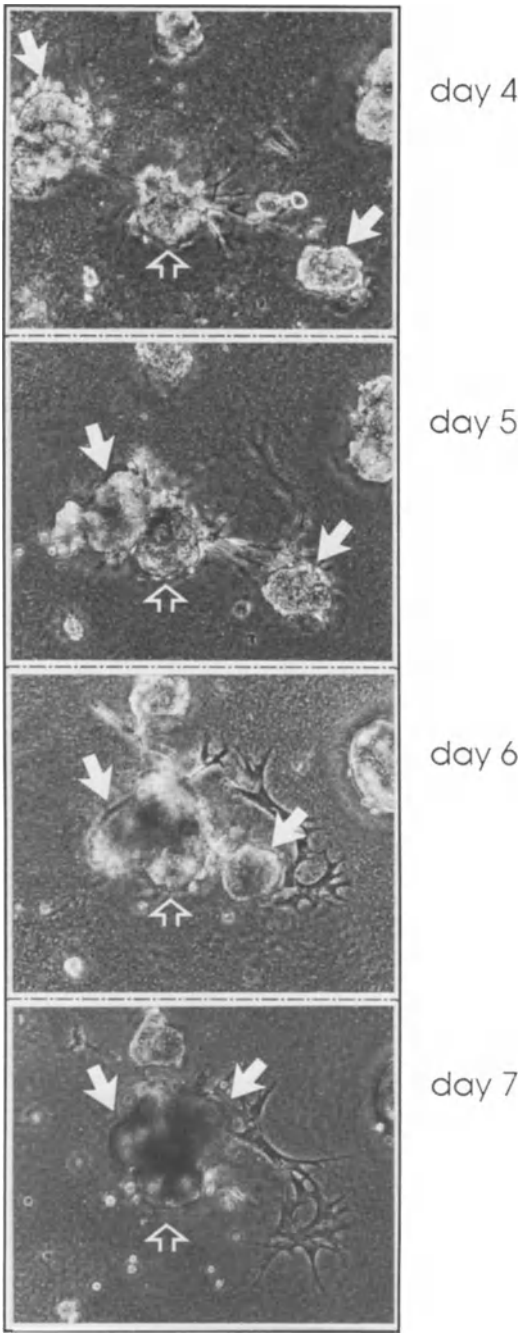

Figure 5. A typical sequence of events depicting the morphogenesis of a spherical structure cultured on the EHS-derived BM matrix. Daily photographs of the same area of the dish show three aggregates (designated by different arrows), which amalgamate over a period of four days to form a single alveolar-like structure.

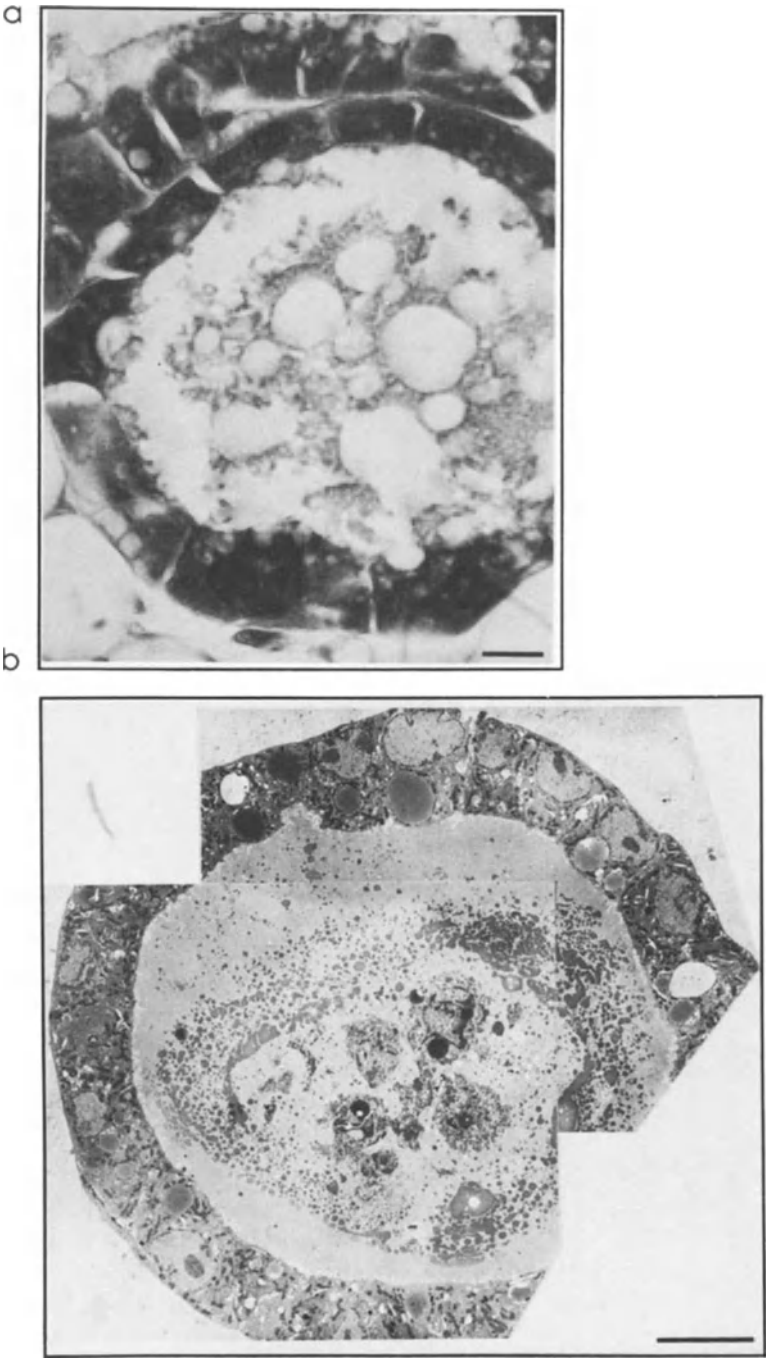

Figure 6. Light micrograph of a cross-section of an alveolus in a lactating mouse mammary gland, stained with hematoxylin and eosin (a). On a similar scale is an electron micrograph through a cultured spherical structure, showing that the EHS-derived BM matrix induces cultured mammary epithelial cells to form normal alveolar architecture (b).

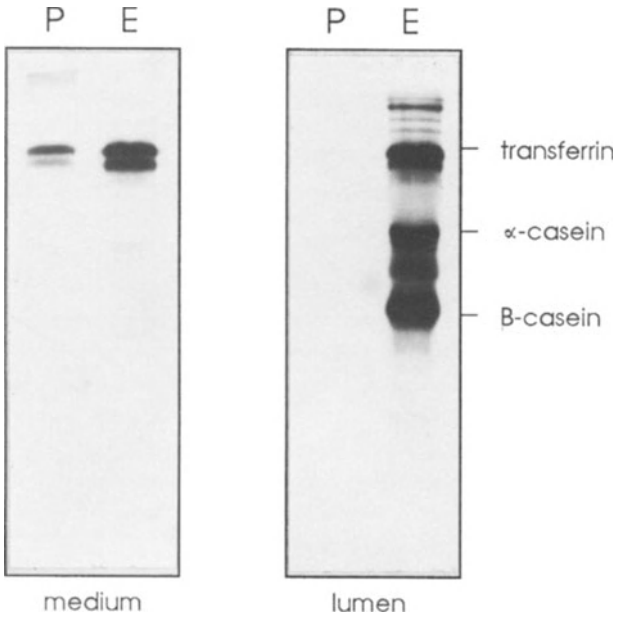

Figure 7. Immunoprecipitates of newly synthesized milk proteins, resolved by SDS–PAGE, demonstrate that caseins are secreted into the lumen of spheres cultured on the EHS-derived BM matrix (E), but that transferrin and lactoferrin are secreted bidirectionally into both the lumen and medium compartments. Note that caseins are more sensitive to substrata than is transferrin. In this experiment, cells cultured on plastic (P) synthesized almost no caseins.

a type I collagen gel that is subsequently floated. The extent of differentiated function in cells cultured under these different conditions was compared by analyzing the steady-state levels of milk-specific mRNAs (figure 8). A dual Northern blot illustrates comparable levels of β–casein and transferrin mRNA to those present in the mammary gland (figure 8a, M), when cells were cultured on the exogenous substrata that induce shape changes and polarization (floating gels, F; BM matrix, E). Further, the total levels of secreted milk proteins in the medium of floating gel cultures could be as high as those shown in figure 7 for the lumenal-secreted proteins of cells grown on EHS-derived BM matrix [not shown].

Since it is clear from these results that ECM/shape effects have a profound influence on the function of these cells, it was necessary to investigate whether the formation of alveolar structures on EHS-derived BM matrix reflected any additional change in functional differentiation that is not seen in the floating gel cultures. A significant protein in rodent milk, whey acidic protein (WAP), is not expressed in culture on floating collagen gels [7, 8] or in any other culture conditions, except where cells make alveolar structures on the BM matrix. Under the latter conditions, significant levels of WAP mRNA are present (figure 8b) [11]. The protein itself is also made and secreted vectorially into the lumen [not shown].

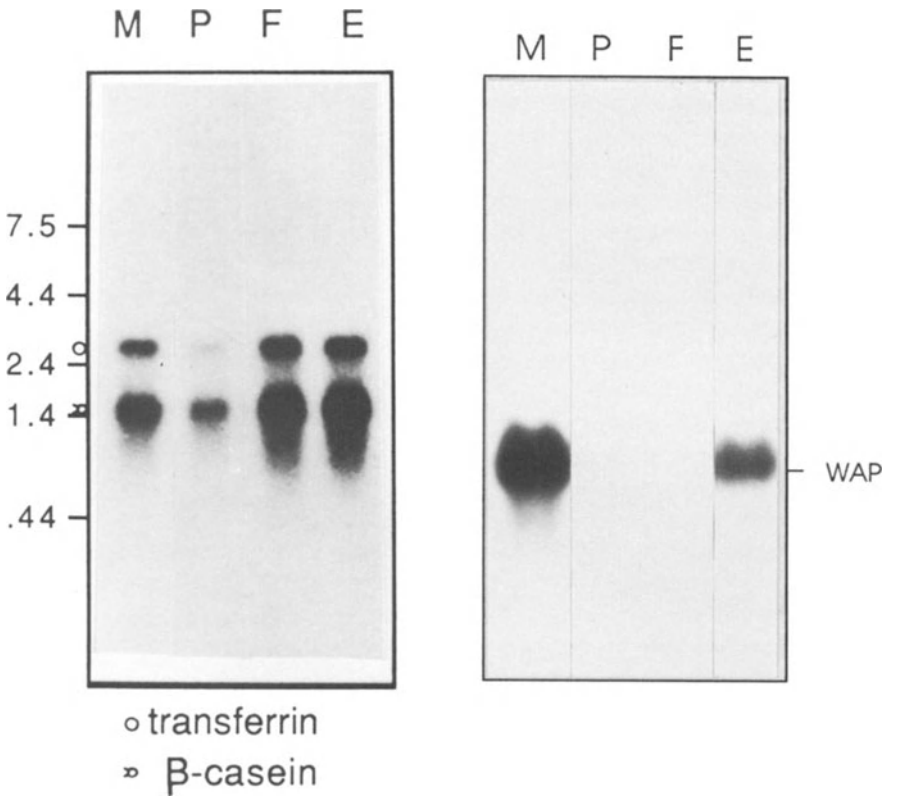

Figure 8. Northern blots of mRNAs coding for transferrin and β–casein (left panel) show that cells cultured on floating collagen gels (F) and on the EHS-derived BM matrix (E) function equally well and to the same extent as the tissue (M) from which the cells were derived. Substantial loss of function occurs when cells are plated onto plastic (P). On the other hand, WAP is expressed only by cells cultured on the BM matrix that are able to form alveolar structures (right panel). [Right panel is rearranged from Chen and Bissell, 1989.]

Thus it has been possible to develop culture systems in which cells dissociated from the mid-pregnant mammary gland are able to acquire new morphological and polarized characteristics and a functional capacity. In one instance, when cultured on a BM matrix, the cells underwent morphogenesis and formed structures that resembled alveoli in the mammary gland. Furthermore, the alveolar structures, in contrast to cells cultured on plastic or on floated collagen gels, were able to impart additional information, in that the cells could now accumulate WAP mRNA and secrete the protein. These results indicate that the extracellular environment influences epithelial cell function at the levels of morphogenesis, cytodifferentiation, and functional differentiation.

We therefore asked how the cells cultured on both the stromal and the BM matrices were able to reacquire their differentiated phenotype, given the

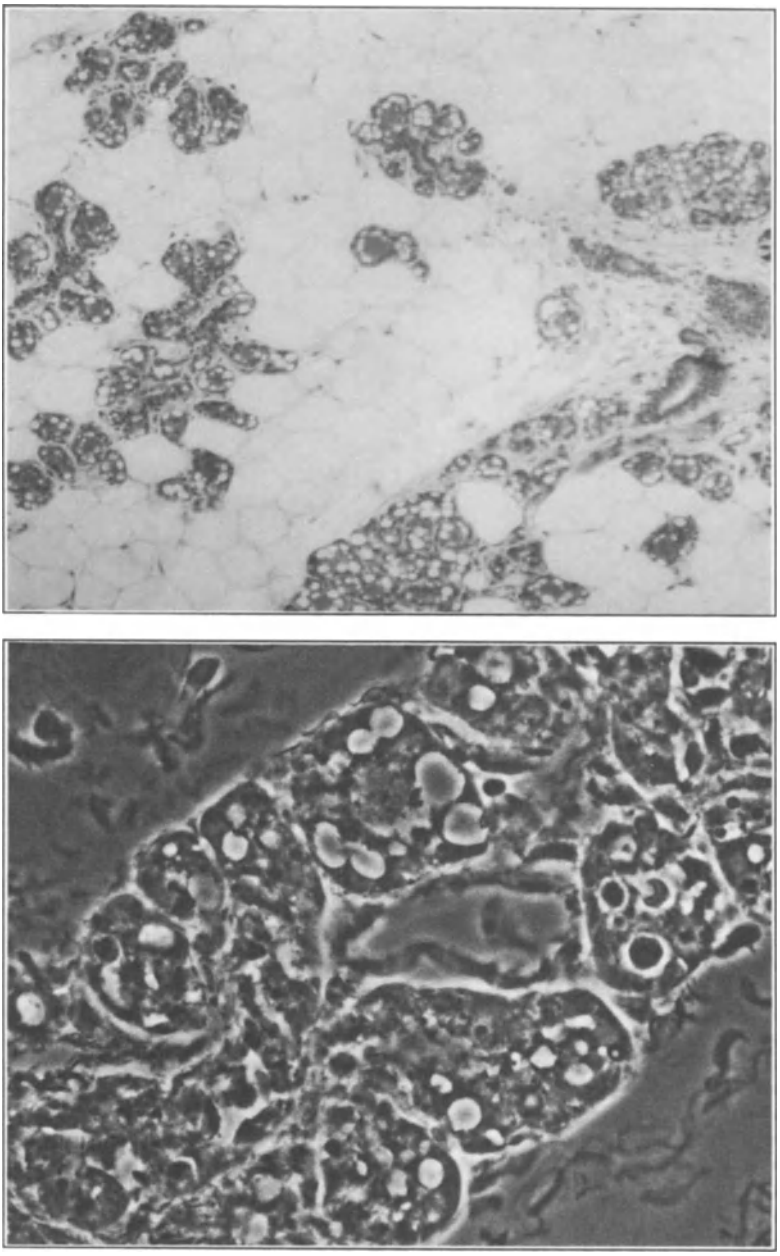

Figure 9. Thick sections through a mid-pregnant mammary gland show the gross structure of developing alveolar buds (a. stained with hematoxylin and eosin), surrounded by a laminin-containing BM (b. a dual exposure in which cells are viewed by Nomarski optics and the laminin visualized by rhodamine fluorescence. In this black and white reproduction of a color original, the laminin staining is restricted to the white lines surrounding individual alveolar buds) Mag: a × 150; b × 490.

diverse nature of these substrata. It is apparent from the in vivo situation that clusters of alveolar cells in the mid-pregnant gland (figure 9a) are surrounded by a BM, stained in figure 9b for the presence of laminin. Consequently, in the gland itself the epithelial cells are not in direct contact with stroma, but they interact with a BM. What then is the nature of the signal from the stromal collagen, which allows differentiation to occur?

On closer examination, it is apparent that cells grown on top of collagen gels and floated into the medium are also sitting on a BM situated between the cells and the collagen (figure 10a) [12]. Thus in a fundamental way, this culture system mimics the situation provided by the EHS-derived BM matrix and the alveoli in vivo. The floating gel system, however, allows investigation of whether the appearance of this BM correlates with the onset of lactogenic function, because the cells plated onto a flat collagen gel are initially squamous (figure 10c) and have little or no BM (figure 10b). After flotation, the cells not only undergo shape changes (figure 10c) and become polarized, but they also deposit a BM (figure 10b). Functional analysis (figure 11) of these cells indicates that while the steady state levels of β–casein and transferrin mRNAs in freshly isolated epithelial cells (To) are high, they are lost in culture after one day. After floatation at day 2, the cells on collagen gels reacquire their high steady state levels of β–casein and transferrin mRNA. The restoration of function therefore correlates with the shape changes and appearance of the BM. In contrast, cells cultured on plastic do not recover functional differentiation (figure 11), correlating with the lack of an organized matrix of BM components [not shown] [12]. It is reasonable then to conclude that BM plays a significant role in the establishment of mammary cell function; i.e., in addition to changes in shape and polarity, cells cultured on floating collagen gels acquire a BM and thus sense their environment in a manner similar to the cells placed on an exogenous BM. This is shown diagramatically in figure 12.

The production of BM by cells on collagen gels raises another important and related issue: What regulates the production of these ECM components? To answer this question the steady-state levels of mRNAs for laminin and type IV collagen were measured by Northern analysis (figure 13). Cells from mid-pregnant gland (M) or freshly isolated epithelial cells (To), which contain high levels of milk mRNAs, have low levels of mRNA for laminin and type IV collagen. By the time a BM has been deposited by cells cultured on floating collagen gels (Fl) and when they make milk products, the levels of ECM mRNAs are low again. However, on plastic substrata (P), where the cells do not make milk and do not lay down a BM, there is a substantial induction of both type IV collagen and laminin mRNAs.

In summary, our current model is that the BM or its components, in conjunction with the shape changes that accompany the establishment of polarity, are the positive modulators of functional differentiation. One or both of these signals feed back negatively on the synthesis of mRNAs for ECM components, such as laminin and type IV collagen.

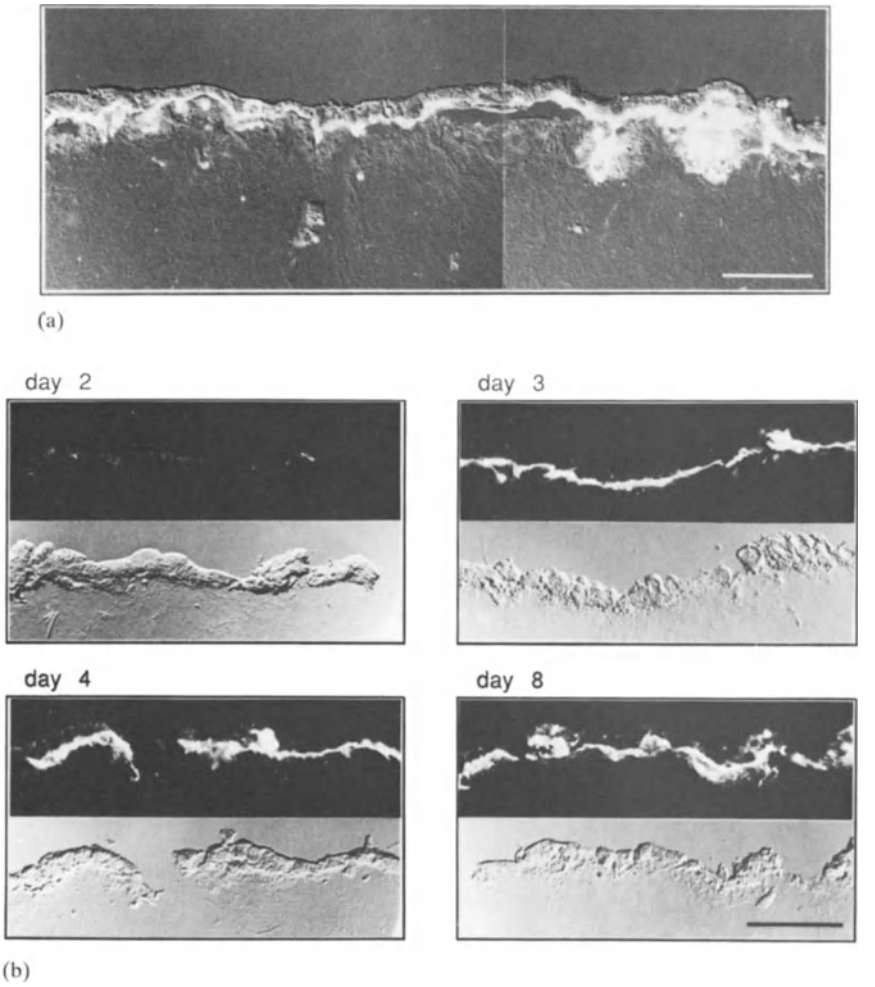

Figure 10. Thick transverse sections of cells cultured on collagen gels stained by immunofluorescence for the presence of type IV collagen (a: photographed simultaneously with Nomarski optics) illustrate the spatial relationship between the cell layer and its BM (bar = 50 μm). Similar sections, stained for the presence of laminin, show that the BM is deposited only after the gel is floated at day 2 (b). [From CH Streuli and MJ Bissell, 1990, reproduced with permission.] At a lower magnification, sections stained with Wrights/Geimsa (c) show the gross changes in cellular organization that occur after the gel is floated into the culture medium at day 2. A 35 mm diameter gel contracted to 36% of its surface area within 24 hours of release (day 3) and was only 16% of its original area at day 6. The increased local density is due to gel contraction rather than cell growth (bar = 200 μm).

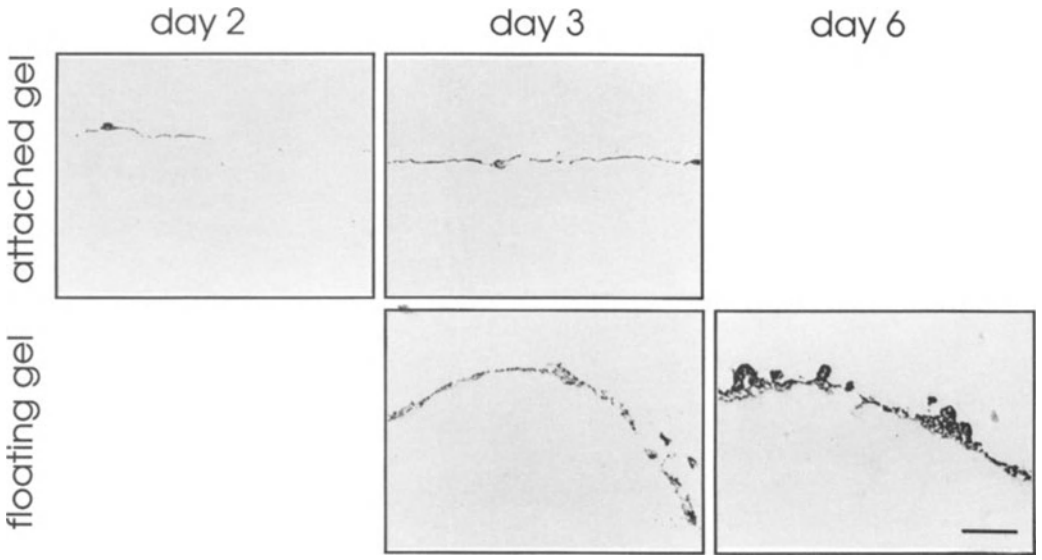

Fig. 10(c).

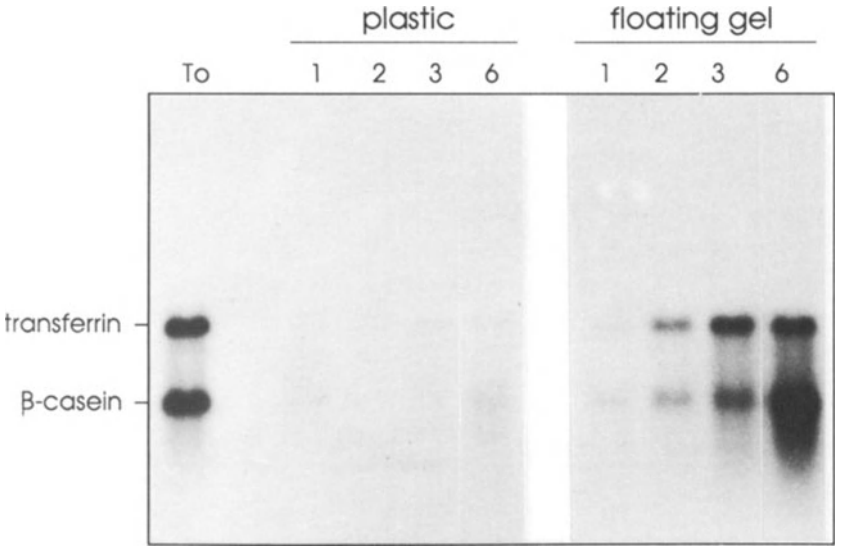

Figure 11. A time course of milk protein mRNA production by cells cultured on different substrata shows that only on collagen gels and after floatation (at day 2) do they function to the same extent as cells (To) freshly isolated from the mammary gland and harvested just before plating out.

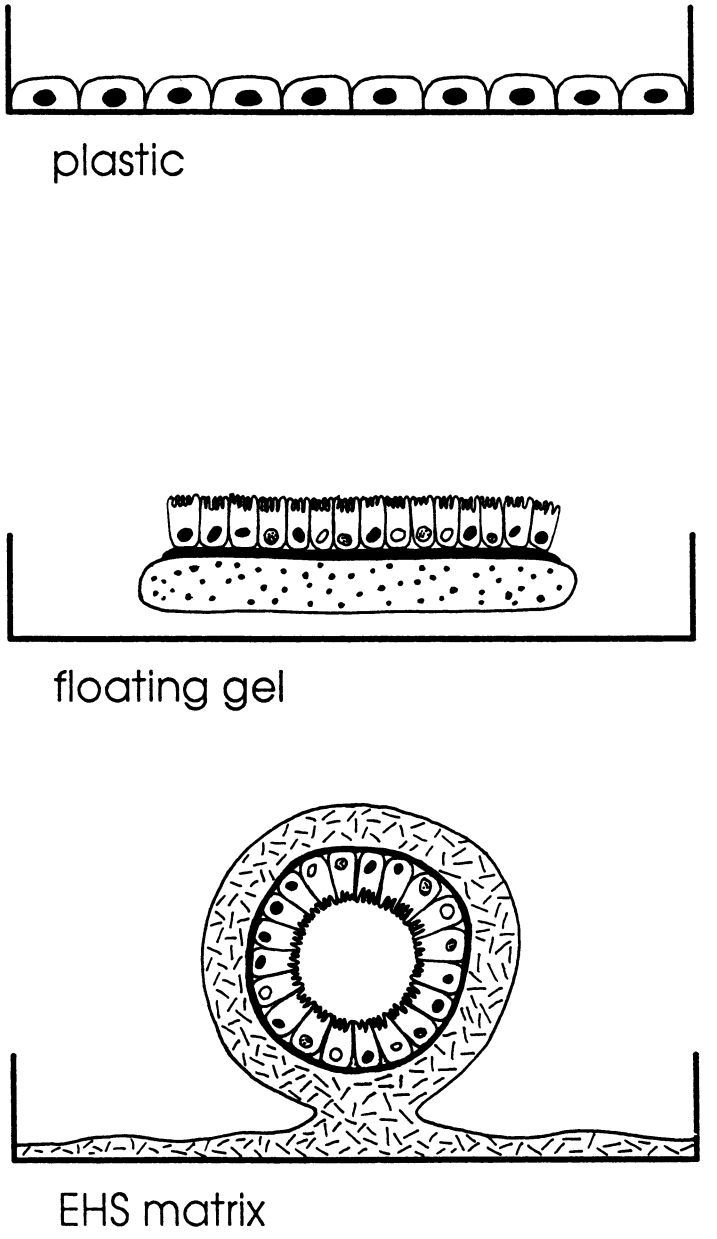

Figure 12. Scheme showing the relative differences in ultrastructure between cells in various culture conditions: it is only on the floating gel or on the EHS-derived BM matrix that the cells are both polarized with apical microvilli and basal nuclei and are associated with a basement membrane (illustrated by the thick black line). Our model suggests that both these conditions are required for functional differentiation to occur.

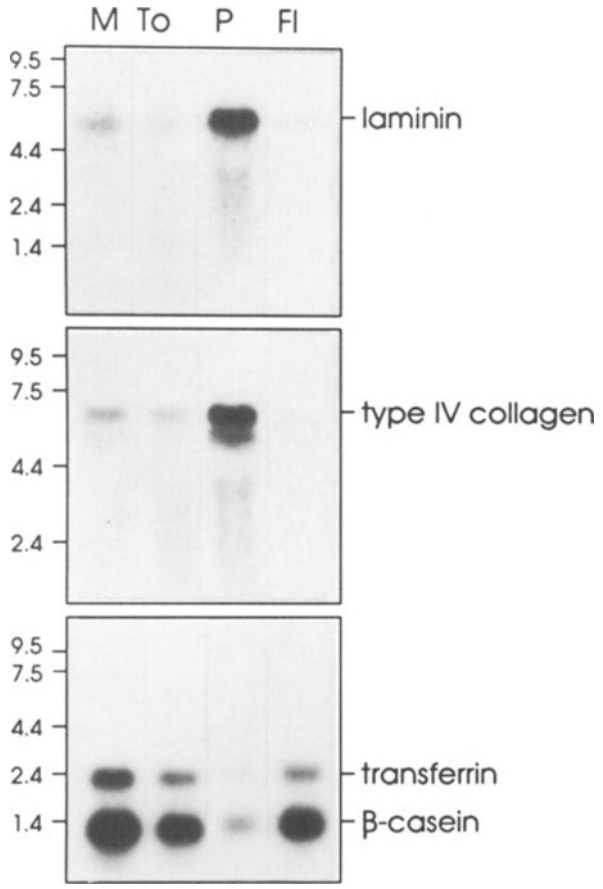

Figure 13. Functional mammary epithelial cells from intact gland (M), from freshly dissociated tissue (To), and from 6-day floating collagen gel cultures (FL) have high levels of mRNAs for transferrin and β–casein but low levels of the mRNAs for laminin (B1 chain) and type IV (α1) collagen. This trend is reversed in nonfunctional cells cultured for 6 days on plastic (P). [From Streuli CH and Bissell MJ, 1990, reproduced with permission.]

Having demonstrated the profound influence of BM on gene expression in the mammary gland, it should be pointed out that this appears to be a universal mechanism. EHS-derived BM also influences functional differentiation in other cell types, including Sertoli cells [13], Schwann cells [14], hepatocytes [15, 16], alveolar lung type II cells [17], endothelial cells [18], and uterine epithelial cells [19]. How does the matrix so profoundly affect gene expression? Our working hypothesis, stated many years ago [20, 21], is that transmembrane receptors for ECM components, including members of the integrin family, reorganize the cell via attachments to the cytoskeleton, providing a continuum from matrix to nucleus. Understanding the exact mechanism by which the signals from ECM are transduced and translated into

information that in turn regulates differentiation is now a most significant challenge for cell and molecular biologists working in this area.

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18. Tenascin in mammary gland development: From embryogenesis to carcinogenesis

T. Sakakura, A. Ishihara, and R. Yatani

Introduction

The enormous progress in immunology and molecular biology has encouraged the development of many ingenious techniques that now make it possible to analyze various biological phenomena at the molecular level. For example, pieces of immunoglobulins or fragments of oligonucleotides have been used as probes to demonstrate the presence and location of their molecular partners in both cells and tissues. Such capabilities represent an important advance in cell biology and in the fields of animal biology and pathology, where a large amount of knowledge has already been accumulated.

In cancer research, a conceptual breakthrough came with the idea that cancers are caused by either the activation of transforming genes or the inactivation of antitransforming genes, both of which are found in normal cellular DNA sequences. This enables us to consider that cancers may arise from intrinsic changes of cells rather than from extrinsic stimulations to the cells. Widespread acceptance of this model, and other technological innovation, has served to redirect cancer research toward studies of molecular genetics during this decade. Under these circumstances, any nonneoplastic cells that are present near the neoplastic cells have been largely ignored. However, to physicians and pathologists and also to the patients, cancer is not merely a mass of genetically abnormal cells, but a complex lesion made up of cancer cells and its stroma cells. They include fibroblasts, nerves, blood vessels, macrophages, and various blood cells, and perhaps even some normal epithelium. Any interactions between different types of cells (for example, between cancer cells and fibroblasts or endothelium or macrophages; fibroblasts and endothelium; or one cancer cell and another cancer cell) and further interactions with many types of cells have been shown to exert a controling influence on the local growth patterns of specific tissues. An awareness of interaction effects is important and necessary for a deeper understanding of the biological behavior of cancer. Fortunately, data from classical animal biology research has been turned to good use in modern

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molecular biology, helping to demonstrate the discovery of new changes of the cellular genes.

Questions as to the mechanism by which cells communicate with other cells have been considered and studied by cell biologists for a long time. Interactions between epithelium and mesenchyme have been well documented and shown to play important roles in the various developmental events, such as determination, growth, morphogenesis, and functional differentiation in many organs [1–3]. The molecular mechanism of these interactions may possibly be explained in four ways (figure 1). (a) small molecules are infected, either from epithelium to mesenchyme and/or vice versa, through the plasma membranes by direct contact; (b) diffusible substances, such as hormones, growth stimulating or regulating factors, and/or other factors, are secreted by these cells to influence each other in auto-paracrine fashion; (c) cell surface contact of epithelium and mesenchyme initiates biochemical cascades in the cytoplasma, connecting the signal transduction system of the cells; and (d) extracellular matrix substances mediate the interaction between the epithelium and mesenchyme. Of these four, the relative importance of the last mechanism has been increasing in the light of modern cell biology. The major groups of extracellular matrix substances are collagens, glycoproteins (such as fibronectin and laminin), and proteoglycans. Among these, type IV collagen, laminin, and proteoglycans are synthesized by mammary epithelium, while fibronectin is produced by fibroblasts [4–9]. Tenascin is an extracellular matrix glycoprotein [10]. The name was coined in 1986 to describe the characteristic appearance of a new antigenic substance in the dense mesenchyme surrounding the epithelia of mammary tumors and embryonic glands. Subsequent experiments have suggested that tenascin synthesis may be induced in the mesenchyme by interactions with embryonic or malignant epithelia [11–13]. This chapter describes the distribution of tenascin in mammary glands, and its possible role in embryogenesis and tumorigenesis.

Tenascin

In 1984 a novel extracellular matrix glycoprotein, 'myotendinous antigen,' was reported [14, 15]. This is the epitope recognized by M1 monoclonal antibody, which appears early in chick limb morphogenesis at sites connecting developing muscle fibers, tendons, and bone. Biochemical characterization of this molecule revealed that it consists of several disulfide-linked subunits with molecular weights of 150–240 kd, and sulfated glycosaminoglycans. In subsequent experiments, rat mammary glands were analyzed by immunohistochemistry. Myotendinous antigen was found in the dense mesenchyme surrounding the growing epithelium of embryonic and neoplastic mammary glands but not in the proliferating epithelium of pregnant rats. After examining the histologic sections of these materials, a new name, 'tenascin,'

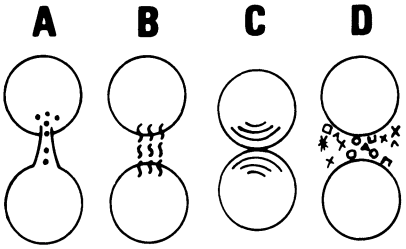

Figure 1. Possible molecular mechanism of tissue interactions.

was proposed for myotendinous antigen [10]. The biological functions of tenascin are not clear yet, but in a culture system the following activities have been reported.

Biological activities

Hemagglutination Since cellular fibronectin was demonstrated to be a hemagglutinin a hundred times more active than plasma fibronectin, hemagglutination was considered to be a biological activity of cellular fibronection [16]. In subsequent experiments, however, an oligomeric protein contaminated in a cell surface fibronectin preparation was isolated and named as 'hexabrachion' because of its electron microscope photographs [17]. Moreover, it was demonstrated that the hemagglutination activity of cellular fibronectin was due to this contaminate, hexabrachion. Like Concanavalin A, chicken tenascin causes hemagglutination of formalin-fixed sheep erythrocytes. This activity is much stronger than that of cellular fibronectin [10]. Human tenascin isolated from normal fibroblast cultures [18] or from malignant tumors also has hemagglutination activity [19].

Growth stimulation of tumor cells Growth analysis of tumor cells cultured in the presence of various extracellular matrix substances as a substrate indicates the stimulating effect of tenascin [10]. When rat mammary tumor cells were cultured for three days in medium containing 10% fetal calf serum and then switched to serum-free medium for two days, the cells stopped growing, regardless of whether the cells were cultured on substrates, such as fibronectin, laminin, type I collagen, or type IV collagen. When cultured on tenascin, however, the subsequent growth of cells was promoted. Molecular analysis of tenascin's gene gives us a hint of why there is growth-promoting activity. The structure of tenascin revealed the presence of a domain consisting of 13 epidermal growth factor-like repeats, which may be the domain causing the growth promoting effect [20].

Cell attachment and detachment A variety of experiments has suggested that tenascin is not directly involved in cell attachment. Mammary tumor cells cultured on tenascin-coated plate attached much less effectively than

did similar culture grown on plastic or on plates coated with other extracellular matrix substances [10]. Further, tenascin was considered to be a molecule that permitted detachment of growing cells from their substrate, since a tenascin substrate stimulated the migration of cultured neural crest cells [21]. On the other hand, cytotactin [22] and J1 [23], which are both indentical to tenascin, were originally reported as adhesion molecules of the neuron-glia during development of the cerebral cortex. Therefore, there is an apparent paradox between fibroblast tenascin and brain J1/cytotactin, although they have been characterized to be identical in terms of both cDNA sequences and electron microscope photographs [20, 24, 25]. Moreover, in various cell cultures, substrate tenascin and medium tenascin have displayed different effects on the migration, attachment, and morphology of the cells [21, 26]. Cytotactin also affected the attachment of neuron or glia to a polystyrene substrate, depending on assay conditions. Inhibition occurred when the cells were plated by gravitation, while promotion occurred when the cells were plated by centrifugation [27]. On the basis of these results, the hypothesis may be formulated that tenascin exerts a wide range of effects on cell differentiation. In tissue, tenascin binds to fibronectin, proteoglycans [18], and probably many other substances, making a three-dimensional conformation in the extracellular space. Tenascin also metabolizes rapidly in response to the environmental changes that are induced by various growth factors, hormones, etc. During the rapid metabolism of an extracellular matrix complex, a variety of information will be expressed.

Similar molecules

Within the past several years, tenascin-like molecules have been discovered independently by various laboratories.

GMEM (Glioma-mesenchymal extracellular matrix) GMEM is an antigen detected by immunohistochemistry and found in human glioblastoma but not in normal brain. By radioimmune binding assay, it is detected on the surface of various cultured cells, such as glioma and fibroblast. This antigen was recognized by monoclonal antibody 81C6 obtained from a hybridoma, using mouse spleen cells immunized with human glioma cell line U–251MG [28]. The immunochemical studies demonstrated that GMEM is a high-molecular-weight macromolecule composed of M_r 230,000 disulfide-bonded glycoprotein subunits [29].

Myotendinous antigen (a previous name for tenascin) Myotendinous antigen was found to be a new extracellular matrix glycoprotein bearing an epitope that was recognized by monoclonal antibody M1. M1 was selected from hybridoma libraries prepared from mouse spleen cells immunized with chick type V collagen. Myotendinous antigen appears during early limb morpho-

genesis, while in vitro it is released by cultured fibroblast cells. Biochemical analysis demonstrated that this molecule is a large glycoprotein complex consisting of several disulfide-linked subunits estimating the molecular weights from 150 to 240 kD [14, 15].

Hexabrachion Hexabrachion is a large oligomeric glycoprotein of the extracellular matrix. It was found as a contaminate of cellular fibronectin by use of the rotary shadowing electron microscope. This molecule has a six-armed structure as the name implies, while fibronection is dimeric [17]. The structure of myotendinous antigen and GMEM protein have subsequently been demonstrted to be six armed [25].

J1 J1 is a glycoprotein originally defined by a specific polyclonal antibody prepared in rabbits against the 160 kD component, which is a member of the L2/HNK–1 family and considered to be important for neuron-glia adhesion. Western blot analysis of mouse cerebella membrane preparations using polyclonal antibodies demonstrated that J1 is composed of four components of M_r, 160,000, 180,000, 200,000, and 220,000 [23]. The latter two are immunochemically indistinguishable from tenascin [30].

Cytotactin Cytotactin is an extracellular matrix glycoprotein, which is isolated from embryonic chicken brains, is structurally related to polypeptides of M_r 220,000, 200,000, and 190,000 [22]. Its structure, as seen by the electronmicroscope, is six armed, the same as hexabrachion and tenascin [25]. Immunohistochemical studies showed that cytotactin appears in various neural tissues during development and is present in the basement membranes of a variety of nonneural tissues, including smooth muscle, lung, and kidney [22, 31]. Biochemical analysis revealed the binding of cytotactin to cells and to other extracellular matrix proteins, including a chondroitin sulfate proteoglycan and fibronectin [27]. Recently, its amino acid sequences were determined and the gene was cloned [24, 32]. Within the sequence, EGF-like repreats, fibronectin-homologous type III repeats, and an area of β–subunit of fibrinogen homology was found. The tissue culture studies of chicken neural crests indicated that cytotactin is a poor substrate for the crest cells and is inhibitory for cell migration [33]. On the basis of these results, the function of cytotactin has been suggested to be involved in cell adhesion, regulation of cell migration, and pattern formation.

Distribution of tenascin in the tissues

As described above, within the past six years and independently in several laboratories, several new extracellular matrix glycoproteins have been purified and some cDNA cloned. The immunological corss-reactivities, the electron microscope photographs, and the cDNA sequences of these poly-

Table 1. Distribution of GMEM, J1, cytotactin, and tenascin (myotendinous antigen) in normal and abnormal tissues.

Name	Recognized by	Source	Animal	Positive staining tissue	References
GMEM	Mab 81C6	U-251MG human glioma cell line	human	stroma and endothelial basement membranes of tumors (glioblastoma, melanoma, fibrosarcoma, Wilms' tumor, ovarian carcinoma) liver sinusoid (embryonic and adult) spleen red pulp sinusoid (embryonic and adult) kidney medullary tubule interstitium (adult) glomerular mesangium (adult)	[28]
J1	Mab 12/HNK-1	adult mouse brain	rat embryo	interstitial space between muscle cells intramuscular nerve fiber	[47]
			rat and mouse embryo	perichondrium surrounding Meckel's cartilage cartilage of developing bones of the cranial base developing membrane bones of the cranium and mandible dense mesenchyme of budding molar teeth	[30]
			mouse neonate	interbarrel septae of somatosensory cortex	[48]
Cytotactin	Mab NC-1	embryonic chicken brain	chicken embryo	basement membranes of the developing neural tube and natochord neural crest pathways surrounding the neural tube and somites	[31]

				central nervous system (all areas) smooth muscles basement membranes of lung and kidney	
Tenascin Myotendinous Antigen	Mab M1	embryonic fibroblast	chicken embryo	endomysium of muscle myotendinous junctions and tendon fascicles smooth muscles of aorta gizzard and lung buds limb buds	[14]
	Polyclonal antibody		rat embryo	dense mesenchyme of mammary glands, hair follicles, and teeth	[10]
				condensing mesenchyme of cartilage and bone anlage	[49]
				perichondrium periosteal and endosteal surface neural crest pathways	[50]
			adult rat	stroma of mammary tumors	[10]
				granulation tissue in healing wounds	[13]
			mouse embryo	kidney mesenchyme at the early tubulogenesis	[12]
				gut mesenchyme	[51]
				mammary gland mesenchyme	[11]
				urogenital sinus mesenchyme at the sites of prostatic buds and vagina formation	[52]
			adult mouse	gut stroma close to the migration pathway of renewing epithelium	[51]
				stroma surrounding mammary end buds stroma of mammary tumors stroma of metastasized tumors	[10]
			amphibian	neural crest pathways	[50]
				condensed mesenchyme of endocardium and aortic arch	[53]
			human	stroma of mammary cancers (adult)	[41]

peptides have revealed that they were identical. Immunohistochemical studies of these molecules have demonstrated the characteristic distribution with site and stage specific expression. As summarized in table 1, the localizations of these molecules are highly specific, suggesting that the role of these molecules is important for embryonic development because they aid in morphogenesis, pattern formation, tissue remodeling, stabilization, boundary formation, and so on.

Tenascin in mouse mammary gland development

This chapter describes the appearance and localization of tenascin during mammary gland morphogenesis and tumorigenesis, and its metastasis in mice. Mouse mammary gland is one of the most studied organs in animal biology research.

Fetal and neonatal development There are several papers based on histological observations describing the sequential changes of mammary gland morphogenesis [34, 35]. In 10–11-day fetuses (Vaginal plug = 0 day), the enlargement of a single-layered ectoderm appears as a line extending from the anterior to posterior limb buds. These enlarged epidermal cells then migrate to the proper sites, forming five pairs of mammary buds in 12-day fetuses. The mammary epithelium forms a lens-shaped structure at the beginning and changes to a bulb shape with a narrow neck at day 14 of gestation. Surrounding the epithelium, mammary mesenchyme exists, which is not a distinct tissue type at 10–12 days of gestation but which forms a dense mesenchyme composed of a few layers of fibroblastic cells. Tenascin appears in the mesenchyme at day 14 of gestation, localized in the limited area immediately surrounding the epithelium (figures 2 and 3).

In female fetuses, the mammary buds elongate by rapid cellular proliferation, forming mammary sprouts late on day 16. Tenascin is present in the dense mesenchyme but not present in the mesenchyme surrounding the top of the growing sprout. At day 17, the sprout grows downward, penetrating mammary fat pad precursor tissue. In the following few days until birth, the epithelium keeps growing and ramifying to form a mammary gland tree with about 15 to 20 branching ducts. At this stage, tenascin disappears but is still slightly positive in the mesenchyme around the nipple duct.

In male fetuses, the testes start to produce androgen late on day 13, and the male phenotype in the mammary gland is determined. As a result of androgen action, the dense mammary mesenchyme is further condensed and the epithelium decreases in volume; then the stalk connecting the mammary rudiment with the epidermis becomes very narrow and finally ruptures. The gland rudiment, detached from the epidermis, shows poor growth or even degeneration. There are clear strain differences. For instance, in BALB/c mice about half of the males completely lack mammary glands

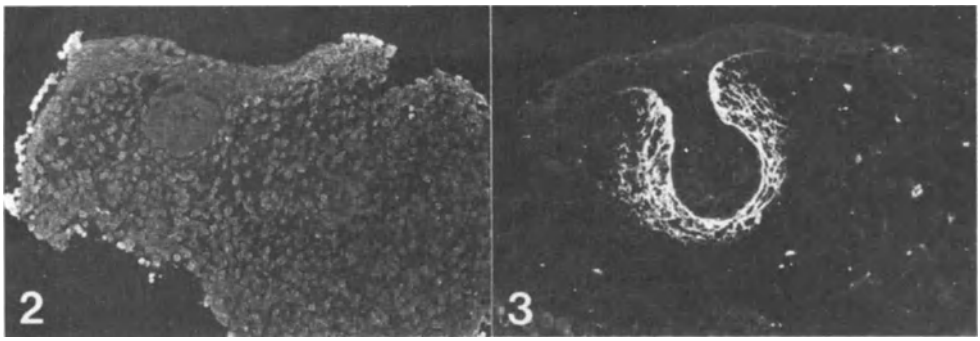

Figure 2 and 3. Indirect immunofluorescence polyester wax section of embryonic mouse mammary glands. Tenascin is absent in the gland of 13-day embryo (2) and appears on day 14 in the dense mesenchyme immediately surrounding the epithelium (3). (×80.) [From reference 11.]

and the other half have poorly developed mammary glands buried in the fat pad as a blind duct. In GR mice, males lack nipples but have normally developed mammary glands; and C3H mice are between BALB/c and GR. Tenascin appears in the dense mesenchyme in the same manner as in the female mammary gland. There is no indication that tenascin expression is related to androgen function.

Juvenile development From the time of birth up to the start of puberty, mammary glands increase in volume to keep pace with general body growth but do not change morphologically. At the approach of puberty, the mammary ducts start to grow rapidly, resulting in the formation of end buds and the elongation of ducts. This happens usually during the third to fourth week. The histology of end buds is characterized by the presence of a monolayered epithelium, the cap cells covering the tip of the end dubs along the flank and neck regions, where it is continuous with the myoepithelium [36]. In the stroma of the end buds, the tip or cap region is rich in hyaluronate, while the flank region is rich in chondroitin sulfate [37].

Tenascin is absent in the stroma of mammary glands at the prepubertal stage (figure 4), while in postpubertal glands it is present in the mesenchyme surrounding the end bud. At the beginning, tenascin appears at the tip of end bud (figure 5), then shifts to the flank region (figure 6). As the end bud elongates, tenascin starts to disappear from the tip and is present only in the neck region and in the clefts between branches (figure 7) [11]. The sites of the stroma where tenascin expression occurs are the same mesenchymal areas where sulfated glycosaminoglycans have been demonstrated [37]. Glycosaminoglycans may play an essential role in morphogenetic development of the growing epithelium [38, 39], and further, tenascin is supposed to be binding to proteoglycans in the native form [18]. These findings suggest that the interaction of tenascin and proteglycans or glycosaminoglycans

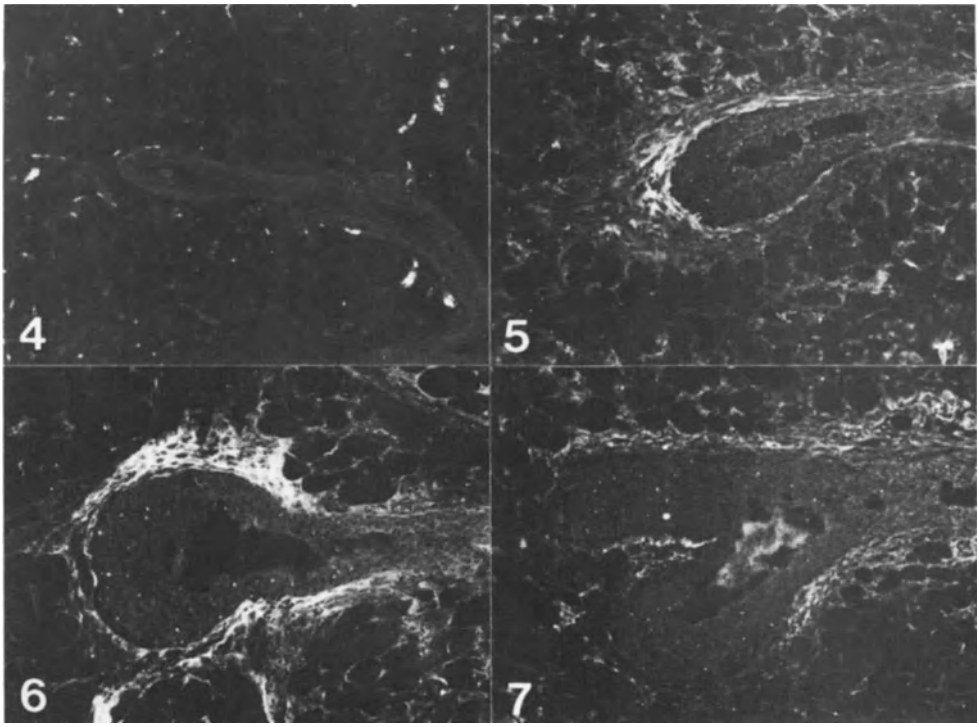

Figure 4–7. Distribution of tenascin in juvenile mouse mammary glands. Tenascin is absent in the mesenchyme of 2-week-old mammary gland (4). Tenascin appearance starts from the top of the end bud (5) and moves to the neck region (6), then is detected only in the cleft and flank region (7) as the end bud growth occurs. (×80.) [From reference 11.]

makes a complex molecule in the extracellular space and plays an important role in the establishment of the interductal spacing, in cooperation with other extracellular matrix substances, such as collagens, fibronectin, and laminin. And a variety of combinations between these various molecules could function as a stabilizer or an agitater of mammary gland development.

Pregnant, lactating, and involuting mammary glands During pregnancy the ducts become much longer, with numerous lateral bud outgrowths, which then form the intralobular ducts of the future lobes. At the terminal end and the lateral walls of these ducts, more buds appear and proliferate to develop alveoli, giving the hyperplasia of the gland parenchyma. Following parturition, the cells are stimulated to secrete milk. During the entire period of lactation, the epithelial parenchyma predominates, with the fatty stroma appearing very slight in amount. After weaning the involution proceeds rapidly. Tenascin is not present in the stroma throughout any of the procedures: proliferation, differentiation, or involution of the mammary glands.

Preneoplastic and neoplastic development A hyperplastic alveolar nodule (HAN) is considered to be preneoplastic and appears before a spontaneous mammary tumor developes, in certain strains of mice, including C3H and GR (40). Histologically, HAN resembles the mammary gland at the late pregnant or early lactating stage. It is composed of proliferating alveolar cells and there is slight secretion. HAN produces HAN when transplanted into the mammary gland fat pad of which epithelial components have been removed previously, and further, almost all HANs then give rise to tumors in the fat pads. Thus HAN is understood to be a lesion in which cells have been transformed and have a higher sensitivity to lactogenic hormones. Tenascin is not present in the stroma of HANs.

Most mammary tumors in mice are hormone independent and are capable of growth even in male hosts following subcutaneous isografting. Histologically, adenocarcinoma is the most frequent. The well-differentiated type of adenocarcinoma manifests an acinar structure. Poorly differentiated types show papillary or intracanalicular growth and cyst formation. Tenascin reappears in the stroma of all these mammary tumors, surrounding the acinar and tubular nests, and also in capsular connective tissue of the tumor nodules (figure 8).

Metastasized tumor Mouse mammary tumors frequently metastasize to other organs, particularly to lung and lymph nodes. Tumor cells that come through the capillary wall settle down and proliferate in their new environment. Tenascin appears in the stroma immediately surrounding the tumor epithelium (figure 9).

Tenascin in human breast

Comparative studies of malignant and benign mammary tumors by immunohistochemistry have revealed that tenascin is a stromal marker for epithelial

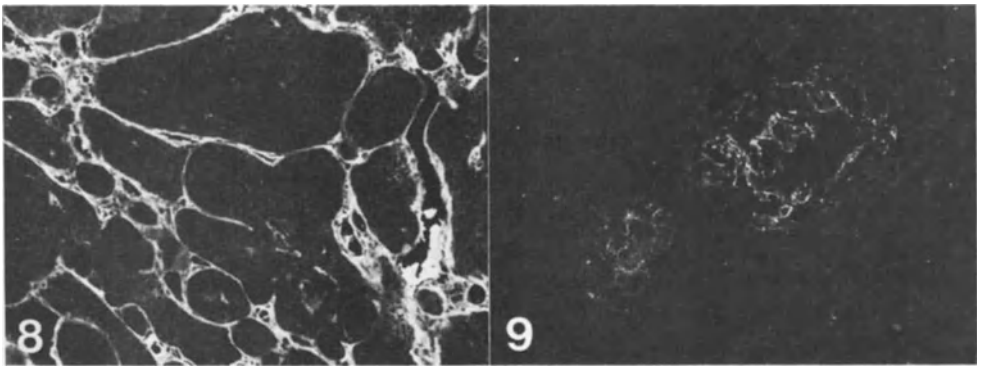

Figure 8 and 9. Distribution of tenascin in mammary tumors. Tenascin is present in the dense mesenchyme of mammary tumor (8) and the lung metastases (9). (×80.) [From reference 11.]

malignancy [41]. It has been suggested that the appearance of tenascin in the stroma is due to an interaction between stroma and cancer cells [11]. Therefore, malignant transformation of epithelium seems to be characterized by the capacity to stimulate the stroma cells to produce tenascin. However, tenascin is also expressed in fetal and juvenile mammary glands, and granulation of healing wound is another positive tissue [13]. Several normal tissues, such as kidney mesenchyme, bone marrow stroma, and smooth muscle of the gastrointestinal tract, are tenascin positive [18]. The distribution pattern of tenascin in the various tissues supports the idea that tenascin appears in the stroma as a result of histological disorders of the tissues, such as cancers, but that this mechanism does not account for all appearances of tenascin.

A monoclonal antibody has been produced against human tenascin [18] and is available for the histological analysis of surgical materials. The results of immunohistochemistry of human breast tissues are summarized (table 2). As seen in the table, tenascin was not detected in proliferating mammary lesions, such as mastopathy and lactating mammary gland (figure 10), while in malignant invasive ductal carcinoma, tenascin appeared in most cases (figure 11). About 90% of the lesions were positive, and more than half of them were highly positive. Malignant but noninvasive tumors showed less tenascin expression (figure 12); Out of 32 lesions, 12 were negative, 17 were slightly positive, and only 3 were highly positive. This incidence is similar to the results from benign breast tumors, such as gynecomastia and fibroadenoma (figure 13). The stroma of mucinous or medullary carcinoma expressed tenascin much less than did ductal carcinoma. Both of these tumors appear to have a better prognosis than does invasive ductal carcinoma. These results suggest that tenascin is a stromal marker for the invading capacity of epithelium and for an unfavorable prognosis.

Table 2. Tenascin expression in human breast tumors.

Histological classification (WHO)	No of lesions	Tenascin expression		
		−	+	++
Benign				
Gynecomastia	8	4	3	1
Fibroadenoma	9	5	4	0
Mastopathy (nontumor)	7	7	0	0
Lactating (nontumor)	1	1	0	0
Total	25	17	7	1
Malignant				
Noninvasive				
Intraductal carcinoma	32	12	17	3
Invasive				
Ductal carcinoma	85	8	33	44
Mucinous carcinoma	5	2	3	0
Medullary carcinoma	36	4	30	2
Others	1	0	1	0
Total	159	26	84	49

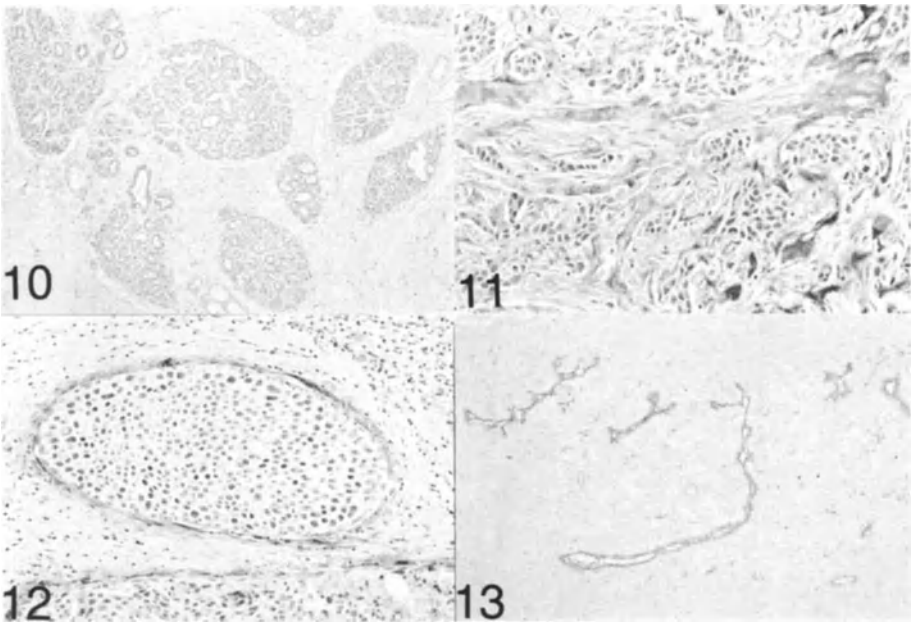

Figure 10–13. Enzyme immunohistochemistry of human mammary glands. Tenascin is strongly positive in the connective tissue of invasive ductal carcinoma (11) and weakly positive in the dense mesenchyme immediately surrounding the noninvasive intraductal carcinoma (12), but appears neither in mastopathy (10) nor in fibroadenoma (13).

Induction of tenascin in mesenchyme

During mammary gland development from embryogenesis to carcinogenesis, tenascin is expressed in the stroma closely surrounding the epithelium of the fetal, juvenile, and neoplastic mammary glands. A question as to whether it appears as a course or a consequence of the interaction between epithelium and mesenchyme is biologically important. The following two findings indicate that tenascin synthesis is induced by either fetal or neoplastic epithelium. If the epithelial component of embryonic mammary gland is isolated and then cultured on a feeder layer from a fat pad, the epithelium penetrates the cultured fat pad, then undergoes mammary gland morphogenesis forming elongated ducts with end buds at the tip [42]. Closely surrounding the explanted epithelium, a halo is formed by the dense feeder cells after two days in culture. Tenascin accumulates in this area but not in the peripheral site of the feeder layer (figure 14) [11]. This experiment indicates that tenascin synthesis is induced in the mesenchyme by interacting with the embryonic epithelium. The other finding is the induction of tenascin by neoplastic epithelium [11]. CMT315 is a cell line that originated from a C3H mammary tumor. Subcutaneous injection of these cells into BALB/c nude mice gives rise to tumors. Each tumor contains fibroblastic mesenchyme

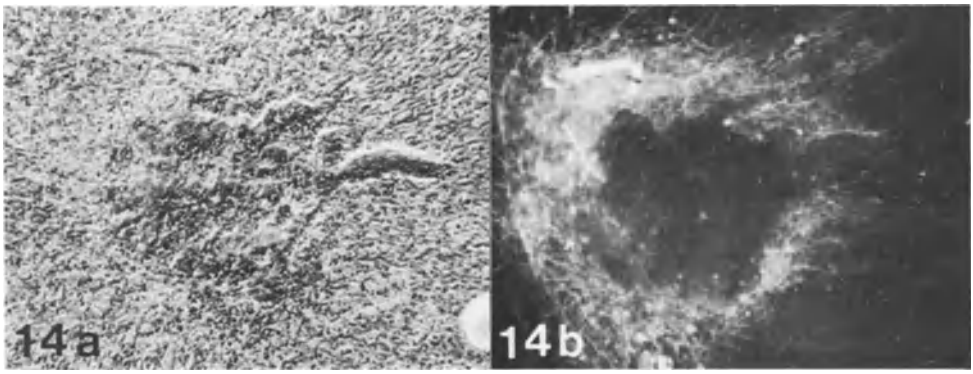

Figure 14a and 14b. Tenascin in mammary morphogenesis in vitro. Tenascin is detected only in the feeder cells surrounding the cultured mammary epithelium. The other cells are not stained by antitenascin. (a) Phase-contrast microscopy. (b) Indirect immunofluorenscence of the same tissue. (×80.) [From reference 11.]

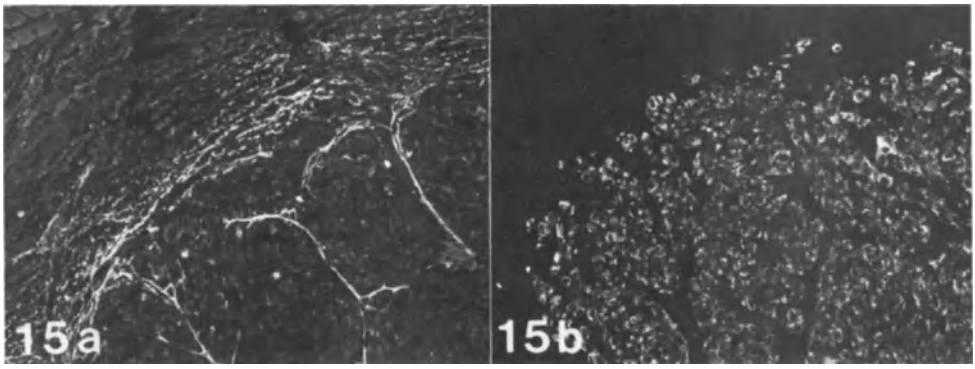

Figure 15a and 15b. Distribution of tenascin and CSA [43] in the tumor induced by subutaneous injection of C3H mammary tumor cells into a BACB/c nude mouse. Tenascin is detected in the mesenchyme surrounding the tumor (a). These mesenchymal cells are not stained by anti-CSA (b), indicating clearly the BACB/c origin. (×80.) [From reference 11.]

and is surrounded by dense connective tissue. The tumor cells originate from the C3H line, but the fibroblasts must be derived from the BALB/c nude host (figure 15). The cells on which tenascin are detected are from the BALB/c nude because they do not express a C3H-specific antigen [43], and this result indicates that the tumor cells induce the BALB/c mesenchyme to produce tenascin.

Other reports also suggest this idea, such as tenascin expression in newly formed granulation tissue induced by epidermis in wound healing [13], and tenascin's appearance in the mesenchyme of developing kidney [12].

Role of tenascin in breast cancer

As described above, tenascin was found to be a kind of oncofetal antigen of the stroma. Immunohistochemistry demonstrated tenascin in the mammary gland mesenchyme that closely surrounds the epithelia of both embryonic and neoplastic origin [10]. In the proliferating glands during pregnancy, however, tenascin expression was not observed. In distinguishing between sections of malignant and benign human mammary tumors, tenascin was found to be a good marker (41). Both in vivo and in vitro experiments suggest that tenascin is induced by embryonic or malignant epithelia [11]. On the basis of these findings, we postulate that tenascin appears as a result of interactions between epithelium and mesenchyme during embryogenesis, and recapitulating embryogenesis appears in tumor development.

Subsequently, immunohistochemical studies have shown that tenascin and similar substances can be demonstrated in many other tissues besides embryonic and tumor mesenchyme (see table 1), including a wide variety of animal species and many organs. The expression of tenascin is restricted to the mesenchyme of developing tissues undergoing physiological and/or pathological changes, and it appears to be responding to specific tissue change. This is different from other extracellular matrix substances.

The biological function of tenascin has not been elucidated yet. However, several findings in culture studies suggest such activities as cell attachment, detachment, migration, proliferation, and morphogenesis. The results of immunohistochemistry experiments also support these possibilities. The fact that tenascin is more abundant in breast cancers of unfavorable prognosis with invading tendency than in the noninvading cancers of better prognosis suggests that the role of tenascin in cancers may be related to the migration and invasion of cancer cells. If we compare the functions of tenascin with those of fibronection [44], laminin [45], and collagens [46], we are surprised to find that so many functions are similar among the extracellular matrix substances. It may be that each substance has no unique function but can express differing informations depending on a three-dimentional architecture that these substance join in building. Tenascin may be the cement that appears in the case of tissue emergency.

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19. Stromal-epithelial interactions in normal and neoplastic mammary gland

Sandra Z. Haslam

Introduction

Mesenchymal-epithelial interactions are critically important during embryonic development for normal organogenesis. These tissue interactions are thought to be reciprocal and are clearly involved in many fundamental developmental mechanisms, such as morphogenetic movement [1–3], cellular proliferation [4–7], and cell death [8]. Although many organs are fully functional before birth, other organs, particularly those of the reproductive system, undergo major morphogenetic changes and cyclic expression of functional differentiation postnatally over extended periods.

In the case of the mammary gland, true morphogenetic processes occur cyclically during estrous or menstrual cycles, pregnancy, and lactation and are hormonally regulated. During lactational involution, the epithelial and stromal elements degenerate and later reform from rudimentary precursors. Thus epithelial renewal, morphogenesis, and differentiation must be tightly regulated to meet the functional demands of successive reproductive cycles. It is likely that these events proceed by the same basic mechanisms that are operative during embryonic development and may be dependent upon stromal-epithelial interactions in the adult. In this context, the abnormal cell proliferation and loss of hormonal regulation that often occurs in mammary cancer is likely to involve a change or loss in the normal regulatory interactions between mammary stromal and epithelial cells.

The mammary gland is composed of epithelial, adipose, and fibrous connective tissues. The relative proportion of each tissue type varies with the species of origin and/or the developmental state of the gland. The term 'stroma' herein refers to the adipose and fibrous connective tissues of the postnatal gland and is derived from embryonic mesenchyme (i.e., embryonic connective tissue). This review will examine the roles and underlying mechanisms of stromal-epithelial interactions in normal mammary gland growth and differentiation and in mammary neoplasia. Because rodent mammary gland has been studied most extensively in this regard, studies in mice and rats provide the main focus for this review.

M. Lippman and R. Dickson (eds.), REGULATORY MECHANISMS IN BREAST CANCER.

Embryonic mesenchymal-epithelial interactions in normal gland

During embryonic development in mice, mesenchymal-epithelial interactions are evident at day 11, the time at which mammary gland anlagen is first identifiable and progressive changes in mesenchymal-epithelial interactions occur rapidly in the embryo [9]. The embryonic mesenchyme both induces organotypic morphogenesis in the fetus and in the male and mediates androgen-induced regression of the epithelium [10]. A reciprocal regulation of differentiation occurs such that the mammary epithelium induces the expression of androgen receptors in the mesenchyme in both males and females. The mesenchyme, in response to testosterone, then induces the regression of the mammary epithelium in the male [11, 12]. At day 14, two different mesenchymal components are identifiable. The first is the fibroblast mesenchyme that directly surrounds the epithelial rudiment, and the second is an underlying fat pad mesenchyme. These two mesenchymes give rise to the fibrous and adipose stromal components of the gland, respectively [13]. Steroid autoradiography has shown that in the 16-day female fetal mammary gland, the cells that possess estrogen receptors are restricted to the fibroblast mesenchyme and are not present in either the epithelial rudiment or the fat pad mesenchyme [14]. Interestingly, injection of estrogens in the neonatal period can result in numerous epithelial malformations [15]. Thus the fibroblast mesenchyme appears to be an estrogen target tissue capable of estrogenic responses that affect the epithelium.

In a number of classic embryonic-adult tissue recombination studies, Sakakura and colleagues have demonstrated that adult mammary stromal fat pad retains mesenchyme-like inductive ability capable of inducing embryonic mammary epithelium to undergo characteristic morphogenesis in vivo [16]. Furthermore, adult mammary epithelium retains the ability to respond to the inductive influences of embryonic mesenchyme [17]. However, the two types of embryonic mesenchyme behave differently when tranplanted into adult mammary gland. The fibroblast mesenchyme, the fibroblastic stromal precursor, induces ductal hyperplasia, whereas the fat pad mesenchyme induces normal epithelial glandular architecture [13].

The molecular mechanisms underlying mesenchymal epithelial inductive interactions are not well understood. Grobstein's studies suggested that the induction of epithelial development is mediated by the extracellular matrix that is laid down by specific mesenchyme [18]. In order to understand how each of the embryonic mesenchymes might produce different morphogenetic patterns, Sakakura et al. analyzed the extracellular matrix molecules produced by the different mesenchymes [19]. They showed that the fibroblast mesenchyme produces only fibronectin, whereas the fad pad mesenchyme produces laminin and heparin sulfate proteoglycan. Laminin and heparin sulfate proteoglycan are also components of the epithelial basement membrane. Sakakura has hypothesized that the molecular composition and structural features of the mammary gland basement membrane provide the specific conditions necessary for mammary epithelium to undergo normal

morphogenesis. Thus the extracellular matrix products of the fat pad mesenchyme might affect the basement membrane assembly, and this in turn determines the branching pattern of the mammary gland.

Recently another extracellular matrix glycoprotein, tenascin, has been identified in embryonic mammary gland and appears to be specifically produced by the fibroblast mesenchyme but not by the fat pad mesenchyme [20]. A cDNA clone for tenascin has been isolated that encodes for a protein sequence of 632 amino acids containing 13 consecutive epidermal growth factor (EGF)-like repeats [21]. The function of the EGF-like region is unknown but could be the domain causing the hyperplastic growth-stimulating effect that is observed when mammary mesenchyme is transplanted into the adult gland.

Embryonic mesenchymal-epithelial interactions and mammary cancer

That embryonic mesenchymal-epithelial type interactions might be important in mammary tumorigenesis has also been investigated. DeCosse et al. [22] reported that a mouse mammary tumor line could be induced to differentiate when cultured transfilter from mouse mammary mesenchyme in vitro. Sakakura et al. [23] found that when ductal hyperplasias produced by embryonic mammary mesenchyme were transplanted into mammary tumor virus-positive C3H mice, they were 12 times more likely to form tumors than was transplanted normal mammary tissue. Most recently Sakakura and her colleagues have also shown that tenascin is a stromal marker for epithelial malignancy in the adult mammary gland [24]. As described above, tenascin is an extracellular matrix molecule produced by embryonic fibroblast mesenchyme, and it is not found in the normal mature mammary gland [20]. However, a survey of benign and malignant mammary tumors in mice, rats, and humans has shown that tenascin was present only in stroma of malignant but not benign tumors from all species [24]. Further experimental studies in mice have demonstrated that tenascin can be induced in the adult mammary gland experimentally either by injecting tumor cells or by transplanting embryonic mammary epithelium into adult stroma. Tenascin appears to be associated with the proliferation of embryonic epithelium as it starts to develop morphogentically, but not with cellular proliferation leading to cytodifferentiation such as seen in adult mammary gland during pregnancy [20]. Thus the presence of tenascin in malignant mammary tumors is indicative of the disruption of adult regulatory stromal-epithelial interactions resulting in the regression to a more dedifferentiated, embryonic state.

Postnatal stromal-epithelial interactions in normal gland

The retention of inductive activity in adult mammary stroma and the maintenance of the ability of adult epithelium to respond to inductive mesenchymal influences provide suggestive evidence that stromal-epithelial

interactions may be active in the postnatal gland. More direct evidence is derived by examining the growth behavior of the postnatal mouse mammary gland. Postnatal ductal elongation occurs at puberty and is the result of proliferative activity in the mammary stroma and in specialized epithelial structures called end buds [25]. Transplantation experiments have demonstrated that an adipose stromal matrix is an absolute requirement for ductal development [26]. Transplantation to any nonmammary site of adipose stroma also will support mammary epithelial growth, which argues against a requirement for specific mammary adipose stroma. However, subcutaneous transplantation or transplantation into any other stromal matrix will not support normal mammary growth. If epithelium is transplanted into an aritificial collagenous matrix (collagen gel) within the mammary adipose stroma, the epithelium will produce atypical spike-shaped outgrowths. However, when these spikes grow beyond the collagen and come into contact with adipose tissue, normal end buds form [27].

In the nonpregnant state, the adipose tissue constitutes the major portion of the gland. During pregnancy and lactation, the epithelial component of the gland increases concomitant with a depletion of the adipose tissue. After involution, there is degeneration and loss of the secretory epithelium and the adipose tissue is restored. Thus cyclic changes in mammary adipose tissue composition occur in relation to mammary gland function. Accumulation of glycogen, the activity of glycogen synthetase, and lipogenic rate in mammary adipocytes are modulated during pregnancy and lactation to channel nutrients to the mammary epithelial cell [28]. These changes appear to require the presence of mammary epithelium, indicating interactive behavior and possible metabolic cooperativity between epithelial cells and adipocytes. Similar interactive phenomena may also take place at other mammary gland developmental states.

The fibroblast-derived tissue component of postnatal mammary stroma may also play a role in the regulation of epithelial function. During ductal elongation as the end bud proliferates and penetrates the adipose stroma, the subtending newly formed duct is surrounded by a sheath of fibrous connective tissue; this surrounding stroma is rich in fibroblasts [29]. Extracellular matrix molecules, particularly glycosaminoglycans, are known to be important in the regulation of branching growth [2]. Intense chondroitin sulfate synthesis is observed along the newly formed duct in association with the fibroblasts. It has been hypothesized that changes in glycosaminoglycan turnover are controlled locally by stromal cells and are assoicated with either growth or cytodifferentiation and tissue stabilization [30]. Postnatal ductal elongation has also been shown to be accompanied by DNA synthesis in unidentified stromal cells close to the growing end buds [29]. This proliferation of the stroma is a response to the proliferating epithelium and does not occur in the absence of epithelial proliferation [29]. Thus this is further evidence of the reciprocal interactions between epithelial and stromal cells of the postnatal gland.

In the human mammary gland, the structural relationship between the epithelial and stromal components have also been examined. The interlobular stroma that is directly adjacent to the epithelium is composed of a dense fibrous connective tissue. Ozzello has studied the ultrastructural features of the constituents of that portion of the human breast in which the epithelial structures and stroma come into contact and has termed this zone the epithelial-stromal junction [31]. The epithelial-stromal junction includes the plasma membranes of epithelial and myoepithelial cells, intercellular spaces, lamina lucida, basal lamina, fibrillar connective tissue and ground substance beneath the basal lamina, and a layer of delimiting fibroblasts. The latter make an almost continuous layer of attenuated cytoplasm enveloping ducts and ductules. Alterations in the ultrastructural features of the epithelial-stromal junction have been observed in various manifestations of mammary dysplasia [31]. These alterations include straightening of the plasma membranes, straightening of the basal laminae, increased number of elastic fibers, increased number and compactness of collagen fibers, and partial to total loss of the layer of delimiting fibroblasts. Ozzello has hypothesized that the epithelial-stromal junction acts as a functional unit in the transport of materials to and from the epithelium, thus taking an active part in the normal functions of the mammary gland and in the evolution of mammary dysplasia.

The intralobular stroma of the normal resing human mammary gland is comprised of fibroblasts, adipocytes, blood vessels, and mononuclear cells; in contrast with the interlobular stroma, which has fewer cells separated by more compact collagen. Eyden et al. [32] have studied the ultrastructural features of the interlobular stroma and have found that the interlobular fibroblasts ultrastructurally resemble the delimiting fibroblasts of intralobular stroma and appear to from close contacts with the delimiting fibroblasts via close plasma membrane juxtapositions. They suggest that it would be more appropriate to extend the morphophysiological unit applied to delimiting fibroblasts to include the entire population of interlobular fibroblasts.

Further evidence that appropriate stromal-epithelial interaction may be required in order to express normal function in human breast comes from the transplantation studies of Sheffield and Welsch [33]. In these experiments enzymatically dissociated normal breast tissue was injected into epithelium-free mammary stroma of athymic nude mice. Appropriate growth-promoting mammotropic hormones were also provided. The human tissue was readily accepted and maintained, and the epithelium formed duct-like organoids. However, no expansive growth was observed. By contrast the hormonal treatment caused extensive proliferation of the host mouse glands. One explanation offered for the lack of human tissue growth was that the mouse mammary stroma may not be the appropriate stromal environment for human tissue. In this context, Horgan et al. [34] have demonstrated that when human fibroblasts and MCF–7 mammary cancer cells are transplanted in nude mice, there is a significant increase in tumor take and growth rate over that observed when MCF–7 cells are transplanted alone. The fibroblasts

from benign or malignant breast tissue or skin appeared to be equally effective in stimulating growth, whereas substitution of other cell types was not effective. Also of interest was the observation that killed fibroblasts were able to increase tumor take, but not tumor growth. The authors speculate that the killed fibroblasts may act to provide a substratum effect similar to that observed in cell culture. However, the requirement of live fibroblasts for growth to occur indicates that they may secrete growth factors that stimulate tumor cell growth.

Stromal-epithelial interactions and hormonal regulation

The cyclic processes of growth and differentiation experienced by the postnatal mammary gland are hormonally regulated. The most readily detectable hormone-induced changes occur in the epithelial compartment of the gland; however, there are changes in the stromal compartment as well. In the rodent gland, as described above, there is depletion of the adipose stroma during pregnancy and lactation, with replenishment upon lactational involution. In the human breast, morphologic changes in mammary stroma have also been identified during the menstrual cycle [35]. The principal stromal change observed was the premenstrual edema and loosening of the interlobular connective tissue. An increase in the acid mucopolysaccharide content of the intralobular stroma has been demonstrated during the menstrual cycle and may also account for the alteration in connective tissue structure [36]. The cyclic changes in mammary stroma in both rodent and human mammary gland may be attributed to mammotrophic hormone-induced changes.

The major hormones involved in the postnatal growth and morphogenesis during the ovarian cycle and during pregnancy are estrogen and progesterone [37]. More recently a role for EGF in mammary gland growth and development has been proposed [38]. According to current models of hormone action, initial interaction of hormones and hormone receptors are required to initiate the cascade of events that result in the biological response. This has led us to consider that modulations in stromal-epithelial interactions might be mediated by modulations in tissue hormone receptor concentration.

Analysis of estrogen receptor distribution between the epithelial and stromal cell compartments in normal mouse mammary gland has revealed that in the mature nonpregnant mammary gland, the stroma contains 50% of the estrogen receptors present in the gland [39]. It is interesting to note that estrogen receptors have also been identified in all other adipose tissue tested [40]. This may be the reason that all adipose tissue can support mammary gland growth. During pregnancy, the total estrogen receptor content per cell decreases significantly, with a specific decrease occuring in the epithelial compartment (figure 1). During lactation there is a further decrease. However, this is due to a decrease in stromal estrogen receptor

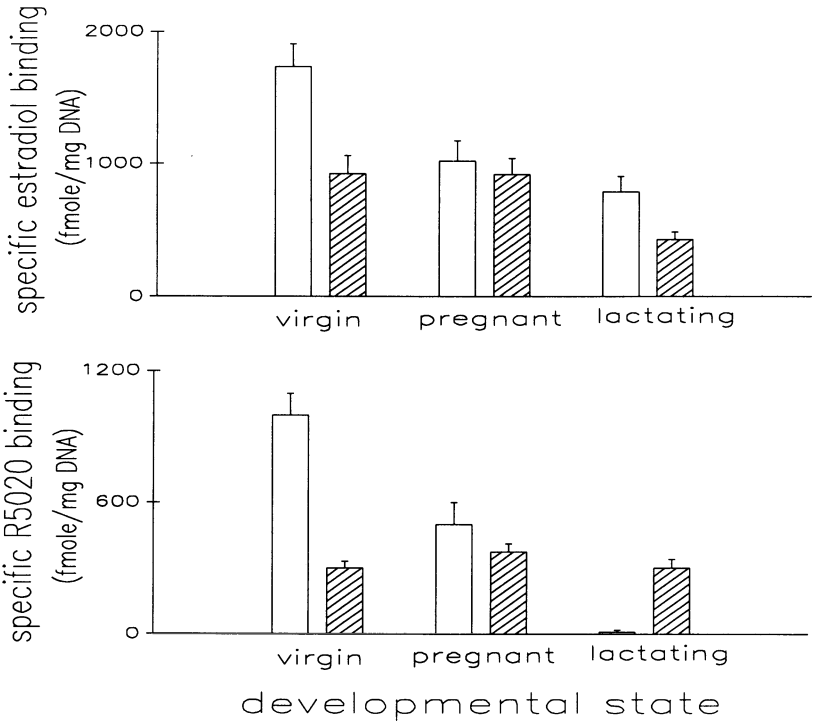

Figure 1. Distribution of estrogen and progesterone receptors in intact mammary gland versus mammary stroma at different developmental states. Estrogen and progesterone receptors were quantitated in intact gland (□) or epithelium-devoid mammary stroma (■) by ligand-binding assay methods [39, 41].

[41]. Thus modulations of estrogen receptor concentration occur in both epithelial and stromal cells at different stages of mammary gland development.

Recently estrogen receptors have also been detected in epithelial cells of normal human breast tissue, using a monoclonal antibody to the human estrogen receptor [42]. Using this method, stromal cells appeared to be devoid of estrogen receptors, indicating a possible species difference with regard to stromal cell estrogen receptor localization.

In the case of progesterone receptors, only 20% of the receptors in the mouse mammary gland are associated with stromal cells [39]. In the mature mouse, rat, rabbit, and bovine mammary gland [41], progesterone receptor concentration changes with the developmental state of the gland. In all cases, progesterone receptors are highest in concentration in mammary tissues of nonpregnant, nonlactating animals. With the onset of pregnancy, progesterone receptor concentration decreases and is generally undetectable or absent during lactation (figure 1) [43]. The modulation in progesterone receptor concentration appears to take place in the epithelial cell compartment [41]. Progesterone receptors present in epithelial cells are under

estrogenic regulation, whereas the stromal progesterone receptors are estrogen independent [41]. At present very little is known about the cellular distribution of progesterone receptors among the epithelial and stromal cell compartments in human breast tissue. Steroid autoradiographic analysis of estrogen and progesterone receptor localization in mouse mammary stromal cells revealed that receptors are localized in an unidentified cell type that more closely resembles an undifferentiated mesenchymal cell than either an adipocyte or fibroblast [Haslam, unpublished observations]. The functional significance of the presence and modulations in the concentrations of stromal estrogen and progesterone receptors in normal rodent mammary gland is not clear. However, recent studies on the proliferative effects of estrogen and progesterone in normal adult nonpregnant mouse mammary gland have revealed that, as in epithelial cells, mammary gland stromal cell DNA synthesis is also stimulated by both estrogen and progesterone [Haslam, unpublished observations]. Other studies have shown that estrogen stimulates the proliferation of human preadipose cells in culture [44]. Estrogen has been shown to promote adipocyte hyperplasia in adult mouse mammary gland [45].

EGF receptors have been detected in normal mouse mammary gland and, like estrogen and progesterone receptors, their concentration undergoes modulations during postnatal development [46]. Beginning at weaning, there is a constant decrease in EGF receptors with increasing age. During pregnancy the receptor level increases, followed by a decrease during lactation. It is not known if changes in EGF receptor concentration occur in epithelial or stromal cells. In the immature mammary gland, autoradiographic studies using ^{125}I–EGF have shown that epithelial cell labeling is seen only in the outermost cell layer of growing end bud. However, heaviest labeling in the gland is seen in stromal cells adjacent to growing ducts [38]. In our own studies on mature mammary gland, only stromal cell labeling is observed [Haslam, unpublished observations]. The significance of epithelial cell labeling in cells of the end bud seems straighforward, since EGF has been shown to directly promote end bud DNA synthesis [38]. The role of stromal cell EGF receptor is less clear. EGF is known to stimulate mouse fibroblast cell division [47], and the flank region of the end bud is characterized by aggregations of fibroblsts that elaborate large amounts of collagen I and extracellular matrix components [30]. Thus the effect of EGF on mammary stromal cells in both the immature and adult gland may be to stimulate and/or modify the synthesis of extracellular matrix components, which in turn may influence epithelial cell proliferation and morphogenesis.

Stromal-epithelial interactions in vitro

The experimental approach for identifying the role of stromal-epithelial interactions in relation to hormonally regulated growth and function in the

postnatal mouse mammary gland is difficult in the whole animal, thus this probelm has also been addressed using various cell culture systems. Epithelial cell interactions with two cell types, mammary stromal fibroblasts and adipocytes, have been analyzed using in vitro models.

Mammary stromal fibroblasts form a collagenous sheath around mammary ducts and separate them from the subadjacent adipose tissue. Thus fibroblasts are the stromal cells closest to the epithelium. These cells are also responsible for contributing stromal extracellular matrix components, such as collagen I and fibronectin, to the extracellular space and to the epithelial basement membrane. As suggested above, it is possible to speculate that mammary fibroblasts may play a regulatory role in epithelial function based upon their contribution to extracellular matrix composition [see chapter 17]. A paracrine effect on epithelium via local production of growth factors is also a potential mode of action of stromal fibroblasts.

Using a two-dimensional monolayer culture system in which mammary stromal cells and colonies of normal epithelium were allowed to confront each other, McGrath [48] showed that epithelial cell growth was inhibited by confrontation. However, epithelial cell growth was reinitiated by addition of estrogen. Contact between stromal cells and the epithelium was critical for the response to estrogen.

We have extended the investigation of mammary epithelial cell fibroblast interactions in order to elucidate the underlying mechanisms involved. Our studies have specifically addressed the interaction of the mammary fibroblast with the epithelium and have examined the response of these cells to two well-characterized effects of estrogen in vivo, namely estrogenic regulation of progesterone receptor levels and cell proliferation [49, 50]. When mixed cultures containing both epithelials cells and fibroblasts were exposed to estrogen, there was a threefold increase in progesterone receptor concentration. When cultures containing only epithelial cells were similarly tested, no stimulation of progesterone receptors was detected. We established that the absence of reponse in epithelial cultures is not due to a loss of estrogen receptors in the epithelial cells [49]. Furthermore, the response observed in mixed cultures is not due to an increase in progesterone receptors in the fibroblast subpopulation [49]. Thus the estrogenic regulation of epithelial cell progesterone receptors in vitro was specifically associated with the presence of mammary stromal fibroblasts.

The favorable effect of fibroblasts on mammary epithelial cell estrogen-induced responses can be explained in several different ways. One possibility is that fibroblasts provide some unidentified soluble factor. Another way that fibroblasts might act is to modify the culture substratum, since the physical nature and chemical composition of culture substratum is known to be important for the expression of mammary epithelial cell differentiated function in vitro [51]. Alternatively, direct contact between epithelial cells and fibroblasts may be required to facilitate estrogen responsiveness. Based upon results from coculture experiments with epithelial cells and fibroblasts,

we have shown that in the case of progesterone receptor regulation, fibroblasts need not be alive or even physically present, since conditioned medium is effective in promoting progesterone receptor response [50]. Pretreating dishes with collagen I was equally effective, which suggests that fibroblasts most likely act through a substratum effect.

Parallel studies to assess the effect of mammary fibroblasts on estrogen-induced epithelial cell DNA synthesis demonstrated that fibroblasts need to be metabolically active and in close contact with epithelial cells to promote DNA synthesis [50]. Furthermore, in the presence of mammary epithelial cells, fibroblast DNA synthesis is also stimulated by estrogen.

Interestingly, the morphology of both epithelial cells and fibroblasts is altered when cocultured. When epithelial cells are cultured alone, colonies of cells with rounded contours are observed. When epithelial cells are cultured with live fibroblasts, there are several notable differences. Colony contours are stellate in shape, and advancing strands of epithelial cells are frequently observed. When grown separately, fibroblasts appear to have a random orientation. By contrast, when cocultured with epithelial cells, fibroblasts appear more elongated and assume a characteristic orientation of parallel arrays between epithelial colonies. These results suggest that there is a bidirectional interaction between the two cell populations

From these studies we have concluded that there appear to be at least two different mechanisms by which mammary fibroblasts affect estrogen-specific responses in the epithelial cells. In the first case, estrogen-dependent progesterone receptor stimulation appears to be mediated by fibroblasts via extracellular matrix. In the second case, mammary fibroblasts need to be metabolically active and in close contact with epithelial cells in order to promote estrogen-dependent DNA synthesis. Furthermore, the reciprocal effect on fibroblast DNA synthesis and cell morphologies strongly indicate interaction between the two cells types. These results clearly demonstrate stromal-epithelial interactions in vitro and may be indicative of the role and nature of stromal-epithelial interactions in vivo. In other studies [52], conditioned media from mouse mammary fibroblasts has been shown to stimulate DNA synthesis in normal and neoplastic mouse mammary epithelial cells in primary culture. The growth-promoting activity in conditioned medium was described as a growth factor distinct from EGF. Similar results have been obtained using stromal cells from rat mammary glands [53].

Interactions of mammary epithelium with adipocytes in cell culture have been investigated. Using the 3T3–L1 cell line, which can exist as preadipocytes or fully differentiated adipocytes, Levine and Stockdale [54] have shown that DNA synthesis in mammary epithelial cells from mid-pregnant mice is highest in the presence of adipocytes. The adipocyte factor is present in both conditioned medium and substatum-attached material derived from adipocytes. The authors conclude that adipocyte effects on extracellular components may be responsible for mediating the increase in epithelial proliferation. Adipocytes have also been shown to promote mammary epi-

thelial cell differentiation in cell culture [55, 56]. When cultured on either preadipocytes or adipocytes in the presence of lactogenic hormones, mammary epithelial cells from nonpregnant, pregnant, lactating, and involuting mice were all capable of producing milk proteins. Ultrastructural studies revealed that the formation of a basement membrane occured only under coculture with preadipocytes or adipocytes. No differentiation was observed with swiss 3T3 cells, newborn foreskin fibroblasts, or substrate-attached material from adipocytes. Thus these results indicate cell–cell interaction was required for the effects observed on differentiation. However, other studies have shown that providing reconstituted basement membrane is sufficient to allow greater than 90% of mammary epithelial cells to express differentiated function in culture [57]. Other studies have also shown that linoleate metabolites enhance the in vitro proliferative and lactational responses of mouse mammary epithelial cells [58, 59]. Thus a metabolic role of mammary adipose stroma may be a key to adipocyte effects in vitro.

Adult stromal-epithelial interactions and mammary cancer in adult tissues

While most studies of mammary neoplasia have been primarily concerned with epithelial structures of the mammary gland, it is well known that the stroma of the human breast undergoes morphologic and chemical changes in physiologic and pathologic conditions of the gland [60–62]. Over the years these stromal changes have also been investigated in order to have a better understanding of the role of stroma and stromal-epithelial interactions in the genesis and progression of mammary neoplasia. The early studies focused on light and electron microscopic evaluations of various dysplastic and neoplastic conditions of the human mammary gland.

Desmoplasia is the common host response to epithelial tumor invasion and is classically described as proliferation of fibroblasts with excessive collagen production [63]. The precise mechanism and significance of the desmoplastic response are still under investigation. A common feature of the desmoplastic reaction is that the proliferating fibroblasts express morphological features similar to smooth muscle and have been classified as myofibroblasts. These cells typically arise in response to injury and participate actively in tissue repair phenomena [64]. This has led to the suggestion that desmoplasia might represent an exaggerated response of tissue remodeling associated with malignancy [63]. Recently the features of stromal cells in desmoplastic reactions in human mammary tumors have been investigated using a mouse monoclonal antibody to α-smooth muscle actin [63]. Cells composing the desmoplastic reaction were found to express α-smooth muscle actin in all 11 breast carcinomas examined, whereas no immunostain was demonstrated in the stromal cells of 7 normal breast tissue samples. Three of 9 cases of fibrocystic disease showed a minority of positively stained stromal cells, generally in association with epithelial hyperplasia. All 7 cases

of sclerosing adenosis, 3 of 4 cases of diffuse papillomatosis, and all 3 intraductal papillomas exhibited a majority of immunoreactive stromal cells. Numerous stromal cells in 3 of 11 circumscribed fibroadenomas analyzed expressed low amounts of α-smooth muscle actin. The factor(s) responsible for smooth muscle differentiation of mammary stromal cells are presently unknown. However, the consistent observation of myofibroblasts in a wide range of mammary dysplasias demonstrates an altered interaction between mammary epithelium and stroma. The detection of this previously unsuspected stromal cell phenotype in nonmalignant mammary tissues might help in characterizing the various mammary dysplasias that may be premalignant or early malignant lesions of the breast. Furthermore, it is possible that aberrations in normal stromal-epithelial interactions may be predisposing factors to mammary cancer.

In animal experimental model systems, aberrations in stromal morphology in mammary neoplasia have also been observed. The distribution of basement membrane and connective tissue proteins in 7,12–dimethylbenzanthracene (DMBA)-induced rat mammary tumors was examined using immunocytochemical and ultrastructural techniques [65]. In the normal gland, laminin is restricted to a thin basement membrane surrounding each duct, whereas type I collagen fibers are distributed throughout the stroma. In DMBA-induced tumors, each cluster of tumor cells is surrounded by interstitial collagen and an abnormally thickened basement membrane. The authors draw attention to the importance of the presence of an appropriate substratum for normal proliferative and functional response to be obtained in vitro. They suggest that abnormalities in the basement membrane and extracellular matrix in vivo may alter attachment characteristics of overlying cells, partially freeing them from some of the restrictions of normal cell growth.

Another approach to investigating the desmoplastic response in mammary cancer was taken by Russo et al. [66]. In these studies cloned mouse mammary adenocarcinoma cells were innoculated into epithelium-devoid mouse mammary stroma in vivo. In response to the innoculated tumor cells, the host stromal response was characterized by an intense fibroblastic reaction producing large masses of collagen. The tumors cells also lacked basal lamina, which suggests that aberrations in basal lamina might influence epithelial-stromal interactions.

The role of extracellular matrix and desmoplastic response has also been investigated in vitro [67]. When newborn skin fibroblasts are cultured on matrix derived from human mammary carcinoma cell line ZR–75–1, an increased growth rate and fourfold increase in cell density is observed. Interaction with the matrix also stimulates the level of collagen and elastin synthesis. No such effect was seen when fibroblasts were plated on their own preformed matrix, on matrices of other cell types, on various type-specific collagen gels, or in media conditioned by ZR–75–1 cells. Furthermore, direct contact between fibroblasts and tumor matrix was required for

the mitogenic response to occur [67]. The authors conclude that stimulation of stromal cells to proliferate by adjacent breast tumor matrix may be the basis of the desmoplastic reaction associated with breast cancer. Furthermore, these results demonstrate that epithelial cells may produce an extracellular matrix factor(s) that affects stromal cell behavior.

Mammary stromal cell influences on tumor cell immunogenicity and metastatic potential have been demonstrated using a spontaneously arising mouse mammary tumor [68]. Transplants of tumor to syngeneic mice behaved differently when transplanted to the subcutis versus the epithelium-devoid mammary stromal fat pad. Specifically, transplants to mammary stroma exhibited increased metastatic potential from 0% to 35%. Furthermore, there was an increased expression of class I major histocompatibility antigens and mammary epithelial cell antigenic markers. These observations suggest that mammary stroma may provide an environment that either selects distinct tumor subpopulations or induces a phenotypic change leading to tumor progression and the generation of metastatic subpopulations.

Familial clustering of breast cancer indicates that there may also be a genetic component of predisposition to breast cancer [69]. The stromal components of breast cancer are generally considered to be normal, and it is the epithelial component that is designated as cancerous. However, several reports have indicated that fibroblasts derived from epithelial tumors may exhibit behavioral characteristics in vitro commonly associated with the expression of a transformed and/or fetal phenotype [70–72]. It has been reported that skin fibroblasts from patients with breast cancer respond in an abnormal way to three biological parameters in vitro: anchorage independence, colony formation on monolayers of normal human epithelial cells, and increased saturation density in overcrowded culture conditions [71]. Studies of growth curves and DNA synthesis of skin fibroblasts from cancer patients have revealed that even at confluency there is a significant fraction of cells still cycling. This is in distinct contrast to fibroblasts obtained from normal donors [73].

Skin fibroblasts from breast cancer patients have also been shown to exhibit increased migratory behavior, similar to fetal fibroblasts, when placed on three-dimensional collagen gels [71]. Using this migration assay, more than 90% of patients with familial breast cancer and 50% with sporadic disease had fibroblasts that exhibited fetal behavior. Furthermore, identification of these characteristics in skin fibroblasts has been shown in a number of instances to precede the discovery of the disease [72]. Recently it has been reported that skin fibroblasts with fetal characteristics are present in about 67% of clinically unaffected first-degree relatives of patients with hereditary breast cancer [74]. In daughters of patients with hereditary breast cancer, the cumulative lifetime risk of the disease developing is 50%. It has been postulated that the continued presence of fetal-like fibroblasts in the adult increases suceptibility to the development of an epithelial tumor [74]. Recent data shows that increased fibroblast migration is due to the autocrine

action of a factor that increases hyaluronic acid biosynthesis [75]. These results support the concept that aberrations in stromal function and abnormal stromal-epithelial interactions may contribute significantly to the neoplastic process. An abnormal stromal extracellular matrix, especially hyaluronic acid, may be a permissive factor for aggressive tumor cell behavior, such as invasion and progression.

Studies of epithelial-stromal interactions in vitro have been undertaken to elucidate the mechanisms underlying abnormal interactions. Several recent reports have provided evidence that the growth of human breast cancer cells is under autocrine influence of growth factors produced by tumor cells [76]. These studies have demonstrated that human breast cancer cells can secrete autostimulatory growth factors either constitutively or under the positive control of estrogen. The possibility of paracrine influences whereby surrounding stromal tissues secrete factors affecting the growth of neighboring epithelial cells has also been suggested. Adams et al. [77] have reported that conditioned medium derived from normal fibroblasts inhibited the growth of human mammary carcinoma cell line MCF–7 in culture. In contrast, conditioned medium from benign- and malignant-derived breast fibroblasts significantly enhanced the growth of MCF–7 cells. Also investigated was the effect of fibroblast-conditioned medium on 17β–dehydrogenase activity of the MCF–7 cells, since this enzyme is of crucial importance because it converts estrone to 17β–estradiol thereby determining the tissue availability of biologically active estrogen. It was found that enzyme activity was stimulated twofold to ninefold by fibroblast factors from both normal and cancerous tissue. The authors conclude that a similar stimulation of enzyme activity in closely associated breast epithelial cells can occur in vivo thereby potentially affecting estrogen-dependent tumor cell proliferation. Further studies investigating the nature of the enzyme activity-stimulating factor in fibroblast-conditioned medium have revealed that the factor is a 50 kD polypeptide [78]. This polypeptide exerts its effects by increase in the V_{max} of the cytosolic $NADP^+$/NADPH-dependent 33 kD enzyme, whereas the V_{max} of the 68 kD NAD^+-dependent enzyme is reduced. The identity of the polypeptide is not known. It is possible that it is related to one of the recently discovered growth factors, since some of these have been shown to affect 17β–estradiol dehydrogenase activity in human breast adipose tissue [79].

Another way in which stromal cells can influence normal and neoplastic mammary epithelial cells is by direct transfer of informational molecules across cell membranes by specialized channels, such as gap junctions [80]. One study examined the relationship between metastatic capacity of tumor cells and their intercellular communication with normal fibroblasts [81]. It was found that the frequency of intercellular communication between weakly metastatic clone and fibroblasts was significantly higher than that between highly metastatic clone and fibroblasts. These results indicate that normal fibroblasts may regulate the growth characteristics and the metastatic capacity of tumor cells by intercellular communication.

Summary and conclusions

In the postnatal normal mammary gland, cyclic patterns of proliferation, morphogenesis, differentiation, and regression occur and are hormonally regulated. Mesenchymal-epithelial interactions are critically required during embryonic development for normal morphogenesis. It has been postulated that similar stromal-epithelial interactions are required for postnatal growth and differentiation. The experimental evidence from in vivo and in vitro studies for the existence of postnatal stromal-epithelial interactions have been reviewed herein. Several mechanisms have been proposed to explain the underlying basis for the observed interactions. As depicted in figure 2, it has been proposed that stromal cells can influence epithelial cell behavior (1) by modifying the molecular and/or physical composition of the extracellular matrix, (2) by production of soluble growth factor-like molecules, and (3) by

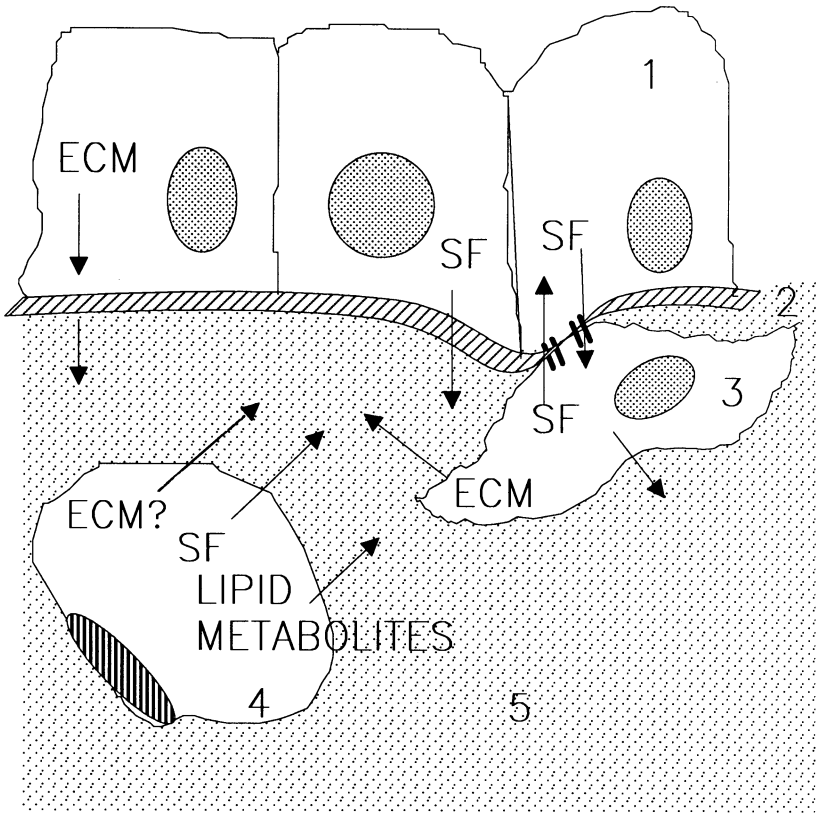

Figure 2. Proposed mechanisms underlying stromal-epithelial interactions in mammary tissue. The epithelial cell (1), basement membrane (2), fibroblasts (3), adipocyte (4), and interstitial extracellular matrix (5) are the major physical components of the gland. Bidirectional influences are believed to be mediated via soluble growth-like factors (SF), the production and modification of extracellular matrix components (ECM), and lipid metabolites.

direct contact and transfer of informational molecules via gap junctions. Furthermore, the interactions are bidirectional such that epithelial cells are capable of influencing stromal cell behavior.

In this review the role of stromal-epithelial interactions in tumorigenesis and mammary cancer have also been considered. There is good evidence to indicate that aberrations in stromal-epithelial interactions occur in the course of the neoplastic process and involve the same mechanisms and interactive phenomena as described for normal cells. Of particular interest is the hypothesis that one component of the genetic predisposition to breast cancer in humans is due to a stromal cell defect.

The precise identification of the mechanisms by which stromal cells influence the neoplastic process may lead to novel therapeutic approaches and agents for the treatment of breast cancer. It might be possible to influence the proliferation and/or differentiation of neoplastic epithelial cells indirectly by a primary effect on neighboring stroma.

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20. Extracellular matrix metalloproteinases in tumor invasion and metastasis

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In recent years, significant progress has been made in understanding the structure, function, and regulation of gene expression of secreted metalloproteinases that participate in the degradation of the mammalian extracellular matrix (ECM). At the same time, several lines of investigation have produced circumstantial evidence implicating these proteases in tumor growth, invasion, and metastasis. A more detailed discussion of the properties of these proteases is outside the scope of this review, but we summarize here the available data concerning the possible role of these enzymes and plasminogen activators in tumor development and metastasis.

It is clear that some tumors are more metastatic than others, and as shown by Fidler and Kripke [1], some cells within any given tumor have a greater metastatic potential than others, suggesting that highly metastatic variant cells preexist within the primary tumor. It appears that a metastatic cell must have features that permit it to detach from its parent tumor, invade and disrupt epithelial and endothelial basement membranes and the other compontents of the ECM, enter lymphatic vessels, and invade the target organ. Whether a single tumor cell or a part of the whole tumor is involved, such processes are likely to be accompanied by perturbation of the surrounding tissue. ECM restructuring in general is a cooperative multistep process involving a localized degradation of the existing ECM, rearrangement of macromolecules, cytoskeletal changes, cell translocation, and deposition of new ECM compontents [2–5]. Although each of these steps is controleed by a variety of molecular mechanisms, the initial step may depend on the presence of secreted proteinases capable of degradating ECM macromolecules. The ability of tumor cells to secrete such enzymes may in turn be required to initiate cell invasion and metastasis [5–13]. This concept is supported on the microscopic level by the fact that connective tissue degradation is typically seen at the margins of invading tumors.

Role of type IV collagenase in tumorigenesis

For a tumor cell to invade, it must acquire the ability to traverse a major connective tissue barrier, the basement membrane, a complex structure

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composed of collagenous and noncollagenous glycoproteins and suflated proteoglycans. The major component in all basement membranes is type IV collagen, which accounts for 40% to 65% of the total protein. Laminin and fibronectin are present in all basement membranes, while some also contain unique components, such as type VII collagen, a major component of anchoring fibrils [14] found at the dermal-epidermal junction and important in attaching epidermis to dermis. Type V collagen was initially thought to be associated with basement membranes, but its relationship to this structure is unclear [15]. None of these basement membrane components, with the exception of type VII collagen [16], are susceptible to cleavage by interstitial collagenase, whose major function is to degrade types I–III collagen (17). An enzyme obtained from metastatic murine tumor cells was initially demonstrated by Liotta and colleagues [18, 19] to be capable of degrading type IV collagen. Subsequently, in a variety of systems, the metastatic potential of a number of animal and human tumors was shown to correlate with their ability to degrade type IV collagen [13, 19–21]. In addition (as discussed below), there appears to be a correlation between the increased expression of type IV collagenase (and perhaps stromelysin) with the acquisition of the malignant phenotype and with the enhanced metastatic capability of some tumor cell strains.

The nature of type IV collagenase was recently established [22] when we determined the complete primary structure of a single major 72 kD ECM metalloproteinase secreted in a latent form by human bronchial epithelial cells in response to transformation with H–*ras* oncogene (TBE–1). When activated, this enzyme catalyzes the cleavage of basement membrane type IV collagen in a pepsin-resistant portion of the molecule. Interestingly, the enzyme also cleaves gelatin, type V collagen, fibronectin, and type VII collagen [22, 16]. The enzyme does not degrade the interstitial collagens (types I–III) or laminin. Unlike expression of interstitial collagenase and stromelysin, the level of expression of type IV collagenase cannot be further increased by treatment of normal human fibroblasts with the tumor promoter 12–0–tetradecanoylphorbol 13–acetate (TPA). This is in agreement with the absence of the TPA-responsive cis-element TRE [24], which mediates TPA induction of interstitial collagenase and stromelysin transcription [24–28].

Of particular importance was the observation that this mealloproteinase is identical to the ECM enzyme known as gelatinase, which was previously isolated from normal human skin fibroblasts and explants [29, 30], from SV40-transformed human lung fibroblasts, and from the human melanoma cell strain A2058. We also concluded that this enzyme was most likely the human analogue of type IV collagenase detected in the murine tumors, which has the same molecular mass. It has subsequently been confirmed from protein sequence data [31] that this 72 kD type IV collagenase is identical to the type IV collagen-degrading enzyme identified previously in human tumor cells [18, 19].

Liotta and colleagues [32] initially demonstrated that murine melanoma and sarcoma cell strains with the highest incidence of metastasis produced markedly increased levels of type IV collagen-degrading enzyme activity when compared to tumorigenic but nonmetastatic spontaneously transformed mouse fibroblasts. Further studies showed [33] that transfection of NIH/3T3 cells with malignant human tumor DNA-containing activated *ras* oncogenes induces the metastatic phenotype in these cells. This induction was associated with an increase of about sixfold in type IV collagenolytic activity and with invasion of human amnion basement membrane in vitro, when compared to parent or spontaneously transformed NIH/3T3 cells.

Evidence has been presented by Zucker et al. [34] that at least some types I and IV collagen-and gelatin-degrading activity form an integral part of the cell membrane in human small-cell lung cancer and pancreatic cancer cell strains. These proteases have not been further characterized, but the authors suggest that perhaps a single membrane-associated metalloproteinase is responsible for all the collagenolytic and gelatinolytic activity. This concept is based on their studies of the metastatic mouse melanoma cell strain B16–BL6 [35], from which a 59 kD metalloproteinase is shed from the cells as part of intact membrane vesicles and has types I and IV collagenolytic and gelatinolytic activities. The relationship of these enzymes to the collagenases already well characterized (see above) and the presence of membrane binding sites for members of the collagenase gene family have not been demonstrated. In contrast, Eisenbach et al. [36] found that type IV collagenolytic activity in several tumor cell strains was present primarily in the cytoplasmic fraction, with little activity associated with purified plasma membranes. The question of membrane-associated collagenolytic activity raised by these studies is of importance, since localized ECM degradation is frequently seen at the margins of invading tumors.

Murine cell hybrids derived from fusion of cells from two metastatic cell strains and by fusion of metastatic and nonmetastatic cell strains express higher levels of type IV collagenolytic activity than does either parent cell strain, and they are associated with an enhanced ability to produce metastasis in both nude and syngeneic mice [37]. Fusion of normal cells with metastatic cells produced hybrids in which both type IV collagenolytic activity and metastatic capability were suppressed, while tumorigenicity was maintained. Since type IV collagenolytic activity and metastatic ability appear closely related, it was suggested that type IV collagenase expression may be genetically linked to the metastatic phenotype.

Basement membranes also contain noncollagenous glycoproteins, such as laminin, which plays a role in the attachment of tumor cells to the basement membrane. Because laminin was found to potentiate the metastatic capability of tumor cells [38], the possibility that it might regulate type IV collagenase production in malignant cells was examined [39]. In the human melanoma cells, laminin increased the secretion of type IV collagenolytic activity two to three fold. Using a laminin fragment that contained the receptor-binding

domain but lacked the other matrix attachment domains resulted in an even greater (6-fold) increase in enzyme activity. Monoclonal antibody to the laminin receptor completely blocked the stimulation of type IV collagenolytic activity by laminin. In contrast, fibronectin, another cell attachment ECM protein, had no effect on type IV collagenolytic activity, suggesting that the effect was specific for laminin. Although laminin increases the expression of type IV collagenolytic activity in metastatic cells, it does not appear to do so in nonmetastatic tumor cells that express type IV collagenase activity constitutively [40].

Although the studies described above suggest that type IV collagenase plays a role in tumor metastasis, it should be emphasized that in these early studies enzyme activity was assessed using preparations of either crude or ammonium-sulfated culture medium following trypsin activation, so that the relationship of enzyme activity to levels of type IV collagenase protein and mRNA was not determined. The enzymatic mechanisms required for the activation of type IV collagenase are unknown. The fact that trypsin is required for activation of the enzyme in crude culture medium is of interest, since pure type IV collagenase is only poorly activated by trypsin [22], most probably due to the absence of a conserved sequence of three basic amino acids (present in both interstitial procollagenase [41] and prostromelysin [42] [KRR and RRK, respectively]) at the site of first trypsin cleavage, which initiates their activation [43]. Organomercurials, on the other hand, can activate type IV procollagenase most likely by initiating an intramolecular autoproteolytic reaction (similar to that observed with procollagenase [43], resulting in the loss of 6 kD from the molecule [22, 24]. Since all ECM metalloproteinases are secreted as zymogens that are then subject to extracellular activation, perhaps the ability of trypsin to activate type IV collagenase in crude culture medium is an indirect one, mediated by an as yet undefined pathway of activation.

In contrast to the above studies, Teale et al. [45] assessed the secretion of type IV collagenase from cell strains derived from lung metastases that developed following resection of a primary herpes simplex virus–2-transformed hamster fibrosarcoma. These cell strains possessed varying metastatic potential, and even though they all secreted significantly more type IV collagenolytic activity than did normal untransformed cell strains, there was no correlation between enzyme levels and their metastatic potential. In fact, the highest level of type IV collagenolytic activity was present in a nonmestastatic cell strain. The reason for the difference between this study and the previous work indicating a correlation between metastatic potential and type IV collagenolytic activity [13, 19–21] is unknown, but perhaps type IV collagenolytic activity is rate limiting for some but not all metastatic cell strains.

There are several other lines of evidence that indicate that metastatic potential may indeed be associated with the enhanced expression of type IV collagenase. Bonfil et al. [21] used human bronchial epithelial cells immor-

talized with adenovirus 12–SV40 and subsequently transfected with H–*ras* oncogene, to evaluate the development of neoplastic changes intratracheally in de-epithelialized rat tracheas xenotransplanted subcutaneously into nude mice. Using this technique, a cell strain was obtained that produced malignant tumors in this xenotransplantation model system, which were invasive using a reconstituted basement membrane (Matrigel-coated polycarbonate filters) and metastatic when injected into athymic mice. A direct correlation was observed between the development of the malignant phenotype and increased type IV collagenolytic activity and mRNA levels using the 72 kD type IV collagenase-specific probe [22]. In addition, a more highly invasive and metastatic cell strain was obtained from these tumor cells, produced after their subcutaneous injection into athymic mice. These cells showed even higher levels (a twofold increase over the injected cells and an almost sixfold increase over the parent tumor) of type IV collagenase activity and mRNA levels, which correlated with their greater invasive and metastatic behavior. This suggested that the presence of H–*ras* oncogene in these tumor cells was essential for their invasive capability and for the induction of type IV collagenase.

These studies were extended by Ura et al. [46] to a series of immortalized human bronchial epithelial cells (see above) containing a variety of oncogenes (v–H–*ras*, v–ki–*ras*, and a combination of c–*myc* and c–*raf*). Tumor-derived cell lines obtained by injecting the original immortalized human bronchial epithelial cell strains into nude mice showed enhanced invasive and metastatic abilities when compared to the original parental cell strains. Invasiveness and, to a lesser (but significant) extent, metastatic potential were strongly correlated with increased levels of type IV collagenase mRNA and enzyme activity; this was felt to be related principally to the *ras* oncogene. Except for one cell strain in which an increased level of type IV collagenase mRNA was associated with enhanced in vitro invasiveness but was only moderately invasive and metastatic in vivo, there was a highly significant correlation between in vitro invasiveness, in vivo metastasis, and type IV collagenase gene expression. The increase in type IV collagenase gene expression was also strongly correlated with decreased levels of type IV procollagen mRNA. In several nonmetastatic cell strains, the expression of type IV procollagen mRNA was increased two to four fold when compared to a highly metastatic cell strain. It was suggested that the inverse relationship between type IV collagenase and type IV procollagen mRNA levels indicated coordinate regulation of these two genes perhaps by the H–*ras* gene. These findings support the concept that tumor invasion and metastases may be associated with increased degradation and decreased synthesis of ECM.

Further support for the role of basement membrane-degrading enzymes in tumorigenesis are the observations that transfection of the adenovirus E1A gene into *ras*-transformed rat embryo fibroblasts reduced their metastatic potential and that type IV collagenolytic activity was detected in those fibroblasts transformed with *ras* alone but not in those transformed with *ras*

plus E1A [47]. Frisch and colleagues [23] investigated the possibility that the type IV collagenase gene, and perhaps other secreted protease genes, is repressed by the adenovirus E1A gene product. Expression of type IV collagenase (and also interstitial collagenase) was repressed by stable transfection with the adenovirus–5 E1A gene in three human tumor cell lines: H–*ras* transfromed bronchial epithelial cells, TBE–1; fibrosarcoma cells, HT1080; and the melanoma cell strain A2058. In transient expression assays, plasmids containing the type IV collagenase and interstitial collagenase 5′ flanking regions cotransfected into HT1080 cells with a plasmid bearing a functional E1A gene, repressed transcription of the type IV collagenase promoter. In addition, the induction of the interstitial collagenase promoter by TPA was blocked. E1A also repressed transcription from a TK promoter driven by AP–1 binding site enhancer elements (TRE), suggesting that E1A interferes with the AP–1 transactivation pathway. Whether E1A is acting directly or indirectly through another factor (such as *fos*, which interacts with AP–1 protein) is unknown.

It is of interest that concomitant with reduction in expressed levels of secreted proteases, stable E1A transfectants of the highly metastatic human tumor strains HT1080 fibrosarcoma and A2058 melanoma showed a reduced ability to invade a reconstituted basement membrane (Matrigel) in vitro and a reduced metastatic capability following injection into nude mice [23]. Monospecific anti-type IV collagenase antibody inhibited invasion in the in vitro assay, suggesting a possible causal relationship between the effect of E1A on the expression of secreted proteases and the metastatic properties of the transformants. The role of inhibitors of the ECM metalloproteases in preventing in vivo metastasis will be discussed below.

Role of stromelysin and interstitial collagenase in tumorigenesis

Prostromelysin is a member of the ECM metalloprotease gene family constitutively secreted by a variety of human [42] and animal fibroblasts [48, 49] as a major 57 kD form and a minor glycosylated 60 kD form. Stromelysin has a broad substrate specificity degrading proteoglycans, type IV collagen, laminin, and fibronectin and displaying some activity against gelatin and casein [42, 48, 49]. Unlike type IV collagenase, stromelysin degrades type IV collagen principally in its nonhelical region and causes only limited degradation of the triple helical domain [42]. Stromelysin, like type IV collagenase, does not degrade the interstitial collagens. It is unknown whether stromelysin ever acts in concert with type IV collagenase to degrade basement membrane in normal or pathologic states. Human stromelysin has been cloned [42, 50, 51] and has shown 55% homology to collagenase, but it is more closely related to rat transin (a rat homologue of human stromelysin), to transin–2 [52–54], and to human transin–2 [55].

Even though tight coordinate regulation of stromelysin and interstitial

collagenase genes has been observed in rabbit brain endothelial cells, synovium, and macrophages [56], we have shown [42] that regulation of expression of ECM proteases, and consequently thier individual roles in tumor invasion, is cell-type specific and possibly varies among species. For example, in cell strains of fibroblast origin, stromelysin and collagenase are secreted at similar rates, which can be stimulated with TPA. In contrast, human endothelial cells, keratinocytes, melanoma cells (A2058), fibrosarcoma cells, (HT1080), H–*ras*-transformed bronchial epithelial cells, and SV40-transformed fetal lung fibroblasts do not express stromelysin, nor can the expression of the enzyme be induced in these cells by TPA [42]. In comparison, the expression of rat stromelysin (transin) in rat embryo fibroblasts is induced upon transformation with polyoma virus, Rous sarcoma virus, and the activated oncogene H–*ras*, but not by SV40 [52]. Transin–2, a related protein showing 71% homology to rat stromelysin (transin), is also induced by oncogenes in rat cells [28]. It has been shown [52, 54] that the growth factors PDGF and EGF have a positive effect on transin gene expression either by modulating c–*fos*-levels, the degree of phosphorylation, or the amounts of a transacting factor, such as AP–1 or *fos*-related proteins that recognize the TPA response (TRE) consensus sequence. Offringa et al. [57] have shown that stromelysin mRNA levels are markedly reduced by transfection with the adenovirus E1A 5 or 12 gene in NRK cells and in EJ–*ras*-transfromed NRK cells. E1A was found to affect stromelysin gene expression at the level of transcription. However, E1A did not block the induction of stromelysin mRNA in NRK cells by EJ–*ras*, TPA, and presumably growth factors as well, although the induced mRNA levels were much lower than in untransformed NRK cells.

Using a rat stromelysin (transin) probe, which also detects transin–2 but does not distinguish between the two, increased levels of stromelysin (transin) RNA were found in mouse skin squamous cell carcinomas chemically induced by initiation with dimethylbenzanthracene and promotion by TPA, but not in benign papillomas or normal mouse epidermis [53]. Nucleotide sequence analysis of the cloned cross-hybridizing carcinoma cDNA showed a 90% amino acid similarity to rat transin, identifying it as the mouse homologue of rat stromelysin (transin). The increased levels of stromelysin in these carcinomas is consistent with the notion that increased expression of secreted proteases plays an important role in tumor invasion.

In a subsequent study [58] using a mouse transin cDNA as a probe, these investigators found consistently increased levels of stromelysin (transin) mRNA in mouse squamous cell carcinomas induced by a protocol that gives rise primarily to metastatic tumors — repeated applications of the carcinogens N–methyl–N–nitroso–N′–nitroguanidine (MNNG) or benzo[a]pyrene. Stromelysin (transin) in RNA transcripts was much lower in either benign papillmoas or squamous cell carcinomas induced in mice by MNNG initiation and TPA promotion, which have a low probability of metastasis. An occasional papilloma did show increased stromelysin (transin) mRNA levels, and it

was suggested that this represented a papilloma with an increased probability for malignant conversion.

Surprisingly, lung and lymph node metastatic tumors from primary mouse skin squamous cell carcinomas, induced by repeated application of benzo[a]pyrene, showed strikingly lower levels of stromelysin (transin) mRNA than was present in the primary carcinomas [58]. The lower stromelysin (transin) mRNA levels were explained by a presumed enhanced degree of differentiation (which would be unusual) of the metastatic cells compared to the primary tumor, although no direct evidence was presented that this was indeed the case. They had shown previously [59] that the stromelysin (transin) gene was not expressed in differentiated cells of normal or TPA-treated adult mouse epidermis. Several other possibilities, including dilution by contaminating normal cells or down regulation of gene expression, were also given. This is in contrast to the studies of type IV collagenase, described above, in which the metastatic cells showed much higher enzyme levels than did the parent tumors. Althogh most of the studies relating stromelysin to tumorigenesis and metastasis have been done in rodents, using transin as the model protein [53, 58], it should be noted that the proteolytic activities of transin and human stromelysin have recently been shown to be the same [60].

In studies using a rat stromelysin (transin) cDNA probe to examine RNA from various human tumors [55], six of 13 tumors derived from squamous cells contained RNA capable of hybridizing to this probe. Tumors from other sources were negative. Five of 19 metastatic tumors were also positive. A cDNA library prepared from RNA from the positive tumors screened with the rat transin cDNA probe permitted the identification of clones for human stromelysin, stromelysin–2, and a related protein, pump–1. It is significant that transcripts of stromelysin and stromelysin–2 can be found in some human tumors, particularly those with a squamous component.

Although it seems that proteolytic activity of the tumor cells may be important for their invasive properties, the available data are correlative in nature and do not permit the establishment of a causal relationship between enzyme activity and metastatic potential. The role of stromelysin in tumorigenesis also remains unclear. Although present in some malignant tumors and cell strains, it is absent in others, some of which are highly metastatic.

Interstitial collagenase has been implicated in tumor invasion [6–12], but its role is even less well established than the roles of type IV collagenase and stromelysin. The interstitial collagens (types I and III) are the major structural components of most connective tissues. Although types I and III collagen are the prime targets for interstitial collagenase, this enzyme can also degrade type VII collagen [16] (the major component of anchoring fibrils), which helps maintain stability at dermal-epidermal (epithelial) interfaces. The concept that interstitial collagenase somehow disrupts the collagenous stroma that envelops many tumors and may contribute to their invasiveness is derived from studies of a variety of human tumors, such as basal cell

carcinoma (BCC), squamous cell carcinoma, and malignant melanoma [6–11, 61], and certain animal tumors [61–65].

Initial studies from this and other laboratories [6, 7, 9] showed an increased production of collagenase by organ cultures of BCC and elevated collagenase levels in tissue extracts from these cutaneous tumors [9]. Interestingly, immunohistochemical studies localized collagenase only to the stromal elements surrounding the tumor and not to the epithelial components of the BCC [9]. This led to the hypothesis that the cellular elements of the connective tissue surrounding the tumor had been stimulated to produce increased amounts of collagenase, and this is supported by several studies using animal tumors [11, 62, 66, 67]. Subsequently it was shown [68] that fibroblasts grown in vitro from stromal tissues surrounding BCCs synthesized and secreted significantly increased levels of collagenase when compared to control cell strains. However, this phenotypic trait was not permanent and was expressed for only a few passages following initiation of the primary cultures, suggesting that the tumor produced a product that stimulated adjacent fibroblasts to synthesize more collagenase. It has been shown that extracts from BCCs dissected free from surrounding stroma [69, 70], and the conditioned medium from BCC-derived keratinocyte cultures [26, 18] are both capable of inducing a significant increase in collagenase production by normal human skin fibroblast target cells. Further studies have confirmed that the major collagenase stimulatory factor elaborated by the tumor epithelium has a molecular mass of ~19 kD [69] and is similar or identical to interleukin–1 (IL–1 [72]), which is known to stimulate collagenase production in normal fibroblast cultures [73, 74].

Similar results have been obtained using control human or murine fibroblasts cocultured with a variety of human [75] and animal tumors [76], respectively. Tumor cell strains from lung or breast carcinomas [75], for example, release a factor that can be extracted from the tumor cell membranes and that markedly enhances the production of interstitial collagenase by the target fibroblasts. The amount of this factor was significantly increased when the tumor cells were cultured on ECM deposited by control fibroblasts [76]. Cell membrane extracts from the human lung carcinoma LX–I were used to raise monoclonal antibodies to this tumor cell stimulatory factor and to affinity purify the factor from LX–2 cell membranes [77]. The purified factor is a protein of approximately 58 kD, stimulates collagenase production by control fibroblasts, has been localized to tumor cell membranes by immunofluorescence, and is postulated to be released into the culture medium by proteolytic cleavage.

Other agents in addition to IL–1 stimulate procollagenase synthesis, including tumor necrosis factor–α (TNF [78] and TPA [79, 80–82, 24]. The mechanism by which collagenase (also stromelysin and transin) gene transcription is modulated by TPA is understood, at least in part. The promoter region of these TPA-responsive genes contains a short (8 bp long) TPA-responsive element, TRE [24, 25], which serves as the binding site for

the transcription factor *jun*/AP–1 and for associated *fos* or *fos*-related proteins [25–27, 82–88]. As for IL–1, the results obtained using human skin fibroblasts indicate that this cytokine induces the transcription of c–*jun* by mechanisms independent of new protein synthesis [89]. It was suggested that the protein product of the c–*jun*/AP–I complex, c–*JUN*, is required for mediating the effects of IL–1 in the induction of expression of the collagenase gene. Stromelysin and transin also have a TRE, and the induction of the stromelysin gene by IL–1 [90] may well be mediated in a fashion similar to that for the collagenase gene. In addition, there is evidence [91] that the TRE of the collagenase gene mediates the positive regulatory effect of IL–1 on collagenase transcription and that retinoic acid inhibits this effect through the TRE by inhibition of c–*fos* expression.

TNF–α has also been shown [92] to stimulate collagenase gene transcription, which (like TPA and IL–1) appears to be mediated by the TRE of the collagenase gene. TNF stimulates a more prolonged activation of the *jun* gene, in contrast to its transient activation by TPA. TNF, like TPA, causes a transient induction of *fos* mRNA and is able to elicit the expression of the two genes, *jun* and *fos*, whose products interact to stimulate transcription of AP–1 responsive genes, such as the collagenase gene. These studies also suggest that the effect of TNF, like TPA, is mediated by its ability to activate protein kinase C.

The product of early adenovirus oncogene EIA (see above) represses the constitutive expression of the interstitial collagenase gene and blocks TPA induction of this gene in certain tumor cell strains [23]. Certain model systems in which oncogenes come into play may be helpful in delineating the significance of ECM metalloproteases in tumorigenesis. For example, murine papilloma cell strains established from DMBA-treated mouse skin or DMBA-initiated mouse skin papillomas, express an activated H–*ras* gene and produce premalignant squamous tumors when transplanted on nude mice but not when injected subcutaneously into nude mice. When stably transfected with *fos*, which seems to complement the action of an activated H–*ras* gene, the papillomas undergo malignant conversion [93]. This is in contrast to transfection with EIA, which does not result in malignant conversion of the papilloma cell strains. It is unknown whether malignant conversion of these cell strains is associated with enhanced production of collagenase or other members of this gene family, but there is a provacative relationship between these agents that modify transcription of collagenase genes and induce tumorigenesis.

It appears that certain tumors, such as BCCs, elaborate cytokines and perhaps other factors that elicit the enhanced synthesis of interstitial collagenase by the surrounding stromal fibroblasts. It remains to be determined to what extent this mechanism is operative in malignant tumors, since many tumor cell strains do constitutively produce collagenase. Whether collagenase takes part in disrupting the basement membrane zone or assists in the degradation of connective tissue elements that remains as a barrier to tumor

cell invasion once they have penetrated the basement membrane is unknown. The interrelationship of interstitial collagenase, stromelysin, and type IV collagenase in such processes must now be determined; but if ECM degradation is required for tumor cells to invade, then the mechanism of activation of the proenzyme forms of these enzymes may be crucial in regulating the malignant phenotype.

It has been shown that activation of procollagenase can occur through the urokinase pathway (described above) whereby tumors might secrete urokinase plasminogen activator (u–PA), which converts plasminogen into plasmin. Plasmin, in turn, is capable of activating both procollagenase and prostromelysin by amino-terminal processing. Activated stromelysin then converts plasmin-activated collagenase into a fully active enzyme perhaps by removal of approximately 15 amino acid residues from the carboxyl end of the enzyme [94]. Support for the concept that such a proteolytic cascade may be involved in tumor invasion is provided by the studies of Mignatti et al. [95], who showed that collagenase, produced by the BI6/BL6 melanoma cell strain and most likely activated through a u–PA–plasmin pathway, is required for the B16 cells to invade through the human amniotic membrane.

Effects of protease inhibitors on tumor invasion

Interaction of ECM metalloproteases with the specific tissue inhibitor of metalloproteases (TIMP) is essential for regulation of their activity in the extracellular space. TIMP is a ubiquitous glycoprotein [96–99, 29] known to inhibit interstitial collagenase, stromelysin, and type IV collagenase. A number of specific protease inhibitors, most importantly TIMP, have been shown to inhibit tumor invasion in vitro and in vivo. Thorgeirsson et al. [100] originally showed that TIMP blocked the invasion of reticulum sarcoma cells (M5076) through human amino basement membrane. Using a modification of this assay, Mignatti et al. [95] demonstrated that TIMP and other agents capable of inhibiting collagenase, such as the chelating agent 1–10 phenanthroline and anithuman skin fibroblast collagenase antibodies, totally block invasion of B16/BL6 murine melanoma cells into the human amniotic basement membrane and stroma. The effectiveness of antihuman collagenase antibodies is difficult to interpret because it was not shown that they cross-react with mouse collagenase. The organomercurial compound Mersalyl, a known activator of procollagenases, stimulated tumor cell invasion into the amnion, suggesting that enzyme activation is an essential feature of this process. In addition, a variety of plasmin inhibitors and antimouse urokinase antibodies (but not antitissue plasminogen activator antibodies) prevented amnion invasion. These investigators suggested that a u–PA plasmin cascade resulting in the activation of interstitial procollagenase is involved in tumor cell invasion. Unfortunately, the role of type IV collagenase in the system was not examined. Schultz et al. [102] showed that recombinant TIMP

(rTIMP) inhibited the invasion of B16–F10 murine melanoma cell through the human amniotic membrane. The rTIMP did not inhibit the cell adhesion step in amino invasion; it actually resulted in an increase in the number of tumor cells bound to the amnion. Of particular importance was the finding that intraperitoneal injections of rTIMP into syngeneic mice significantly inhibited metastatic lung colonization by these cells. The anticolonization effect of rTIMP was found to be due to its action on tumor invasion rather than on tumor growth.

It should be emphasized that TIMP cannot differentiate between the various ECM metalloproteases, so it is not clear which of these enzymes might be responsible for the malignant phenotype. Using a Boyden chamber assay in which a porous filter is coated with a reconstituted basement membrane (Matrigel), Reich and colleagues [102] showed that a number of malignant human (HT 1080 fibrosarcoma) and murine (M2 melanoma) cell lines that invade through basement membrane also secreted increased levels of type IV collagenase. Using the synthetic collagenase inhibitor SC–44463, a peptide hydroxamic acid whose structure resembles the structure around the interstitial collagenase cleavage site in type I collagen and froms a coordinate complex with zinc when bound to the active site of the enzyme, they effectively inhibited type IV collagenase and blocked invasion of the tumors cells through the basement membrane barrier. In addition, this collagenase inhibitor significantly reduced the number of lung metastases that formed in mice injected intravenously with malignant melanoma cells. A variety of serine protease inhibitors and antibodies to plasminogen activators also inhibited invasion of the tumor cells through the reconstituted basement membrane. The concluded that a protease cascade leading to the activation of type IV collagenase by plasmin plays a major role in tumor invasion.

It is important to note that although it has been shown that plasmin may be involved in the activation of interstitial collagenase [94], there is no direct evidence that plasmin can activate the 72 kD type IV collagenase. An additional factor that may be relevant to the activity and/or activation of the enzyme is our recent observation [103] that the 72 kD type IV procollagenase exists in a stable noncovalent complex with a novel tissue inhibitor of metalloproteases, called TIMP–2. The physiological significance of the TIMP-proenzyme complex is unclear. Whether it has to do with the ability of type IV procollagenase to bind to its substrate (unlike interstitial procollagenase, which does not bind to collagen in its zymogen form) or to the control of enzyme activation is a subject for future experimentation.

Perhaps the most significant evidence that ECM metalloprotease inhibitor TIMP plays a key role in tumorigenicity is the study by Khokha et al. [104]. Their observations suggest that the expression of TIMP may be directly related to the suppression of tumorigenicity of immortal murine 3T3 cells. Mouse 3T3 cell strains constitutively synthesizing an RNA complementary to the mRNA encoding TIMP were shown to secrete a decreased amount

of TIMP into the culture medium, which correlated directly with reduced levels of TIMP mRNA. Unlike the parental cells, those cells containing the antisense RNA were invasive in the human amnion invasion assay and were tumorigenic and metastatic in athymic mice. Amnion invasion was prevented by 1,10–phenanthroline, adding further support for the role of metalloproteases in the process.

The vitamin A-related morphogen retinoic acid and its synthetic analogues are agents that can promote differentiation of a variety of tumors and also suppress the transformed phenotype [105–107]. Retinoids are known to inhibit the synthesis of interstitial collagenase in cultured rabbit human and rabbit synovial cells [108, 109] and of both interstitial collagenase and type IV collagenae (gelatinase) in normal human skin fibroblasts [110, 111]. It has be demonstrated [112] that retinoids induce a two to three fold increase in the production of TIMP in a variety of cultured human fibroblasts, mediated by an increased biosynthesis of new inhibitor protein and increased steady-state levels of TIMP mRNA. This was accompanied by decreased quantities of interstitial collagenase mRNA, suggesting that retinoids regulate the expression of enzyme and inhibitor in an inverse manner. Studies by Nakajima et al. [113] show that retinoic acid markedly inhibits the production of type IV collagenase by a highly metastatic rat mammary adenocarcinoma cell strain under conditions in which cell growth was not inhibited. Retinoic acid also inhibited tumor cell invasion through a reconstituted (Matrigel) basement membrane. The role of TIMP in this system was not directly evaluated.

Role of plasminogen activator

The idea that plasminogen activator is involved in tumor invasion and metastasis arose because of the possible role of plasmin as an activator of collagenases (as detailed above) and as a protease capable of degrading certain ECM components, such as proteoglycans, fibronectin, and laminin [for review see 20, 114]. For example, in a chick embryo system, pulmonary metastasis, but not local growth, human Hep–3 carcinoma was inhibited by antibodies specifically blocking the enzymatic activity of human (but not chicken) u–PA [115]. It has been shown [116] that the generation of active plasmin bound to the surface of HT1080 cells is dependent on an interaction between bound active u–PA and plasminogen. The bound plasmin in turn produces more active surface-bound u–PA from bound pro–u–PA. In addition, anti–u–PA antibody, which inhibits the activity of cell surface-associated u–PA on B16 melanoma cells, significantly decreases their metastatic capability when injected into syngeneic mice [117]. The specific immunocytochemical localization of u–PA (in the invasive, transplanted Lewis lung carcinoma) to areas of tumor invasion where active degradation of the surrounding normal tissue was occurring [118] also supports this premise.

Studies by Axelrod et al. [119] suggest that both u–PA and t–PA may be involved in tumor invasion and metastasis. H–*ras*-transformed NIH 3T3 cells expressing very low levels of PA were transfected with a recombinant gene encoding either human u–PA or t–PA and were compared with the parental transformed cells for their ability to invade through a reconstituted basement membrane (Boyden chamber-Matrigel assay, see above) and to metastasize after intravenous injection into nude mice. Invasive activity through the reconstituted basement membrane was significantly enhanced by both types of PA, but this was slightly greater by the cells expressing u–PA. Invasion was inhibited by antibodies blocking the activity of the human PAs and by the serine protease inhibitor EACA. Of further interest was the finding that invasion was associated with the secretion of increased levels of type IV collagenase activity. The increase in type IV collagenase activity correlated with cells expressing high levels of either u–PA or t–PA; the collagenase inhibitor SC–44463 [119] blocked invasion by cells producing either of the human PA types. It appears that the proteolytic events involved in basement membrane invasion may be initiatd by either type of PA. Moreover, following intravenous injection into nude mice, cells expressing high levels of u–PA resulted in a marked increase in the number of pulmonary metastases that could be significantly reduced by simultaneously injecting EACA. Whether the cells expressing recombinant t–PA have a similar effect on metastasis remains to be examined.

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Index