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Advances in Cellular and Molecular Biology of Breast Cancer

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Table of Contents

Preface

The current volume represents the fourth over a period of five years in our series on Advances in the Cellular and Molecular Biology of Breast Cancer. The first three volumes were entitled *Breast Cancer: Cellular and Molecular Biology, Regulatory Mechanisms in Breast Cancer*, and *Genes, Oncogenes, and Hormones*, respectively. Throughout this series, we have tried to take a broad look at cutting-edge topics in basic science research into breast cancer. This attempt has resulted in a wide range of subject material, including rodent and human model systems, oncogenes, suppressor genes, growth factors, hormones, tumor–host interactions, and determinants of metastases. Since our last volume, research in breast cancer has continued to proceed at an explosive rate. We hope the current volume will provide the reader with some of the excitement felt by the editors and authors as we begin to understand this all-too-common disease.

The first section of this book is devoted to the basic processes of proliferation, differentiation, and malignant progression of breast cancer. T.J. Anderson and W.R. Miller lead off with a detailed description of controls on proliferation in the normal human breast and in breast cancer. This chapter strongly emphasizes pathological aspects. The second chapter, by M.R. Stampfer and P. Yaswen, presents a corresponding viewpoint through a presentation of experiments with human mammary epithelial cells in culture.

The second section of the book emphasizes the genetic basis for breast cancer onset and malignant progression. Chapter 3, by M.-C. King and S. Rowell, focuses on the rapidly evolving, very exciting studies attempting to clone a gene responsible for familial, early-onset patterns of breast and ovarian cancers. The next chapter, by D.C. Allred and co-workers, evaluate a different gene, termed p53, whose alteration is responsible for a very small subgroup of familial breast cancers (Li–Fraumini syndrome). In addition, the gene is commonly mututated during progression of breast cancer, to the detriment of the patient: this gene is a tumor-suppressor gene until it undergoes mutation to an oncogenic form. Chapter 5, by H.S. Smith, presents an overview of the tumor-suppressor genes and mutated loci of interest in breast cancer. Chapter 6, by C.A. Encarnacion and S.A.W.

Fuqua, presents recent data on the structure and function of genetic variants of the estrogen receptor in breast cancer. The final chapter of the second section, by K.B. Horwitz, focuses on mutant estrogen and progesterone receptors in the context of malignant progression and of failure of anti-hormone therapy for the patient.

The third section of the book deals with growth factors, receptors, and polyamines in proliferation mechanisms. Chapter 8, by R.P. DiAugustine, presents an overview of the epidermal growth factor and receptor. This family of expanding growth-regulatory molecules continues to capture the interest of investigators studying proliferation mechanisms in malignant mammary glands of both human and rodent. The next chapter, by Y. Yardin and colleagues, provides an update on the erbB-2/neu oncogene, with special emphasis on its newly discovered ligands. Chapter 10, by M. Bano and colleagues, presents data on a relatively new growth and collagen synthesis modulatory factor from human milk and from human breast cancer: MDGF1. The final chapter of the section, by A. Manni, presents studies on the role of polyamine synthesis in mediating diverse proliferative stimuli by steroids and growth factors in breast cancer.

The fourth section of the book is devoted to aspects of metastases and tumor–host interactions. J.P. Thiery and colleagues begin the section with a chapter summarizing the regulation of differentiation and dedifferentiation processes governing cellular transitions between epithelial and mesenchymal morphologies in embryogenesis and breast cancer. The next chapter, by J. Behrens and W. Birchmeier, summarizes the evolving literature on cell–cell adhesion in invasion and metastases. Chapter 13, by H. Kleinman and colleagues, presents data on extracellular matrix–tumor interaction. Specifically, these authors focus on the role of laminin to modulate the growth both of the tumor and of the developing tumor vasculature. The next chapter of this section, by S.L. Schor and colleagues, presents recent studies on the nature of the stromal fibroblasts surrounding tumors and the factors that they secrete. The fifth and final chapter of the section, by N. Brünner and colleagues, presents the rapidly evolving, highly exciting work on the plasminogen activator–inhibitor system in breast cancer proteolytic processes.

The final section presents recent studies on rodent models of breast cancer. Chapter 17, by K. McKenzie and S. Sukumar, dissects at a molecular level the carcinogen-induced rodent mammary cancer model into initiation, promotion, and progression. Chapter 18, by C. Dickson and V. Fantl, presents an update on mammary tumors induced by the mouse mammary tumor virus (MMTV) and on transgenic mouse models. In particular, the authors focus on the evolving role of the Fgf-3 gene, which encodes a growth factor with oncogenic potential for mammary cancer.

Breast cancer continues to menace our society. However, the disease is gradually yielding its secrets at a cellular and molecular level. We hope that

these chapters will be of interest to a widely varying readership, including biochemists, molecular biologists, cell biologists, and a diversity of cancer scientists. Progress is being made.

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I

Proliferation, Differentiation, and Malignant Progression

1. Morphological and biological observations relating to the development and progression of breast cancer

Thomas J. Anderson and William R. Miller

The normal breast

Basic structure and development biology

Mammary glands are basically modified sweat glands comprising a group of ducts descending from the skin in a radial system of dichotomously branching structures. The human female is unique in that the breast develops fully without the stimulus of copulation or pregnancy. Indeed, there are three overlapping phases of activity that can conveniently be termed structural, functional, and differentiated. The first is associated with puberty, the second with ovulatory menstrual cycles, and only the last with pregnancy and lactation. During the structural phase, the lengthening ducts descend into the fibrous disc below the nipple, extending within and along the contours of the fat spaces, giving rise to an inverted tree-like parenchymal structure. A crucial distinction of the human female from the other members of her species is the virginal development of terminal duct lobular units (TDLUs). These units are spherical or conical in shape due to the compact arrangement of multiple short branchings of the smaller blind-ended ductules, usually enclosed in a specialized stroma (figure 1). They form in groups and individually from the sides and ends of major, intermediate, and small ducts, measure well under 1 mm, and are situated most commonly in the outer third of the breast disc and axillary tail, often amounting to tens of thousands in number. The TDLUs consists of several cell types; the parenchymal branching component is composed of two principal cells, the luminal cuboidal or columnar epithelium and an outer layer of myoepithelium, all enclosed by a basement membrane; on the opposite stromal side of the membrane lie fibroblasts and a loose textured collagen containing ground substance, traversed by small blood vessels and a variable component of lymphocytes, macrophages, and plasma cells. This intralobular stroma is usually distinguished microscopically from the denser collagen containing extralobular stroma, which is itself surrounded by fat cells. The adipose tissue composes the bulk of the breast, but it is the parenchymal and stromal components that constitute the responsive elements of the basic

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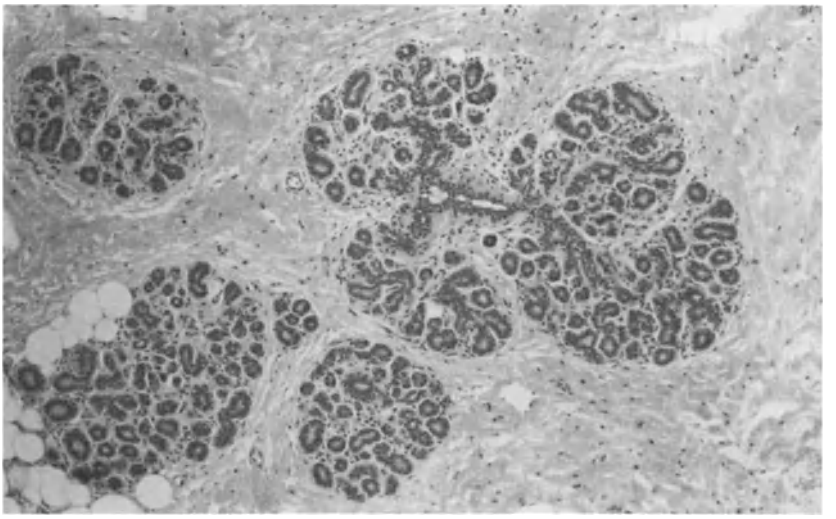

Figure 1. A group of normal resting lobules, with associated extralobular terminal duct and interlobular and extralobular stroma. Field size 1 × 1.5 mm.

functioning unit that will become fully differentiated during pregnancy and lactation.

The point of relevance is that these features make for difficulty in anticipating similarities between humans and rodents in models of carcinogenesis [1], particularly in the context of stem cell populations. The virgin rodent gland has no lobules but has clearly identifiable terminal end buds or caps from which the mature gland, including ducts and lobules, will form in response to copulation and pregnancy. Such stem cell groups would obviously be sensitive targets for exposure to carcinogens. Subgross stereomicroscopy offers the means to search for such structures in human material, which until recently had produced entirely negative reports. The fact that stem cell groups have now been observed [2] is notable, but so also is the rarity of the event, indicating the low level of relevance to the human situation.

What has been observed for many decades is that the TDLU acts as a functional unit, even to the extent of undergoing lactational differentiation in the resting breast (figure 2), giving rise to the feature termed *residual lactational lobule* [3]. However, the change can also be found in the nulliparous breast and to an increased extent in oral contraceptive (OC) users [4]. This must reflect an extreme sensitivity of some TDLUs to systemic fluctuations of prolactin, which can become elevated (though admittedly well below the levels found in pregnancy) in a small proportion of OC users in consequence of pituitary sensitivity to exogenous estrogen. This of course does not deny the existence of stem cells, but suggests an altogether different

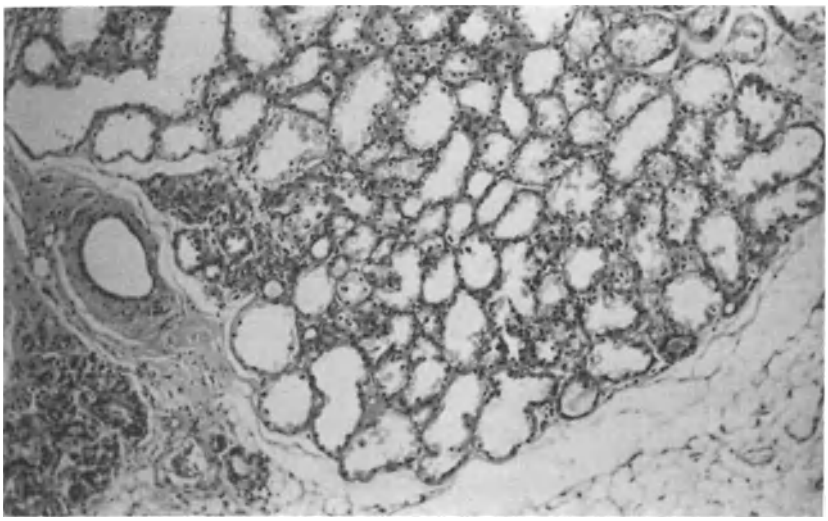

Figure 2. Lobule showing lactational differentiation (compare with accompanying resting lobules). Field size 1 × 1.5 mm.

hierarchy of cell maturation potential among the units of the breast than in other mammary glands.

Two events that are influential in determining risk of cancer in a breast are menstrual cycles (age of onset and total number experienced) and parity, but only in the last five years has there been any measure of understanding of how such events may modulate responsiveness of TDLUs. Menstrual cycles, both naturally and artificially (OC) regulated, induce proliferation of TDLU epithelium that peaks in the last week of cycle [5,6]. Individual cell death and deletion by apoptosis is maximal a few days later [4,7,8], pointing to the need for balance between addition and deletion if focal hyperplastic pathology is to be avoided. Perhaps crucial to an appreciation of the susceptibilities of DNA to mutational events is that a transitory period of stem cell existence takes on minor significance compared to a prolonged period (menarche to menopause) of recurring cyclical episodes of stimulated mitosis, with accompanying potential for genetic alteration (initiation) favoring progressive transformation (promotion) to malignant phenotype. In that context, it is worth noting that levels of proliferation are greater in the terminal duct within the lobule than in the surrounding constituent ductules of the TDLU [9] (Battersby and Anderson, unpublished). Both Muir [10] and Foote and Stewart [11] believed this region to be the source of breast cancer from observational studies, which rather reflects the rate of scientific progress on this topic over 50 years.

A further pointer to the influence of pregnancy, long appreciated by epidemiologists as a factor affecting cancer risk, comes from observations of

postpregnancy involution. There has never been a definable histological characteristic for reliable distinction of nulliparous from previously parous resting breast. Yet a recent report highlighted histological features of prolonged TDLU involution, present in a proportion of recently parous women, that diminished until lost at five years [12]. Such affected TDLUs appeared refractory to stimuli, but the mechanisms underlying this behavior are not understood and can clearly be overcome in the short term if another pregnancy ensues. Nevertheless, the fact that some breasts exhibit this nonreactive behavior to stimuli contributes to understanding the reduced cancer risk associated with early pregnancy [13].

Growth control in normal breast

From the above account, it is clear that the elucidation of the factors influencing the growth and development of the normal human breast will be complicated by the possibility that the diverse cellular types composing the breast may be under different regulations and that these controls may be interactive. It is equally clear that controls on growth may exist at different levels and take different forms. The following discussion concentrates on the evidence for the involvement of endocrine, local (both autocrine and paracrine), and intracellular controls.

Endocrine control. That the human female breast is under the primary control of hormones is self-evident. The organ develops at puberty but only reaches full maturity during the course of pregnancy. Conversely, involutionary changes occur *postpartum*, and the glandular structure shows progressive atrophy after the menopause. These observations point unequivocally to involvement of ovarian and placental hormones in breast development, and it would only seem necessary to identify the particular hormone classes and connect them with a specific process.

The role of estrogen seems central; in girls with gonadal dysgenesis, full breast development may be induced by the simple expedient of administering estrogen [14], although in individuals whose gonadal failure is secondary to pituitary deficiency, it is sometimes necessary to provide pituitary hormones in addition to estrogen. These observations would be totally compatible with the reports that estrogen-secreting tumors in prepubescent girls cause precocious breast development [15] and that estrogen may stimulate breast cell proliferation and preserve glandular structure in postmenopausal women [16]. However, to suggest that estrogen alone can account for breast development would be grossly misleading. The involvement of pituitary hormones, particularly gonadotrophins, in initiating breast development in girls with pituitary lesions has already been referred to. Furthermore, at puberty, breast development in girls usually precedes the secretion of major estrogen by the ovary [17]; indeed, levels of estrogen in pubertal girls with substantial breast development may be no more than in males [14].

Endocrinologists have also been unable to demonstrate a good correlation between degree of breast development and circulating levels of estrogen [18]. Similar anomalies exist with regard to the involvement of estrogen in preserving glandular features in the breasts of postmenopausal women where pharmacological doses of estrogen are required rather than the physiological levels that can maintain breast cancer in the same women [15]. It is necessary to invoke the possibilities that 1) adrenal androgens at menarche may induce pubertal development, 2) testicular androgen may antagonize estrogens in males, and 3) local factors account for the differing hormone responsiveness of the breast between a) individuals at puberty and b) individuals with differing reproductive and menstrual status.

The above observations in general relate to the external appearance of the breast and do not necessarily apply to the parenchymal structures. Because of this, certain researchers, including the present authors, have undertaken studies of TDLUs obtained from the breasts of women undergoing surgical biopsies for benign conditions. These investigations have provided some interesting findings. Firstly, as mentioned earlier, during the menstrual cycle normal breast TDLU epithelium exhibits greater proliferation in the luteal than in the follicular phase [4–7] (in contrast to the endometrium [19]). Secondly, when estrogen levels are high, mitotic activity can be low [20]. This suggests that if estrogen stimulates breast epithelial proliferation, the route is not direct. A possible explanation of these findings is that in the presence of permissive amounts of estrogen, progestins play an important role in regulating proliferation in the breast [21], a view supported by the observations that 1) OCs (particularly progestin-only) increase luteal phase proliferation [4–6], and 2) a marker of progestin induction, namely, the enzyme fatty acid synthetase, increases in parallel with proliferation in human breast epithelium [22]. These observations are not totally compatible with results from more artificial systems, e.g., human normal breast maintained in immunosuppressed mice [23,24] and established cell lines of nonmalignant breast [25], in which estrogens seem to have primary proliferative effects while progestins seem to be largely ineffective.

Since the major effects of steroid hormones are mediated through specific intracellular receptors, measurements of estrogen receptor (ER) and progesterone receptor PgR in normal breast are potentially illuminating. The amounts of ER in normal breast are low in comparison with breast cancer [26]. Nevertheless, it has been possible to show that the incidence and level of ER are reduced 1) in the luteal phase of the menstrual cycle [27,28] and 2) by the use of OCs (predominantly the combined estrogen and progesterone types) [27]. More specifically, immunohistological observation of ER in normal TDLU indicate two different patterns of positive staining, namely, sporadic and uniform [27]. It is only the sporadic pattern that is reduced in the luteal phase of the cycle, displaying classical ER regulation, i.e., down-regulation by progesterone. It is emphasized that the reduced expression of ER during both the luteal phase and OC use is at times

of relatively increased proliferation and would seem to be paradoxical if estrogen is the prime mitogenic stimulus to the resting breast. It is also worth noting that ER scores are significantly reduced in TDLU from recently parous women [27].

Interestingly, PgR levels in normal breast remain relatively constant through the menstrual cycle [27] (cf. endometrium [28]), suggesting constitutive expression rather than induction by estrogen.

In summary, while estrogen has a central role in the development of the normal breast, this appears in part to be permissive, and other factors appear to modify and enhance its action. The parts played by pituitary-derived hormones and progesterone still need to be fully elucidated. Regulation by hormones alone cannot account for the variation in proliferative activity displayed by the breast [20].

Local controls by growth factors. In addition to mediating or modulating the action of steroid hormones, polypeptide growth hormones may be involved directly in the process of proliferation [29]. For example, results from studies of breast cell lines suggest that a variety of growth factors, including EGF, TGF-α and -β, IGFs, and certain others that may be specific to the breast (e.g., MDGF1), are capable of either stimulating or inhibiting growth in culture [30–32]. The evidence that the same factors are active in vivo is more tenuous and largely circumstantial.

Thus, IGF levels peak at puberty and correlate better with breast size than with chronological age [33] or circulating estrogens [14]; specific receptors for IGF may also be detected in the normal breast [33]. The relevance of these observations needs to be substantiated, especially since studies by Laron show that normal breast development may occur in girls with congenital IGF-1 deficiency [14].

Mitogenic stimulation through the epidermal growth factor receptor may also play an important role in growth stimulation of breast epithelial cells. Normal breast epithelium expresses specific receptors for EGF [34], and proliferative indices in explants of normal breast maintained in culture may be stimulated both by EGF and TGF-α [35]. This is consistent with our own findings that EGF may influence levels of total phosphorylation and of specific proteins by particulate fractions of normal breast (see below), but as yet there is no direct evidence relating cyclical variation in these local growth factors to breast response.

Intracellular modulators. Many controls of cellular function and proliferation are primarily intracellular, and indeed the effects of certain hormones and most growth factors, whether acting in an endocrine, paracrine, or autocrine mode, ultimately need to be transduced by a second messenger system. In a situation in which primary influences are unidentified, a plausible strategy is to look for differences in expression of second messenger systems. Since many second messenger systems are mediated through phosphorylation, we

have embarked upon a systemic measurement of phosphorylation status in normal TDLU derived from the breasts of women 1) with differing reproductive and menstrual states and 2) displaying differing degrees of proliferation. These studies have shown that the level and patterns of phosphorylation vary greatly between specimens derived from different breasts. However, phosphorylation is significantly greater in those displaying higher epithelial proliferation. While this may be a casual rather than causal relationship, we have been encouraged to explore one of these phosphorylation systems in more detail, namely, protein kinase A. In particular, we have examined the regulatory subunits that bind cyclic AMP [36]. These cyclic-AMP-binding proteins appear to be ubiquitous, and binding activity has been found in all normal breast specimens that we have examined. Although the level of binding activity is substantially less than that in extracts of breast cancer, there is a wide range of activities between different breast specimens. This variation relates to proliferation in that tissues displaying high proliferation displayed significantly increased levels of binding over those with lower activity. Interestingly, this increase in total cyclic AMP binding was also associated with an increased ratio of type I to type II isoforms (figure 3). This would be consistent with the concept that type I binding programs cells for proliferation, whereas type II binding is involved

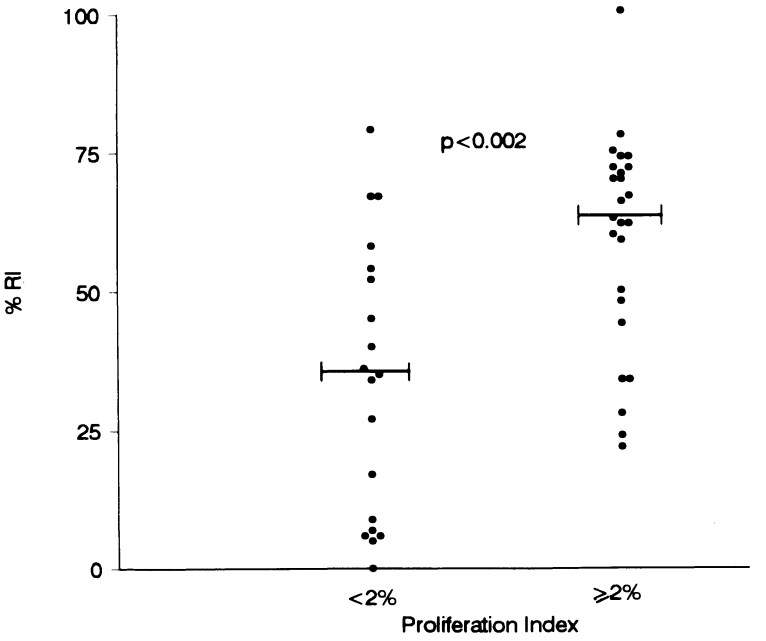

Figure 3. Range of proportional values of R1 cAMP binding protein in normal TDLU with low (<2%) versus high (≥2%) proliferation index.

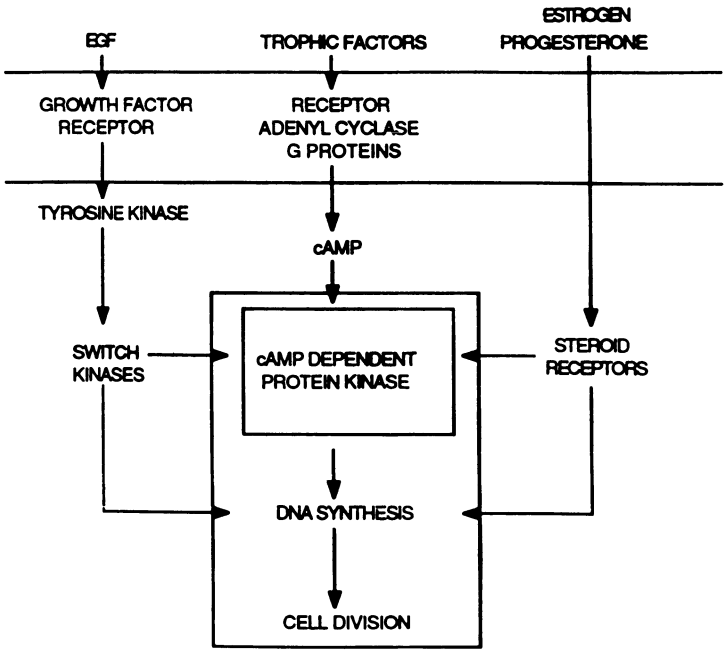

Figure 4. Schematic diagram of possible relationship between extra- and intercellular mediators of cell response (proliferation).

with promoting differentiation [37]. The factors that determine the level and pattern of cyclic AMP binding proteins have yet to be elucidated, but the findings would strongly suggest that agents whose mechanism of action is mediated through cyclic AMP have a role in regulating proliferation within the breast. To our knowledge, corresponding studies in normal breast specimens have not been undertaken with regard to other second messenger systems such as protein kinase C and tyrosine kinase although levels of these kinases appear to be lower in normal as compared with malignant tissue [38,39].

These observations emphasise the possibility that factors other than, for example, estrogen may be primarily responsible for the proliferative status of the normal resting breast, and such a hypothetical scheme is illustrated in figure 4. There is also a corollary of these observations, namely, when surveying the factors associated with the disordered regulation of growth in malignancy, it will be important to consider these same systems.

Breast cancer progression and development

The route from normal to cancer in epithelial structures is accepted from classical teaching to pass through several stages involving degrees of hyper-

plasia leading to carcinoma in situ, thereafter developing into occult invasive cancer and finally to clinical invasive cancer [40]. Access to some human tissues has confirmed this sequence as an appropriate concept, for example, in the cervix and colon. However, there are major limitations to achieving similar confirmation in the breast on account of the complex structure and inaccessibility to repeated sampling of the relevant epithelial structures within the organ. As a result, concepts developed from animal models or derived from studies with cells in vitro have not been substantiated by observations that would more precisely record the natural history of disease.

Pathology and cancer origins

There are three approaches that have been instructive in helping to understand the beginning of breast cancer; 1) the examination of surrounding tissues in cancer-bearing breasts; 2) the follow-up of benign biopsied cases; and 3) the evaluation of malignant lesions detected by mammographic screening of healthy women. These have led to a requirement for clearer discrimination between lesions that are precursors and those that are risk identifiers. The former are *obligate* in local progression to invasion and metastasis (although over a considerably variable time course that could exceed the host's lifetime), while the latter are *nonobligate* [41], but mark an increased risk of (invasive) cancer that could effect either breast.

Cancer-bearing breasts. Subgross microscopy and correlative histology of lesions more common in cancer-bearing breast [42] have clearly illustrated that some proliferative processes involving the TDLU belong to the precursor category. However, judgment of the degree of 'obligation' to progression to cancer is dependent on the histological recognition of specific qualitative cellular characteristics that are unrecognizable on stereomicroscopy. For convenience and historical reasons, these histological appearances are considered in two categories, of which the cytological and pattern characteristics termed *ductal* are regarded as falling within the obligate group, while those with characteristics termed *lobular* are considered examples of the nonobligate lesions. An example of ductal carcinoma in situ is shown in figure 5, and an example of lobular carcinoma in situ in figure 6. The very nature of such probability of progression to invasion is a concept that conflicts with the usual dichotomous decision between benign and malignant diagnosis, but is a more realistic expression of observed biological behavior [43–45]. Furthermore, it acknowledges that several factors, including those within the altered cell, its local environment, and other systems intrinsic to the host, will influence the full expression of the disease process (see below).

Follow-up of benign biopsied cases. Another facet of this puzzle is the terminology applied to denote the middle ground between hyperplasia,

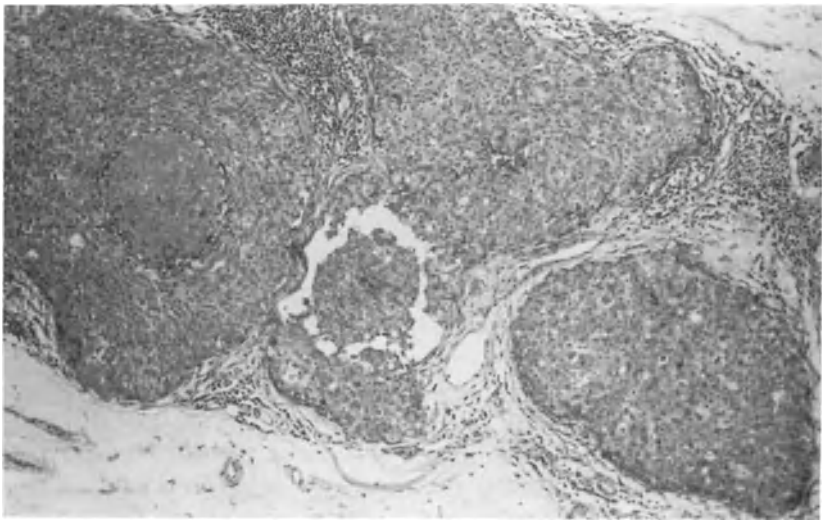

Figure 5. Carcinoma in situ of ductal type arising through expansile growth in TDLU, showing central 'comedo' necrosis. Field size 1 × 1.5 mm.

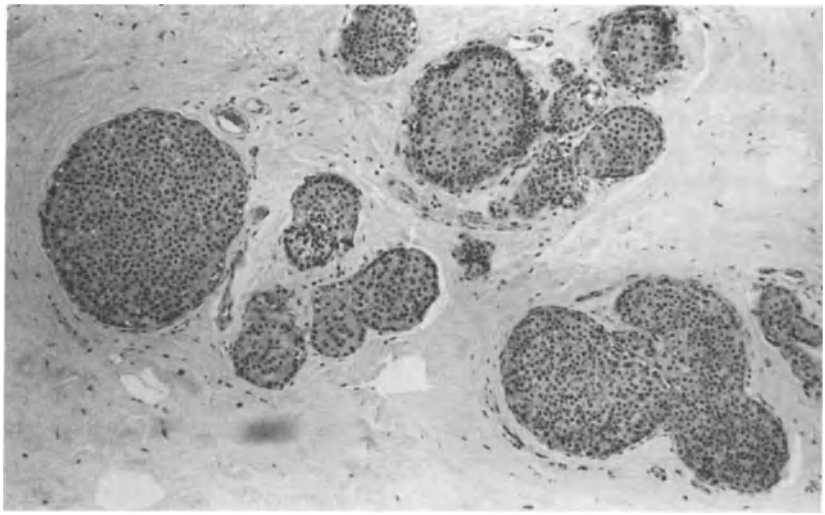

Figure 6. Carcinoma in situ of lobular type has more uniform cell population in evenly spaced placement within units. Field size 1 × 1.5 mm.

which is a reversible regulated disorder of cell proliferation, and carcinoma, which is an irreversible disregulation of cell division associated with a variable capacity for metastatic spread. The terms *epitheliosis* and *papillomatosis* have been employed commonly in the past, but without consistent meaning.

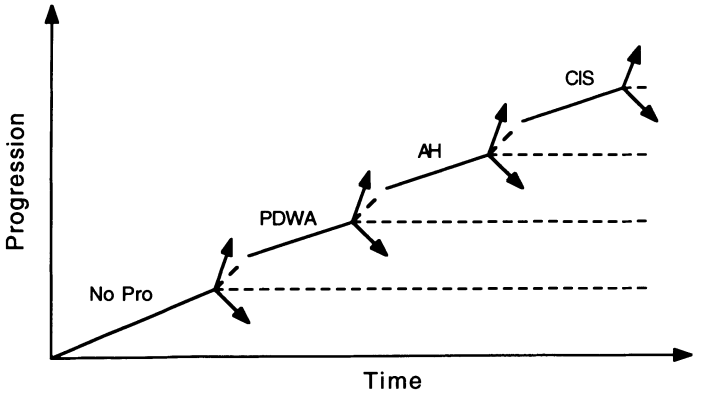

Figure 7. A model to illustrate the incremental stages of histological change with progress to ultimate malignancy (invasive cancer) Each stage is likely to proceed to the next (interrupted line), but may also remain stable (horizontal arrow) or directly develop a clone of cells proceeding to invasion (vertical arrows). The latter event is more likely, with succeeding stages moving to the right.

No Pro = no proliferative disease; PDWA = proliferative disease without atypia; AH = atypical hyperplasia; CIS = carcinoma in situ. Adapted from [110].

Helpful direction was provided in a report of at least 16 years follow-up of 3300 benign cases (from a total of over 10,000) that were histologically reviewed and classified according to defined criteria, during which time 135 women developed invasive breast carcinoma [46]. Only those with proliferative lesions had any risk association, and indeed a risk increase that was clinically significant was restricted to the small percentage (4%) with proliferations possessing some but not the complete histological features of malignancy. The historical perspective on adopting the term *atypical hyperplasia* to describe such processes with relevant identifiable cancer risk in the breast are detailed elsewhere [47–49], and formal recognition of the terminology has been expressed through a Consensus statement form the College of American Pathologists [50]. The modern concept of disease progression, as depicted in figure 7, tacitly acknowledges that some cancers will occur through progressive stages of cellular disorder, while others will bypass these stages, transforming to cancer at any point along the way. Improved consistency in categorization of breast diseases into this model of premalignant conditions will greatly help the characterization of molecular events associated with progression to cancer. This characterization will be necessary to evaluate the similarity, or otherwise, between molecular biological mechanisms of genetic disregulation in the breast (described elsewhere in this book), and those currently believed to underlie the transition from normal to cancer in other sites, such as the colon [51] and cervix [52]. It is already evident that the breast displays more complex anatomical variabilities and physiological interrelationships. The nature and availability

of tissue specimens is such that opportunities to evaluate natural history of breast cancer with anything that approaches a perspective of reality will only pertain where there is a program involving mammographic screening of healthy women. Notwithstanding, this will introduce further aspects of bias [53,54] that must be understood and allowed for when interpreting the observations.

Cancers detected by screening healthy women. It is universally acknowledged that mammography enables breast cancer detection at an earlier moment of natural history, but the 'window' obtained in this preclinical phase, a concept developed by epidemiologists [55], is seldom able to provide a view of sufficient dimension to allow an accurate prediction of all the preceding or generative phases. Thus the yield of a screening exercise contains a mixed population of cancers that requires qualified interpretation [56]. Not all of the noninvasive cancers might have progressed to invasive, metastatic, and life-threatening disease in the host's lifetime. Furthermore, given the limited resolving capacity of the mammographic equipment for invasive cancers less than 5 mm, it is likely that few of the overall yield will be in the 'perinatal or infant' stage of development (figure 8). Such comment is relevant for a realistic appreciation of the time scale of events concerning growth rate in relation to detection of breast cancers [53,57]. It is therefore essential to look beyond the 'size and negative node status,' or chronological stage parameters, as measures of screening effect in order to interpret the biology. This has been done in different ways. From the Two Counties study in

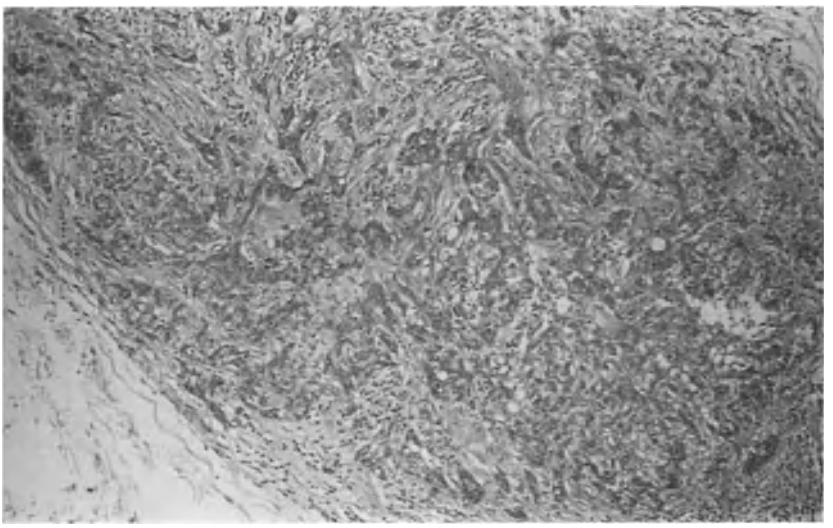

Figure 8. Solitary focus of invasive carcinoma, 1.5 mm diameter, arising in association with carcinoma in situ of ductal type that extended over an area of 18 mm.

Sweden, comparative studies between different populations have indicated that progression with time is likely to lead to a worsening of histological grade [58]. In the context of the breast cancer paradigm [59], which acknowledges that breast cancer will vary in the developmental stage and site from which metastatic cells will disseminate systemically, the Swedish philosophy gives less emphasis to the noninvasive cancer, but more to the detection of invasive cancer below 10 mm with a low proportion (less than 10%) node positive. This view is supported by extrapolations in model systems derived from extensive clinical follow-up studies, used to estimate the range of cancer sizes at which the first systemic metastasis would have occurred [60].

Also from Sweden, but from a different histological perspective, Linell and colleagues [61] are enthusiastic to promote the concept that breast (invasive) cancers can be considered essentially in three categories: 1) tubular and varieties, 2) ductal 'comedo' type, and 3) classical special type (lobular, mucoid, medullary). The first group, which constitutes as much as 35%, is claimed to originate from benign radial scars, with which there are structural similarities; and progression with time involves overgrowth by less differentiated components. The second group arises in the manner predicted from subgross stereomicroscopy [42] through proliferative expansile overgrowth of the TDLU by pleomorphic undifferentiated cancer cells of ductal type. It is worth noting that the term *comedo* is here applied generically, rather than in the customary restricted histological meaning of *focal central islands of necrosis*, and also implies a radiological pattern of tumor growth recognition. The third group arises in different ways, with progression determined by the form of differentiation, invasion, or host response.

Such controversial comment is useful if it promotes awareness that all cancers do not have a common origin. In view of the frequency of radial scars in perimenopausal breasts [62] and lack of cancer risk on follow-up [63], this at least suggests a low level nonobligate process that does not have the proven status necessary for risk notification [50,64]. The association of radiological and histopathological features that brought about the 'lumping' of the above second category is a multidisciplinary exercise with potential for further development, which is likely to have greatest relevance in clinical management. Experience in several years of population screening of healthy women in Edinburgh has encouraged belief in the heterogeneity of invasive cancer development, as well as a strong desire for a better understanding of the interplay of selective processes affecting the nature of diseases detected. As in Sweden, the proportion of tubular and related cancers in the Edinburgh Randomized Screening Project was considerably higher among those screened than in the symptomatic cases of the control population [65], but a substantial proportion of the smallest lesions did not fit this mould. It is of increasing concern that some cancers evolve in a manner that we at present cannot identify in terms of classical pathological processes, with a preceding in situ phase. This is the challenge and the hidden agenda of the left-hand

vertical pathways of figure 7 — that there are histogenetic routes to cancer so far unrecognized in our proliferative spectrum and unrepresented in a benign biopsy population.

Growth controls in the development and progression of cancer

The progression to cancer as described in the preceding section could reasonably be assumed to have resulted from a breakdown of normal controls, which has provided cancers with a growth advantage. The following sections seek to determine whether evidence exists to support this contention. At the outset, two points need to be emphasized: 1) while there are considerable data associating abnormalities in growth controls with the development and progression of breast cancer, this does not prove that the aberration is a causative factor; and 2) there will be a tendency to consider cancer as a single entity, whereas in fact tumors are heterogenous and the factors that control their progression need not be common.

Endocrine controls. The involvement of hormones in the development of breast cancer has been the subject of considerable research, which has been extensively reviewed [66,67]. The etiology of the disease has a strong hormonal component, which is discussed elsewhere within this volume. The most striking observations are that breast cancer does not occur before puberty and that removal of the ovaries, particularly at an early stage, produces a dramatic reduction in the incidence of breast cancer. Conversely, however, most women who develop breast cancer have no overt evidence of endocrine abnormality [66], and no hormone (with the possible exception of diethyl-stilbestrol [68]) possesses the ability to act as a full carcinogen and mutate DNA. The present theory is, therefore, that hormones are not initiators of cancers but rather promote tumors, either by 1) increasing the pool size of susceptible stem cells, 2) stimulating the growth-transformed cells, or 3) advancing occult cancer deposits into overt tumors.

It is worth considering the evidence for each of these possibilities in a little more detail. The effect of hormones on the normal breast has already been considered and attention drawn to the difficulty in identifying stem cells within the breast (see the first section of this chapter). Whether hyperplastic lesions or noninvasive breast cancer are under hormone control or are subject to different regulation is a matter of controversy. For example, certain workers have suggested that cells in atypical ductal hyperplasia and in situ carcinoma are uniformly ER positive as measured by antireceptor monoclonal antibodies [69]; others have suggested that the level of ER and its incidence are substantially lower than those found in invasive carcinoma [70]. However, indirect evidence, such as the reduced incidence of carcinoma in situ after the menopause [71], would suggest an endocrine involvement.

That invasive cancers, whether occult or overt in nature, may be influenced by hormones is much more convincing. Stimulation of breast cancer

in vivo by physiological concentration of estrogens has been reported [72]. Conversely, endocrine deprivation therapy (whether it be ovariectomy or LHRH agonists in premenopausal subjects or adrenalectomy and hypophysectomy in postmenopausal women), aromatase inhibitors, antiestrogens, or pharmacological doses of steroids have all been used successfully to treat breast cancer — although tumor remissions occur only in one third of patients [73]. While these endocrine therapies may affect several hormones or their receptors, there is reason to believe that in most cases their mechanism of action is mediated through estrogen. All the procedures have in common the ability either to reduce cellular levels of estrogen or to antagonize the mechanism of estrogen action [74]. Furthermore, successful endocrine manipulation is invariably associated with cancers that possess specific receptors for estrogen [75]. While estrogen receptors are found in over 70% of breast cancers, it is interesting that there is a tendency for receptors to be associated with well-differentiated early-stage lesions [76]. These levels of receptors are much in excess of those found in the normal breast [26], which might suggest that these cancers have an increased sensitivity to and greater dependence upon estrogen. Up-regulation of estrogen receptors in breast cancer may also be associated with deregulation of controls; for example, it has been more difficult to show that cancer ER levels vary through the menstrual cycle [77], as has been observed in both normal breast [27] and endometrium [78].

The role of progesterone and progestins in the promotion and regulation of breast cancer is much more obscure. High doses of progestins may induce the regression of breast cancers [79], but the mechanism of the effect is not elucidated. It is not even clear if the action is mediated through the progesterone receptor [80], which conventionally is considered to be under estrogen control in most breast cancer [81]. High-dose progestins are capable of down-regulating several classes of steroid receptors [82] and may also suppress estrogen levels [78]. Nevertheless, it has been suggested that there is a change in steroid sensitivity of human breast epithelium during carcinogenesis that involves an increase in estrogen sensitivity and a change in role for pogesterone from one that synergizes with estrogen in normal epithelium to an antagonism of estrogen in malignant tumors [21,82].

While it is clear that certain breast cancers appear to be dependent upon endocrine influences for their continued growth, it is equally apparent that the majority seem hormone independent. Whether at an earlier stage in their evolution these autonomous cancers passed through a hormone sensitive state is uncertain. However, the natural history of hormone-dependent cancers suggest that, under selective pressure, most will acquire a phenotype of autonomy. This provides a precedence for the transition from hormone dependence to independence. It is worth considering briefly what changes in growth regulatory mechanisms may be occurring during the progression to autonomy, although it should be pointed out that 1) such a transition might not involve growth regulation mechanisms per se, and 2)

the most convincing evidence for changes in growth controls with progression to hormone independence came from work in model systems [83].

Insofar as human breast cancers are notorious for their heterogeneity [84], with successful hormone therapy being selective [85], it is possible that endocrine resistance is acquired by the outgrowth of clones of cells that were present at the outset of treatment and that were always inherently independent. This phenomenon is undoubtedly likely to occur and would account for the reported examples whereby an ER+ve tumor recurs as an ER−ve relapse [86]. However, loss of hormone sensitivity can occur without concomitant loss of estrogen receptors [87]. Given this observation, it is necessary to postulate that, during the selective pressure of hormone deprivation, either the ER has become defective (there is evidence for mutated forms of ER [88] but no suggestion that this increases with acquired hormone dependence) or the cancer cells bypass the requirement for estrogen.

Local growth controls. The suggestion that the genesis and the progression of breast cancer is associated with increasing abnormalities in local growth controls is very much in vogue, but it is largely based on evidence obtained from cells maintained in culture and other model systems. It is thus important to review the confirmatory results or otherwise that are derived from observation as clinically derived material.

Perhaps the most convincing evidence that growth factor controls may be abnormal in breast cancer is the finding implicating epidermal growth factor receptors and the analogous protein, encoded by the oncogene c-erbB_2. Both receptors appear to be overexpressed in a minority of breast cancer, this being associated with poor prognosis [89]. The findings associated with c-erbB_2 are especially interesting, for whereas normal breast rarely overexpresses the protein, noninvasive carcinoma frequently does; however, the incidence is lower in invasive cancers [90]. The assumption has been that abnormal expression of the gene is an important step in the process of carcinogenesis within the breast. However, progression from the noninvasive to invasive phenotype expression would seem less dependent upon this growth control; either c-erb$_2$ overexpressing intraduct cancers are less likely to become invasive or these particular growth controls return to normal with progression to invasion.

Interestingly, both at the level of the mRNA [91,92] and protein [93], TGF-α, which binds to the EGF receptor, has been detected in the majority of breast cancers. This finding is to be compared with detection rates in benign breast lesions, which are about 30% [92]. However, while it has been hypothesized that progression from the hormone-dependent to the hormone-independent phenotype might be associated with constitutive production of factors such as TGF-α that are normally under hormone control [94], data from clinical studies do not support this. Thus King et al. [95] showed that levels of TGF-α extracted from ER+vePgR+ve tumors

(likely to be hormone dependent) were not significantly different from ER−vePgR−ve tumors (likely to be hormone independent). Although in contrast to TGF-α, TGF-β is regarded as a negative influence on epithelial cell proliferation [96], levels of TGF-β are usually higher in breast cancers than in adjacent normal tissue [92]. The suggestion that tumors with increased expression of TGF-β have a good prognosis [97] is controversial [98]. Furthermore, although tamoxifen may induce TGF-β production in the breast, the most dramatic effects are seen in stromal cells rather than in malignant epithelium [99].

In terms of other growth factor receptors, those for IGF-1 appear to be higher in breast tumors than in adjacent normal tissue [100]; in breast cancers, binding capacity is exclusively located in the proliferative epithelial compartment [101]. As a result, it has been suggested that the overexpression of IGF receptor is associated with malignant transformation within the breast and that breast cancers may be more sensitive to IGF-like factors. Interestingly, the level of IGF receptors relate to those of estrogen and progesterone [100], which suggest that they may be associated with similar growth controls, all of which confer good prognosis.

Internal controls. There is a wealth of evidence that with the transformation to malignant phenotype, all manner of internal cell cycle controls become unregulated. The finding that many products of oncogenes and tumour-suppressor genes have key roles in signal transduction and transcription of nuclear events [102,103] is compatible with the concept that the carcinogenic process is associated with a disruption of cellular function. If not lethal, these products will lead to uncontrolled proliferation and metastatic growth. It is not the aim of this section to review the involvement of oncogene/suppressor products in breast carcinogenesis and progression; these are reviewed elsewhere. The present authors have taken a closer interest in the possibility that disruption of signal transduction may be associated with the aberrant behavior of breast cancers, and it is this aspect of internal control that is now considered.

Since the primary effects of most growth factors are mediated through tyrosine kinase activity [104], it might be expected that the uncontrolled proliferation of breast cancers should be associated with elevated levels of tyrosine phosphorylation. While there is evidence that this is true, particularly in tumors with a poor prognosis [39], the size of effect is modest. It is possible that what influences tumor behaviour is not general tyrosine kinase levels but the activity of specific tyrosine kinases such as the c-src oncogene product [105]. Nevertheless, the pharmaceutical industry has taken the view that tyrosine kinase is a potential therapeutic target and is developing inhibitors as drugs to treat breast cancers. Only time will tell whether this optimism is misplaced.

Another second messenger system that is attracting interest is protein kinase C. There is abundant experimental evidence that the action of many

tumor promoters may be mediated through this system [106]. Furthermore, it has been shown that this family of enzymes may be elevated in breast cancers as compared with nonmalignant tissues [38]. Whether the activity is correlated with cancer behavior or changes during progression is unknown. However, the observation that tamoxifen-induced regression is associated with a reduction in protein kinase C activity has led to the suggestion that agents that decrease activity will inhibit cancer proliferation [106].

Among the second messenger systems, we have been particularly interested in cyclic-AMP-dependent protein kinase or kinase A. In particular, we have investigated the role of the cyclic-AMP-binding moiety of the complex, the regulatory subunit. Levels of these binding proteins, particularly the RI subunit, appear to be increased in breast cancers as compared with normal and benign breast tissues. Furthermore, in patients presenting with "early" breast cancer, we have found that high levels of binding are associated with poor prognosis [107], the effect being independent of other prognostic factors such as lymph node involvement. Again, the increased binding appears to be associated with a preferential expression of RI to RII [108]. Because of this and similar work in rodent mammary tumors, it has been suggested that strategies designed to increase the levels of RII in comparison with RI will inhibit cell proliferation and retard tumor growth [109]. Results from model systems indicate that this approach can be effective and is worthy of further investigation [37,109]. Certainly these data support the proposal that aberrations in second messenger systems are associated with an increasing degree of malignancy, although whether they are causative of the progression or simply a result of it remains to be delineated.

Concluding remarks

Given the complex nature of the human breast, it is not surprising that despite considerable research there remain gaps in our knowledge of the factors controlling both normal development and the processes associated with transformation to and progression of malignancy within the breast. Problems are compounded by 1) fundamental disparities between the breast and animal mammary glands (which means that observations in the latter are not necessarily relevant to the former) and 2) the potential interaction between different cell types within the breast (which means that results from cell lines can be misleading). Because of these limitations to model systems, the observations from clinical material assume greater importance, even though such tissue is rarely available without some associated pathology and the opportunity for sequential measurements is exceedingly rare. Nevertheless, such analyses must fashion our perceptions of events occurring within the normal "resting" breast and those associated with the evolution of cancer.

Similarly, in terms of control mechanisms, it is clear that these occur in different forms and are effective at different levels. In terms of the normal breast, we have reviewed the crucial role of estrogen in developmental processes, but it is equally clear that other regulatory factors are involved, at least in the response of proliferation. We therefore suggest a 'tonic' function for estrogen, upon which must be superimposed finer controls exerted by other hormones such as progestins, pituitary polypeptides, or local growth factors such as EGF/IGF's. The details of specific influences involved still need to be defined; however, evidence is presented that implicates a second messenger system, protein kinase A, as an example of involvement of other systems that influence proliferation more directly than steroid hormones.

In terms of the development of breast cancer, the concept of transition from normal to malignant that proceeds through defined stages of hyperplasia, atypia, and noninvasive cancers, as has been observed in other organs, has been less easy to confirm. It is therefore difficult to avoid the conclusion that all cancers do not have a common pathway of origin and that there is a heterogeneity in the routes by which invasive cancers develop. If this is the case, then it can be anticipated that any associated changes in growth controls will be equally varied. This accounts for the problems in integrating results into a general thesis. An attempt has been made in figure 9 to develop a rational concept of the general changes in relation to growth control that occur during the development and progression of breast cancers. Etiological evidence points strongly to the involvement of steroid hormones in the development of the majority of breast cancers, and their role would be as promoters rather than initiators. In terms of established breast cancers, most appear (at least initially) to be endocrine dependent. In these lesions, there is a strong indication of increased sensitivity to estrogen, which is accompanied by a phenotypic up-regulation of estrogen receptors; during the transition from normal to malignant, there may also be a change in

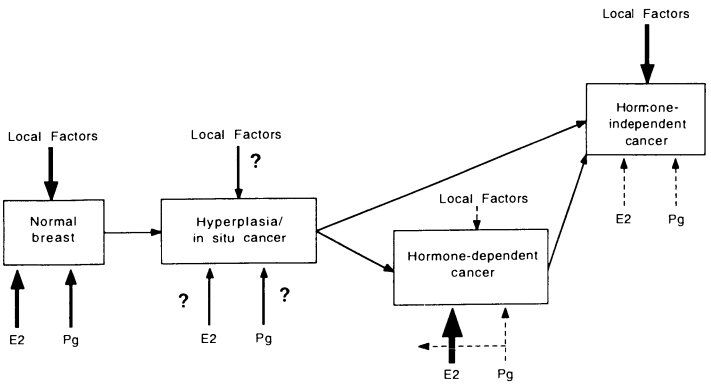

Figure 9. Scheme of proposed influences of steroid hormones and growth factors in the development and progression of breast cancer.

the role of progesterone from one of synergism to one of antagonism of estrogen, but this concept is controversial. Whether all cancers pass through a stage of hormone dependence or whether some move directly to full autonomy is uncertain, and it would be premature to suggest that hormone dependency reflects an 'early' lesion. What is clear is that under the selective pressures of endocrine deprivation therapy, most adopt the hormone-independent phenotype. The role of local growth factors and their receptors in breast cancer development and progression is also ill defined. It may be that enhanced expression of growth factors or their receptors confers a growth advantage. If so, it might be expected that such cancers would appear autonomous. However, while malignant breast tissue tends to have higher levels of growth factors such as TGF-α and -β, there is little to confirm the idea that, within breast cancers, the degree of growth factor expression is related to proliferative activity. In contrast, there is an increasing body of information to suggest that disruption of internal cellular control, as evidenced by changes in second messenger systems, accompanies the transition from the normal to the malignant state and that the degree of system aberration may be related to clinical behavior. As a result, these second messenger systems, which include tyrosine kinases and protein kinase A and C, represent novel targets for therapeutic intervention. It can be seen that our understanding of what factors control the development of normal breast, its transition to malignancy, and the progression of breast cancer is far from complete. However, our knowledge of the events underlying these complicated processes is increasing, and the hope must be that with this, mechanisms will be discovered by which we can 1) identify breasts at high risk of cancer, 2) block the pathways to cancer, and 3) devise more effective treatment for those with established disease.

Acknowledgments

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2. Growth, differentiation, and transformation of human mammary epithelial cells in culture

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Our laboratory has developed a culture system utilizing human mammary epithelial cells (HMECs) in order to facilitate studies on the normal mechanisms controlling growth and differentiation in these cells, and to understand how these normal processes may become altered as a result of immortal and malignant transformation. Underlying these studies has been the assumption that carcinogenesis involves aberrations in the normal pathways of proliferation and differentiation, and that development of optimized culture systems to examine the behavior of normal HMECs will aid in our understanding of the mechanisms of human mammary carcinogenesis. The system we have developed has the following main features: 1) long-term growth in serum-free medium of normal HMECs derived from reduction mammoplasty tissues; 2) two immortally transformed cell lines derived from normal HMECs following exposure to the chemical carcinogen, benzo(a) pyrene (BaP); 3) malignant transformation of the immortal cell lines following exposure to specific oncogenes; and 4) growth factor variants derived from the immortal cell lines either spontaneously or following exposure to the chemical carcinogen, N-nitroso-ethyl-urea (ENU).

Differentiation and carcinogenesis

A relationship between transformation and differentiation is suggested by the fact that cancer cells are often found to reflect specific stages in the differentiation pathway of the organ system from which they arise, and that loss of response to differentiation-inducing agents is one of the earliest observed growth-control aberrations in epithelial cell transformation. In our studies of HMECs in culture, we have defined two different kinds of differentiation. The first type, functional differentiation, refers to those properties of the mammary gland associated with its role in pregnancy, lactation, and involution. Since human mammary tissues cannot readily be obtained in functionally differentiated states, analysis of these properties in culture is extremely difficult. However, since mammary carcinoma cells do not exhibit functional differentiation, the absence of functionally differentiated cells in

R. Dickson and M. Lippman (eds.), Mammary Tumorigenesis and Malignant Progression.

culture does not necessarily limit the usefulness of cultured HMEC for studies of carcinogenesis. The second type of differentiation we have termed *maturation*. This term refers to the developmental history of a cell from a proliferative stem cell population to a cell with diminished reproductive capacity, to a 'terminally differentiated' cell no longer capable of division.

In some epithelial organ systems, such as the stratified epidermis, the pathway of maturation coincides with that of functional differentiation. In simple or pseudostratified epithelial organ systems, the maturation lineage may be more difficult to define, since it is not delineated by obvious positional information. In these systems, the pathways of functional differentiation and maturation do not necessarily coincide. The mammary gland in vivo consists of pseudostratified epithelia, with a basal layer resting upon a basement membrane and an apical layer facing the lumen of the ducts and alveoli. Apical cells display a polarized morphology, with microvilli at the luminal side. The myoepithelial cells, which contain musclelike myofilaments, and which contract upon appropriate hormonal stimuli to cause expulsion of milk, lie in the basal layer of cells. Based upon examination of keratin expression and other marker antigens, it has been proposed for the rodent mammary gland that a stem cell population capable of differentiating into both myoepithelial cells and the apical glandular epithelial cells also resides in the basal cell layer [1].

In order to examine the control of proliferation, cell cycle progression, and differentiation in normal and transformed HMECs, we have measured the effects of key growth stimulators and growth inhibitors and have identified molecules that play a role in HMEC differentiation.

Growth and characterization of normal HMEC types in culture

Large quantities of normal HMECs are readily obtained from reduction mammoplasty tissues. They are isolated and stored frozen as described elsewhere [2,3]. We have used two main types of medium to support HMEC growth — a serum-containing medium, designated MM [4], and a serum-free medium, designated MCDB 170 [5]. Both media contain a variety of growth factors, including insulin, hydrocortisone, EGF, and a cAMP stimulator. MM contains 0.5% fresh fetal bovine serum and 30% conditioned media from other human epithelial cell lines; MCDB 170 contains 70-μg/ml bovine pituitary extract (BPE) as its only undefined element. In MM, there is active epithelial division for 3 to 5 passages at 1:10 dilutions. The cultures acquire a mixed morphology, with larger, flatter, nondividing cells mixed with smaller cells growing with a cobblestone morphology. In MCDB 170, there is initial active cell division for 2 to 3 passages of cobblestone-appearing cells. These cells gradually change morphology, becoming larger, flatter, and striated, with irregular edges and reduced

proliferative capacity. As these larger cells cease growth and die, a small number of cells with the cobblestone morphology maintain proliferative capacity and soon dominate the culture. We have referred to this process, whereby only a small fraction of the cells grown in MCDB 170 display long-term growth potential, as self-selection. The postselection cells maintain growth for an additional 7 to 24 passages, depending upon the individual reduction mammoplasty specimen. At senescence, they appear flatter and more vacuolated, while retaining the cobblestone epithelial morphology.

Most of the studies described below on normal HMEC biology utilize these postselection cells that display long-term growth in MCDB 170. These cells are particularly useful in molecular and biochemical studies, since large batches can be stored frozen, permitting repetition of experiments with cells from the same frozen batch, as well as from the same individual. The postselection cells have doubling times of 18–24 hours, and will grow clonally with 15%–50% colony-forming efficiency. All reduction mammoplasty-derived HMECs thus far examined have shown a normal karyotype [6,7].

In order to characterize the cell types grown in vitro, and to compare them to cell lineages in vivo, we have examined them for expression of potential markers of mammary epithelial cell maturation and differentiation. These include intermediate filaments (keratins and vimentin), the large polymorphic epithelial mucins (PEMs), extracellular-matrix-associated proteins (fibronectin, collagen, laminin, proteases, and protease inhibitors), and milk products (caseins and α-lactalbumin). In vivo, mammary basal cells express keratins 5 and 14, as well as α-actin [8]. Some reports have indicated that a subpopulation expresses the common mesenchymal intermediate filament, vimentin [9,10]. Luminal cells uniformly express keratins 8 and 18, while keratin 19 is present in a subpopulation. Specific epitopes of PEMs are also present. In culture, cells displaying keratin 19 and PEMs have reduced proliferative potential, suggesting that they may represent the least proliferative, or most mature luminal cell type in vivo [11–13]. Only a small fraction of normal mammary epithelial cells in vivo are estrogen-receptor positive, and this positive population is preferentially localized in the nonbasal layer [14,15].

Histochemical and Northern blot analyses of our HMEC cultures have shown that normal primary HMECs grown in MCDB 170 and early passage cultures grown in MM are heterogeneous. Some cells have the basal phenotype — keratin 5/14 and vimentin positive, PEM negative, α-actin positive; other cells show the luminal phenotype — keratin 5/14 negative, keratin 8/18/19 positive, PEM positive. The cells that initially proliferate in MCDB 170 are those with the basal phenotype. However, postselection cells begin to express some properties associated with the luminal cell type, i.e., keratins 8 and 18 and some PEM epitopes. Expression of these luminal properties increases with continued passage in culture, such that the senescent cells uniformly express these markers. Senescent cells continue to express the basal keratins 5/14 and vimentin. Neither keratin 19 nor estrogen

receptor is detected. The senescent, nondividing HMEC may maintain viability for months in culture.

These results have led us to propose that the cells that display long-term growth in MCDB 170 represent a multipotent stem cell population initially present in the basal layer of the gland. With increasing time in culture, these cells are capable of a partial differentiation towards the luminal phenotype. However, the absence of keratin 19 and the presence of vimentin in the senescent cell population suggests that these cell have not acquired the fully mature luminal phenotype. Thus, cellular senescence observed in vitro may follow a biological pathway distinct from maturation or terminal differentiation. The controls that limit the number of times a given cell may complete the cell cycle may be distinct from those that lead to a mature, nondividing, and ultimately nonviable phenotype.

Isolation and characterization of transformed HMEC types in culture

Normal HMECs from specimen 184 were transformed to immortality following exposure of primary cultures to the chemical carcinogen BaP [16]. Selection for transformed cells was based on the ability of the BaP-treated cells to continue growing past the time that the control cells senesced. The two resulting cell lines, 184A1 and 184B5, each show specific clonal karyotypic aberrations, indicating their independent origins from single cells [7]. Some of the karyotypic abnormalities found in 184B5, e.g., 1q22 breaks and tetrasomy for 1q, are also frequently observed in cells obtained from breast tumors [17]. Upon continued passage in culture, these two lines show some genetic drift, but this drift is relatively minimal compared to that observed in most human breast tumor cell lines. Although 184A1 and 184B5 are immortally transformed, they do not have properties associated with malignant transformation. They do not form tumors in nude mice, and they show very little or no capacity for anchorage-independent growth (AIG). Malignant derivatives of 184A1 and 184B5 have been obtained with the use of oncogene containing retroviral vectors and viruses [18–20].

Variants of 184A1 and 184B5 that have altered nutritional requirements were selected for by their ability to grow in medium lacking specific growth factors [21]. Variants capable of growth in MCDB 170 lacking EGF, insulin, hydrocortisone, or BPE arose spontaneously. Variants capable of growth in medium lacking several factors, e.g., EGF and BPE, were obtained after treatment of the cell lines with ENU. None of these variants showed AIG, nor did they form tumors in nude mice.

When cells from breast tumor tissues or tumor cell lines are examined, they display phenotypes similar to those found in the mature luminal cell population. They rarely express keratins 5 and 14, and they nearly uniformly express keratins 8, 18, and 19 and specific PEM epitopes [8,22]. Around 70% of breast tumor tissues also display high levels of the estrogen receptor. Thus breast tumor cells in vivo and in vitro display a phenotype that,

in normal HMECs, is associated with low proliferative potential in vitro. Vimentin is not present in most tumor cells; however, expression is observed in a subset of estrogen-receptor-negative breast tumor cell lines and tissues [23].

Examination of the immortalized cell lines 184A1 and 184B5 for markers of maturation has shown that they express keratins 5 and 14, but at a decreased level relative to normal 184 HMEC, while expression of keratin 18 mRNA is increased. These lines have barely detectable levels of vimentin mRNA. Cell line 184B5 expresses the luminal PEM antigens. We have not been able to detect keratin 19 mRNA in any of these lines. These results suggest that the transformed cells, particularly 184B5, have a phenotype closer to the luminal phenotype than that seen in the normal HMEC, but do not fully resemble breast tumor cells.

The cell lines have also been examined for expression of extracellular-matrix-associated protein and for markers of functional differentiation. In normal HMECs, fibronectin is one of the major secreted proteins [24]. Transformed cells from many different tissue types have been reported to have greatly reduced levels of fibronectin mRNA and protein synthesis. Consistent with this pattern, 184B5, and to a greater extent 184A1, show reduced fibronectin synthesis. Other extracellular-matrix-associated genes, such as laminin and collagen type IV, are not reduced in these cell lines. We have not been able to detect markers of functional differentiation, i.e., caseins and α-lactalbumin, in either the normal or transformed HMECs grown in MCDB 170.

Isolation and characterization of the NB-1 gene

One approach we have taken to characterize the changes that occur as a result of transformation of our HMEC cultures has been to identify genes that are expressed in the normal HMEC, but that are downregulated in the immortal and malignantly transformed cells. Towards this end, selected normal HMEC cDNAs were identified and cloned using probes enriched by subtractive hybridization between the normal 184 cell cDNA and both 184B5 and B5KTu (the 184B5 derivative malignantly transformed by K-ras) cell mRNA [21,25]. Several genes preferentially expressed in normal 184 cells were isolated by this method, including those for fibronectin, keratin 5, and vimentin. Additionally, one 350-base-pair cDNA fragment was isolated that initially showed no similarity to any sequence reported in GenBank. This cDNA hybridized specifically to a 1.4-kb mRNA, designated NB-1, which was expressed in the normal HMEC, but was downregulated or undetectable in the transformed cell lines. Sequence analysis of a full-length NB-1 clone revealed a 447-bp open reading frame with extensive similarity (70%, 71%, and 80%) at the nucleic acid level to the three known human genes coding for the ubiquitous calcium binding protein, calmodulin. The

similarity between the translated amino acid sequence of NB-1 and human calmodulin was 85% over the length of the entire protein.

Using Northern and PCR analysis, NB-1 mRNA has been thus far found only in normal epithelial cells and tissues from human breast, prostate, cervix, and skin. It has not been found in normal epithelial cells other than those from stratified or pseudo-stratified tissues. It was not detectable in nonepithelial cells and tissues, nor in any of the mammary epithelial tumor cell lines that we have examined. Human breast cells obtained from lactational fluids were also negative for NB-1 expression by PCR analysis.

Expression of NB-1 mRNA is not significantly decreased when cells are growth arrested by exposure to anti-EGF receptor antibodies or in senescing cells where proliferation is minimal. It is increased in cells growth arrested by TGF-β and reduced when HMECs are grown on reconstituted extracellular matrix material [21].

In order to study the protein encoded by NB-1 transcripts, NB-1 cDNA was subcloned into a bacterial expression vector bearing an inducible T7 promoter [26]. Lysates from bacterial transformants contained an abundant protein that migrated slightly faster than authentic or recombinant calmodulin in denaturing polyacrylamide gels. Like calmodulin, the recombinant NB-1 protein was stable to heat denaturation, and bound to phenyl-Sepharose in a calcium-dependent manner. These two criteria were used to purify recombinant NB-1 protein from extracts of induced bacteria. The whole purified recombinant protein was then used to develop polyclonal antisera in rabbits. Antisera obtained by this method displayed a strong preference for NB-1 protein over calmodulin. Virtually all remaining calmodulin cross-reactivity could be removed by preabsorption of the antisera with calmodulin-agarose. Based on titrations performed with recombinant protein, the level of endogenous NB-1 protein in 184 HMECs was approximately 100–200 ng/10^6 cells, a level similar to the estimated level of calmodulin in other cultured cell lines [27]. The majority of the protein was present in nonionic detergent-soluble cellular fractions.

Using the NB-1 specific antisera, we have shown that the relative abundance of the 16-kDa NB-1 protein reflects relative NB-1 mRNA levels in various cell types, being most highly expressed in normal HMEC, lower or undetectable in the immortally transformed cell lines, and virtually undetectable in tumorigenic breast and prostate cell lines as well as in normal breast fibroblasts. In contrast, levels of calmodulin protein were nearly constant in the same cell extracts (figure 1).

Indirect immunoflourescence was used to study the distribution of the NB-1 protein in normal finite lifespan and immortalized HMECs. In the normal cells during interphase, NB-1 protein was present diffusely throughout the cytoplasm and, to varying degrees, in the nuclei as well (figure 2A). During mitosis, NB-1 immunofluorescence was particularly bright in regions around mitotic spindles (figure 2B). In 184B5, NB-1 expression was heterogeneous both among different cells and within individual cells (figure 2C).

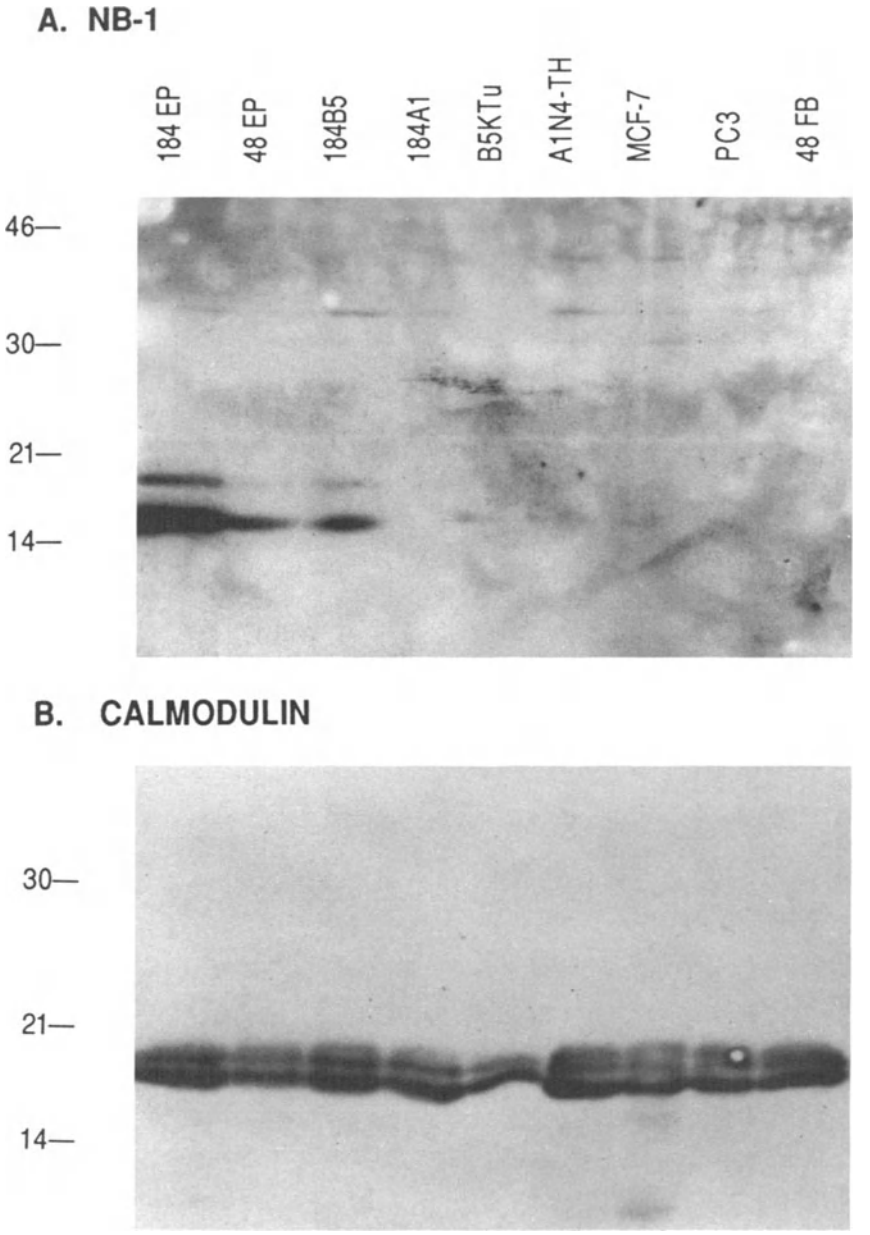

Figure 1. Differential expression of endogenous (A) NB-1 protein and (B) calmodulin in normal and transformed cells. Immunoblots of heat stable cell lysates (10 μg) obtained from confluent cultures of normal HMEC strains (184 EP and 48 EP), immortally transformed HMEC lines (184B5 and 184A1), malignantly transformed HMEC lines (B5KTu and A1N4-TH), a breast-tumor-derived cell line (MCF-7), a prostate-tumor-derived cell line (PC3), and normal breast fibroblasts (48 FB) were performed as described [26].

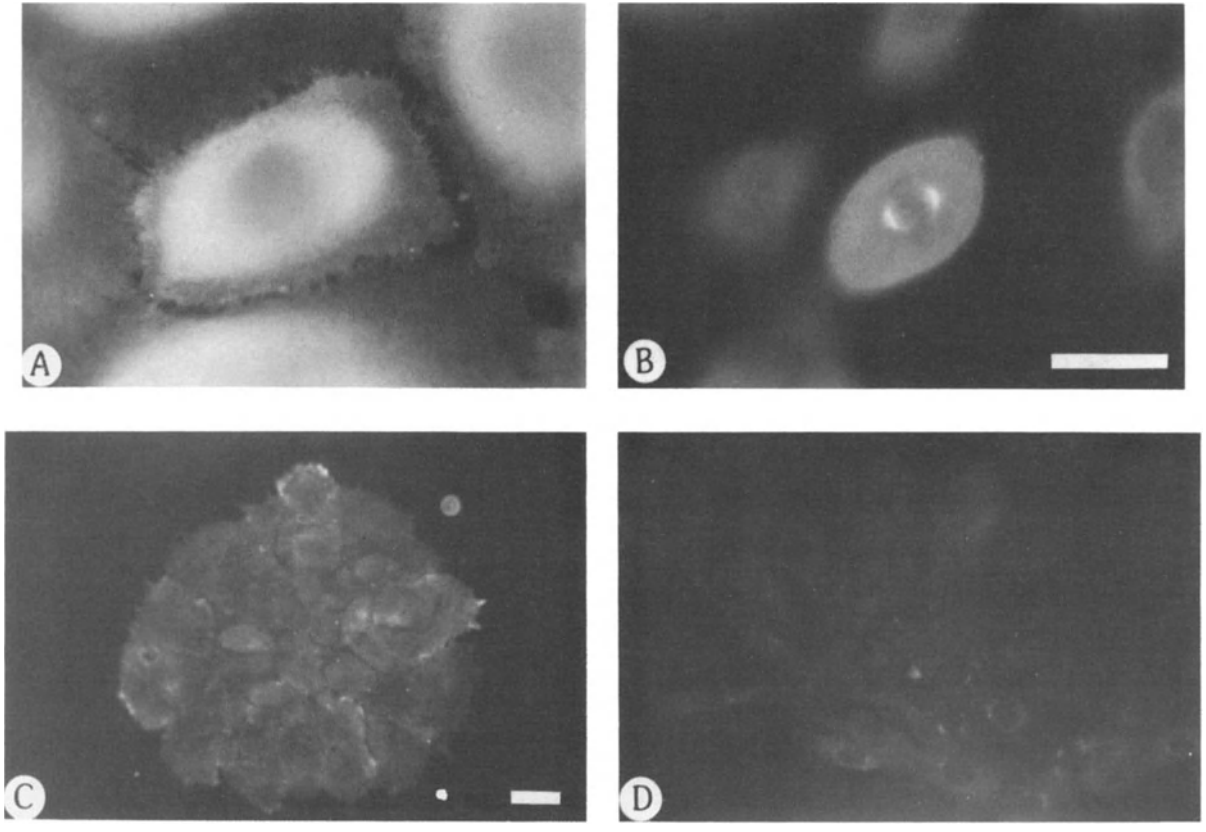

Figure 2. NB-1 immunoflourescence in cultured human mammary epithelial cells. Normal HMEC specimen 184 cells during interphase (A) and during mitosis (B), and immortalized 184B5 cells (C) and 184A1 cells (D). Bars indicate 20 μm. Cell monolayers grown on glass coverslips were fixed and assayed as described [26].

No significant NB-1 immunofluorescence above control levels was observed in 184A1 (figure 2D). The distribution of NB-1 protein in surgical specimens from histologically normal and malignant tissues was compared by immunohistochemistry. In benign breast specimens from nonpregnant, nonlactating individuals, the majority of the basal epithelial cells in small ducts stained intensely positive for NB-1 protein (figure 3A). Luminal cells in the small ducts also stained specifically, although not as intensely as the basal cells.

In larger ducts, staining was mainly confined to the basal epithelial cell layer and was generally less intense than in the small ducts. In all cases,

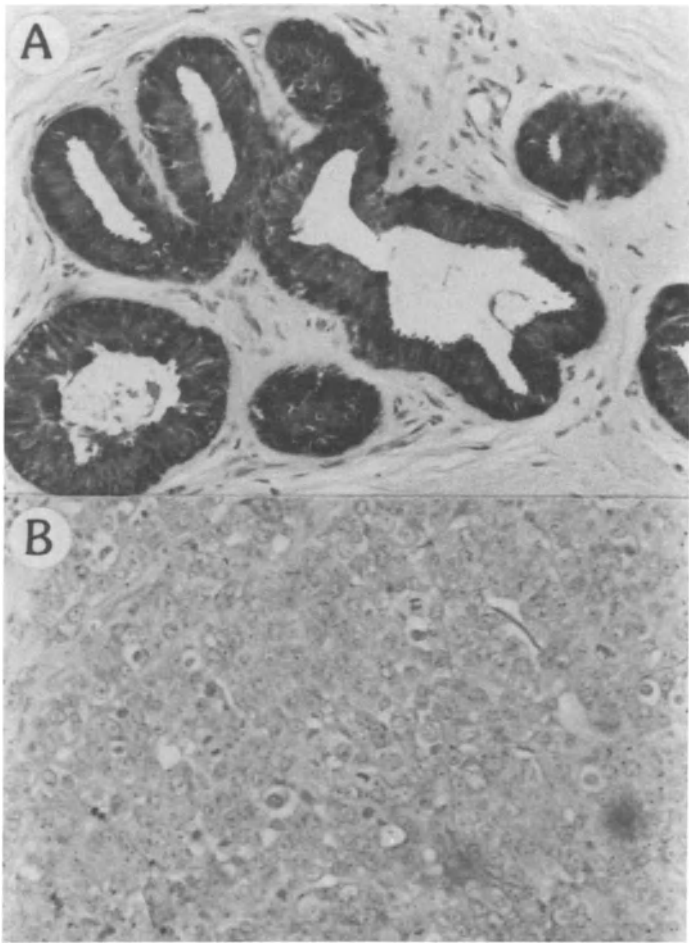

Figure 3. Distribution of NB-1 protein in normal and neoplastic human breast tissues. Immunoperoxidase localization of NB-1 protein in histologically normal human breast (A) and in a ductal carcinoma of the breast (B). Paraffin-embedded tissue sections were deparaffinized and examined as described [26].

distribution of the protein appeared uniformly intracellular. No staining was evident in basement membrane or stromal areas. In contrast to normal breast tissue, sections from six infiltrating ductal breast carcinomas were consistently negative for NB-1 expression (figure 3B). Serial sections of the normal and tumor tissues all showed abundant calmodulin expression.

NB-1 distribution has also been examined in other stratified and pseudostratified epithelial tissue sections [26]. In normal prostate, nearly all the epithelial cells were stained to a similar degree by NB-1 antiserum. In normal cervix and skin, no staining was observed in the basal cell layer. In the cervix, suprabasal cells were intensely NB-1 positive, with the degree of staining diminishing in the more distant upper layers. In the skin, the intensity of NB-1 staining increased from the suprabasal layer until the stratum corneum, which itself was not stained. Thus, in the four different tissues examined, NB-1 has shown distinct patterns of expression. These results suggest that the role of NB-1 may be defined by the differentiated state of the cells where it is expressed, perhaps being involved in the initiation or maintenance of certain differentiated responses.

The initial characterization of genomic DNA corresponding to the NB-1 transcript indicated the unexpected absence of introns. All vertebrate calmodulin genes studied to date contain five similarly placed introns [28]. A literature search revealed the existence of a previously reported human calmodulin 'pseudogene' hGH6, which shared identity with NB-1 cDNA [29]. This gene was designated a pseudogene, since the authors were unable to demonstrate the existence of a corresponding mRNA. Our evidence of expression of NB-1 at both the mRNA and protein levels suggests that NB-1 may be a rare example of an expressed retroposon [30].

External calcium concentration has been shown to affect the proliferative potential and differentiated states of some cultured epithelial cells, including keratinocytes and mammary epithelial cells [31–33]. In normal keratinocytes, increasing calcium concentrations can lead to cessation of proliferation and expression of markers of terminal differentiation. Loss of response to the calcium-induced differentiation signal has been shown to correlate with the early stages of transformation in keratinocyte cultures [34]. The down-regulation of NB-1 expression observed after in vitro and in vivo transformation of HMECs may reflect a consequence or requirement of the transformed state. Possibly, a particular state of differentiation is required for transformation to occur, or the transformed state may be incompatible with high expression of NB-1.

Effects of TGF-β on normal and transformed HMECs

A main focus of our research has been to study the effect of growth factors on normal HMEC proliferation, and compare these data with growth control of the transformed HMECs. In particular, we have examined the effects of

TGF-β and EGF/TGF-α. A long-term objective of these studies is to understand the parameters influencing cell cycle progression in normal, finite-lifespan human epithelial cells, in the hope that this information may facilitate elucidating the aberrations that occur when cells attain immortality and malignancy.

Initial studies with TGF-β examined its effect on the growth of normal and transformed HMECs [35]. Like other normal human epithelial cells, normal HMECs are growth inhibited by TGF-β. While some HMEC strains displayed continued growth for one to two passages following TGF-β exposure, all normal HMECs examined were ultimately growth arrested by TGF-β. Cells closer to senescence showed more rapid growth inhibition at lower TGF-β concentrations compared to younger cells in vitro. Analysis by flow-activated cell sorting and by incorporation of ^{3}H-thymidine into DNA indicated that cells arrested in the late G1 phase of the cell cycle, and that the growth inhibition was at least partially reversible (unpublished data). The extent of reversibility decreased with cell passage in vitro and was relatively asynchronous. Normal HMECs showed distinctive morphologic changes in the presence of TGF-β, characterized by an elongated, flattened appearance.

HMECs that had been transformed to immortality or malignancy had altered growth responses to TGF-β [35]. The 184B5 line maintained active proliferation in the presence of TGF-β, although it showed some decrease in growth rate at TGF-β concentrations greater than 3 ng/ml and displayed some morphologic alterations. We have been able to isolate clonal variants of 184B5 (e.g., B5T1) that are severely growth inhibited by TGF-β. The 184A1 line showed a complex response to TGF-β; most cells were growth inhibited, but all cultures contained a minority population that continued growth indefinitely in the presence of TGF-β, allowing us to isolate 184A1 variants that could maintain growth in the presence of TGF-β. This minority population did not simply represent genetic heterogeneity, since 4 of 4 single-cell clonal isolates of 184A1 showed the same heterogeneity as the parental population. The plasticity in expression of TGF-β growth responses in these HMEC lines has led us to suggest that epigenetic as well as genetic mechanisms may play a role in TGF-β-induced growth inhibition.

Although growth responses to TGF-β vary among the normal and immortalized HMEC lines, all these HMECs showed a similar profile of TGF-β1 receptors, and all expressed specialized responses to TGF-β [36]. In particular, these HMECs exhibited strong induction of extracellular-matrix-associated mRNA and protein species such as fibronectin, collagen IV, and laminin, of the proteases — type IV collagenase and urokinase type plasminogen activator (uPA) — and of the protease inhibitor, plasminogen activator inhibitor 1 (PAI-1) (figure 4 and table 1). The level of overall protein synthesis, especially secreted proteins, was increased following TGF-β exposure, even where cell growth was inhibited. Therefore, the HMECs growth inhibited by TGF-β were not in a resting state. These results indicate

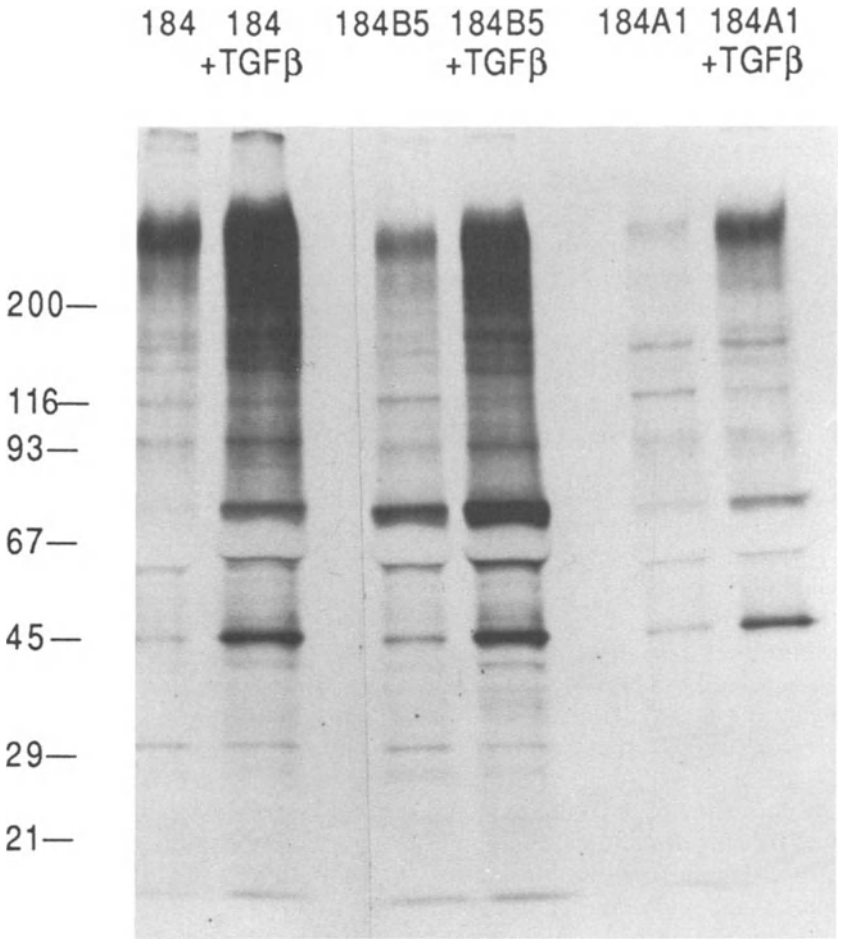

Figure 4. Effect of TGF-β on secreted proteins in normal and immortally transformed HMECs. Sparse to midconfluent cultures of 184, 184B5, and 184A1 cells were exposed to 3 ng/ml of human recombinant TGF-β1 for 72 hours. Control cells received no TGF-β. For the last 24 hours, cells received ^{14}C-leucine (10 μCi in 1.4 ml). An analysis of 15 μl of conditioned medium from each culture was carried out by 4%–20% SDS-PAGE followed by fluorography as described [36]. The prominent band at 220 kDa is fibronentin, as verified by immunoprecipitation with antibodies to human fibronectin. The band at 47 kDa probably represents the 47-kDa PAI-1 protein.

that the effects of TGF-β on cell growth can be dissociated from its effects on specialized responses. Thus, within this one cell type, there must be at least two independent pathways for TGF-β activity, one that leads to cessation of proliferation and one that induces a specific set of cellular responses. It is possible that these epithelial cells' specialized responses to

Table 1. Effects of TGF-β1 on levels of secreted proteins

Cell	184			184A1			184B5		
	+[a]	−	Ratio	+	−	Ratio	+	−	Ratio
DNA synthesis (cpm/dish × 10^3)	17.7	153.8	0.12	66.9	230.5	0.29	110.9	120.1	0.92
% of total protein synthesis in conditioned medium	9.0	5.1	1.8	9.7	5.0	2.2	5.7	2.2	2.6
Fibronection (% of conditioned medium)	23.6	13.9	1.7	2.2	0.8	2.8	17.2	13.8	1.2
Fibronectin (total secreted amount)			12.3			6.2			4.1
uPA (PU/ml)	60	20	3.0	130	52	2.5	0.86	0.26	3.3
PAI-1 (ng/ml)	420	190	2.2	260	80	3.3	840	200	4.2
Procollagenase type IV	+++	++		+++	++		+/−	−	

[a] Sparse to midconfluent cultures were exposed to MCDB 170 containing 3 ng/ml TGF-β and 0.1% BSA for 72 hours or 48 hours (uPA). Control cells received medium containing 0.1% BSA with no TGF-β. To determine DNA synthesis, cells received a two-hour pulse with ^{3}H-thymidine. Data are presented on a per-dish basis. To determine incorporation into conditioned medium and fibronectin, cells were exposed to ^{14}C-leucine (24 hours) or ^{35}S-methionine (5 hours). Incorporation into DNA and into cellular and secreted proteins was measured as described [36]. Fibronectin levels were quantitated by immunoprecipitation with antibodies to fibronectin. Protein incorporation data are presented on a per-cell basis. uPA was assayed by radial caseinolysis, PAI-1 levels were determined by enzyme immunoassay, and collagenase type IV amounts were estimated from a gelatin zymogram as described [36]. Data are presented from equal volumes of conditioned medium.

TGF-β play a role in glandular remodeling, homeostasis, and/or wound healing.

Since both the normal and transformed HMECs express a variety of TGF-β responses, this cell system should provide a convenient experimental model for further studies on the mechanism(s) of TGF-β-induced responses. The immortal cell lines, with their variant cells that display a range of growth responses to TGF-β, allow us to compare closely related cells that do and do not retain proliferative capacity in the presence of TGF-β. These cells can be used to elucidate the mechanisms specifically responsible for TGF-β growth sensitivity, since it may be possible to separate responses that are part of the pathway of growth inhibition from those that lead to induction of specialized functions.

Effects of EGF/TGF-α on normal and transformed HMECs

Initial studies on the effects of EGF/TGF-α on growth of normal HMECs indicated a stringent requirement for this growth factor class for clonal growth [19,21]. However, growth in mass culture proceeded without addition

of exogenous EGF, presumably due to the significant levels of endogenous production of TGF-α [37,38] and amphiregulin [39]. The cell lines 184A1 and 185B5 also required EGF/TGF-α for clonal growth. In contrast to the normal HMECs, these lines were unable to grow in mass culture in the absence of exogenously added EGF. Spontaneous variants of both these lines capable of growth in MCDB 170 minus EGF were then isolated (designated A1NE and B5NE) [21]. These variants represented rare cells in the uncloned population that showed slowly increasing growth rates during the course of 4 to 6 subcultures in medium minus EGF. Cell lines 184A1 and 184B5 were also exposed to ENU, followed by selection of variants capable of growth in MCDB 170 minus multiple growth factors. From these studies we obtained the variants A1ZNEB, capable of growth in medium minus EGF and BPE, and B5ZNEI, capable of growth in medium minus EGF and insulin.

To demonstrate the dependence of the normal HMEC on EGF-receptor signal transduction for growth, cells were exposed to a monoclonal antibody (MAb 225) to the EGF receptor [38]. Blockage of the EGF receptor by MAb 225 led to nearly complete inhibition of cell growth. Recent experiments [40] have shown that MAb 225 produces a rapid, efficient, and reversible G_o growth arrest of normal 184 HMECs. Protein synthesis remains depressed in the presence of the antibody. DNA synthesis decreases 12 hours after antibody addition, and is sharply decreased by 24 hours. Removal of MAb 225 and restimulation with EGF led to a rapid increase in protein synthesis. DNA synthesis increased only after 10 hours and peaked around 18 hours. A one-hour exposure to EGF after MAb 225 removal was sufficient to allow the majority of cells capable of cycling to subsequently enter S-phase. High levels of synthesis of mRNA for the early response genes c-myc, c-fos, and c-jun were observed within one hour of EGF restimulation. Expression of these genes was detectable in normal cycling HMECs, but was decreased while the cells were growth arrested. Synthesis of TGF-α mRNA, which was also inhibited in the presence of MAb 225, was detected by two hours after EGF restimulation. Some mRNA species, such as those for keratin 5 and NB-1, continued to be expressed during the growth arrest. These results indicate that blockage of EGF-receptor signal transduction is sufficient by itself to cause normal HMECs to enter a G_o-like resting state similar to the G_o state described in fibroblasts.

We next examined the immortally transformed HMEC lines for the effects of blockage of EGF-receptor signal transduction on cell cycle progression. Since 184A1 and 184B5 do not maintain growth in the absence of exogenous EGF, it was not necessary to expose the cells to MAb 225 in order to achieve growth arrest. No significant differences were observed in protein or DNA synthesis in 184B5 when cells were placed in medium minus EGF vs. medium minus EGF containing MAb 225 [40]. In most parameters examined, the two cell lines resembled the normal 184 HMEC. Cell line 184B5 had a slightly shorter cell cycle than the normal 184 or 184A1 cells.

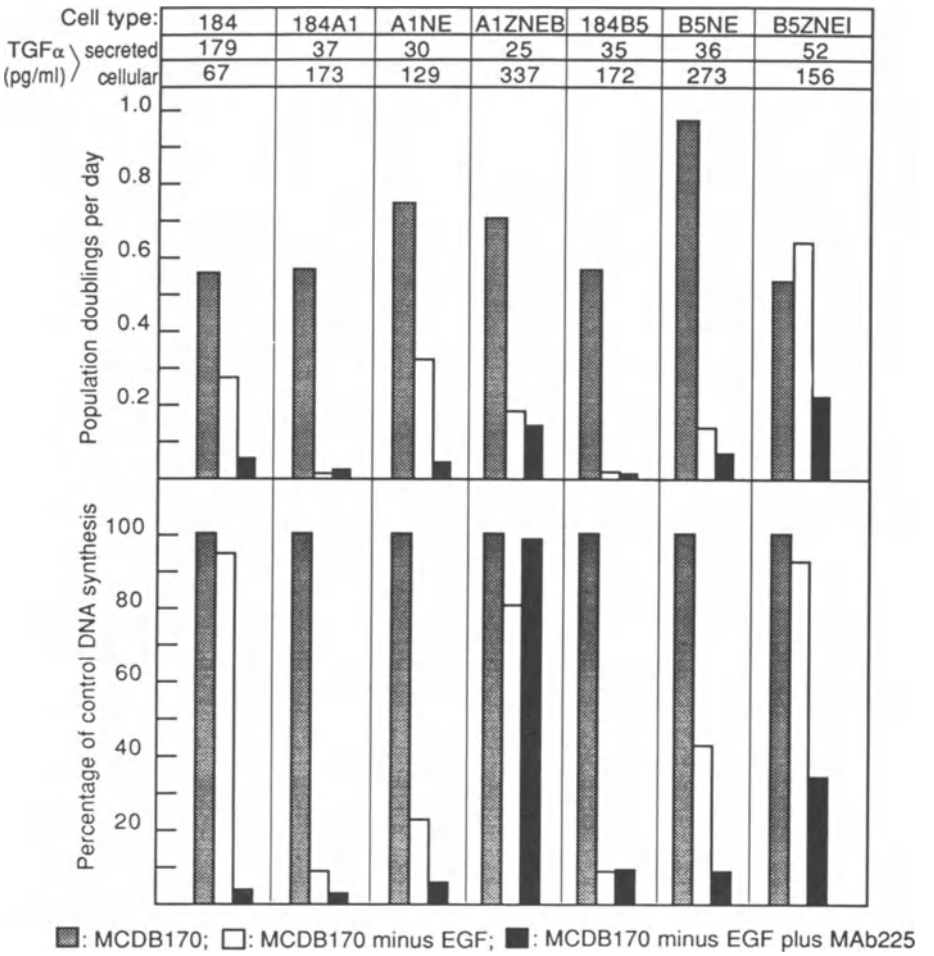

Figure 5. TGF-α production and secretion, and effects of EGF on growth and DNA synthesis of normal, immortally transformed, and EGF-independent variant HMEC. To determine the effect of EGF on growth rates, 0.5×10^5 cells were seeded into 35-mm dishes in either complete medium (MCDB170), medium without EGF, or medium without EGF containing 6 μg/ml of MAb 225. The number of attached cells was determined 16–24 hours later. When control cultures (complete MCDB170) were just confluent, all cell cultures were trypsinized and cell numbers determined by Coulter Counter. To determine the effect of EGF on DNA synthesis, midconfluent cultures that had been grown in complete medium were switched to the indicated medium for 24 hours. For the last 2 hours, cells were exposed to 4-μCi ^{3}H-thymidine in 1.5 ml. Acid-precipitable counts were determined by scintillation counting as described [36]. To determine TGF-α synthesis and secretion, cells were grown in 60-mm dishes until subconfluence. Then the 24-hour conditioned medium was removed, and the cells harvested and frozen. Radioimmunoassays of medium and cells were performed by Robert Coffey, Vanderbilt University, as described [41].

The main difference was the absence of reduced levels of c-myc and c-fos mRNA during growth arrest of 184B5, and to a lesser extent, 184A1.

These data indicate that a synchronous entry into the cell cycle can be easily obtained in these nonmalignant human epithelial cell populations. The method employed — reversal of blockage of EGF-receptor signal transduction — provides cell populations that have not been subjected to conditions that are cytotoxic or lead to metabolic imbalance. Achievement of synchronous, reversible growth arrest will allow examination of the synthesis and activity of specific mRNA and protein species as a function of the cell cycle, and of the effects of potential growth enhancers and inhibitors at different stages of the cycle. The information obtained on the cell cycle controls present in the normal HMEC can then be compared to that seen in cells whose growth control has been altered as a consequence of immortal and malignant transformation.

The ability to growth arrest 184A1 and 184B5 by removal of EGF, without requiring anti-EGF receptor antibodies, facilitates the use of these cell lines in studies of cell cycle control. However, this capacity was puzzling in light of the observed synthesis and regulation of TGF-α mRNA by the cell lines, similar to that seen in normal 184 HMECs. We therefore further investigated the synthesis and secretion of TGF-α protein by our HMEC cultures, as well as the requirement of these cells for EGF-receptor ligands for growth and DNA synthesis. Normal 184 HMECs, 184A1 and 184B5, and the EGF-independent variants of these cell lines were examined (figure 5). Sensitive radioimmunoassays for TGF-α [41] indicated that 184A1 and 184B5 synthesized amounts of TGF-α similar to normal 184 HMECs, but failed to secrete this protein. The ability of the variant lines to maintain growth in the absence of EGF did not appear to be due to increased TGF-α secretion. Although A1NE and B5NE could continue to grow in the absence of EGF, their rate of growth was decreased, and they were still sensitive to MAb 225-induced inhibition of growth and DNA synthesis. It is possible that increased synthesis of another ligand for the EGF receptor, such as amphiregulin, may be responsible for their altered growth properties. The ENU-induced variant A1ZNEB also grew faster in the presence of EGF, but it was no longer sensitive to MAb 225 inhibition. Additionally, it showed significantly increased levels of cell-associated TGF-α protein. One possible explanation for this phenotype is internal or membrane-associated stimulation of the EGF receptor. The ENU-induced variant B5ZNEI showed no difference in growth rate with or without EGF, but was still partially sensitive to MAb 225 inhibition.

Summary

The HMEC system we have developed provides large, standardized quantities of actively proliferating cells from individual specimen donors. The

properties of normal finite-lifespan HMECs can be compared to cells transformed in vitro to immortality or malignancy. These cells are particularly useful for certain kinds of studies of normal and transformed human breast cell biology, e.g., molecular and biochemical analyses requiring large, uniform, proliferating cell populations. However, the inability to sustain in vitro growth of all the cell types found in the breast in vivo limits their utility for studies of cellular differentiation or differentiation-dependent phenomena. Changes in specific cell culture parameters (e.g., medium composition, calcium concentration, addition of extracellular matrix material, use of three-dimensional culture supports) may be necessary to address questions relating to normal cellular maturation and differentiation, and how the state of differentiation affects the cells' capacity to acquire and maintain a transformed state. Since the immortal HMEC lines 184A1 and 184B5 still retain many normal characteristics, they can be useful for some studies of normal cell behavior where the capacity for indefinite lifespan is convenient. On the other hand, the fact that they have acquired some aberrant properties relative to normal HMECs, especially their indefinite lifespan, makes them useful substrates for determining the potential capacity of additional factors (e.g., chemical and physical carcinogens, oncogenic viruses, transfected genes) to induce malignant transformation.

We have utilized this culture system to ask questions concerning control of cell proliferation in normal HMECs. This information can then be used as a baseline for understanding the aberrations in growth control that occur as cells become transformed to immortality and malignancy. We have shown that normal HMEC proliferation is inhibited by TGF-β, whereas immortally and malignantly transformed cells may acquire resistance to TGF-β-induced growth inhibition. Nonetheless, these cell lines that are resistant to inhibition of growth can retain responsiveness to TGF-β induction of specific protein species. The immortal cell lines also retain most of the growth factor requirements of the normal HMECs, including a stringent requirement for EGF-receptor signal transduction in order for cell cycle progression to proceed through G_1. This requirement of the HMEC has enabled us to achieve an efficient G_o arrest that is easily reversible, leading to synchronous entry into the cell cycle. Such synchronized populations will allow us to study questions related to cell cycle progression, and hopefully to identify the key differences in control between normal and transformed HMECs.

In mammary tumor cells, the somewhat surprising observation is their nearly uniform expression of a phenotype that most closely resembles that of the normal mature luminal cell — the cell type that shows the least proliferative potential in culture. Clearly, the tumor cells have acquired a derangement in normal growth control that allows them to maintain active proliferation even though they display the "mature" phenotype. Possibly, cells in a particular state of maturation are more susceptible to carcinogenic transformation. On the other hand, it is possible that the transformed state is either incompatible with the basal cell phenotype, or requires some aspect

of the mature luminal cell phenotype, resulting in changes subsequent to transformation. We are particularly interested now to examine whether the presence or absence of the NB-1 protein that we have found associated with specific states of differentiation, and that is absent in most breast tumor cells, plays a causal role in affecting the mammary cell's capacity to transform or to express a transformed phenotype.

Acknowledgments

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II

Genetic Basis for Cancer Onset and Progression

3. Genetic analysis of breast and ovarian cancer in families

Mary-Claire King and Sarah Rowell

It has been observed for more than a century that breast cancer clusters in families [1]. It is now clear that between 5% and 10% of breast cancer [2,3] and of ovarian cancer [4] can be attributed to inheritance of a gene conferring high risk, followed by genetic changes specific to the target epithelial cells of the breast or ovary. Given its very high incidence, breast cancer may also appear more than once in a family purely by bad luck, not because of inherited susceptibility. Based on molecular genetic analysis, it is now possible to tell women in some very high-risk families whether they have inherited susceptibility to breast and ovarian cancer [5–7]. This information raises difficult questions for women and clinical providers concerning surveillance and treatment options.

A brief summary of the genetics of breast tumor development

The pathway from normal function of breast ductal epithelial cells to invasion and metastasis requires multiple genetic alterations, perhaps between three and six changes that are rate limiting in tumor progression. (Of course, other noncritical changes will accumulate in tumors as well.) Almost certainly, the critical combination of genetic alterations varies for different individual breast tumors, possibly even for different tumors in the same patient.

Inherited alterations

Figure 1 illustrates some of the genetic alterations that may be involved in breast tumor development. The genes shown at the left of the figure — p53, BRCA1, or ESR — represent loci that may be inherited in altered form, leading to greatly increased risk of breast cancer. The evidence for inherited genetic risk is best known for the p53 gene, which is responsible for the Li–Fraumeni syndrome [6]. This rare, familial cancer syndrome involves very high risk of brain tumors and adrenocorticocarcinoma in children and of breast cancer in young adult women who carry a mutant allele of p53.

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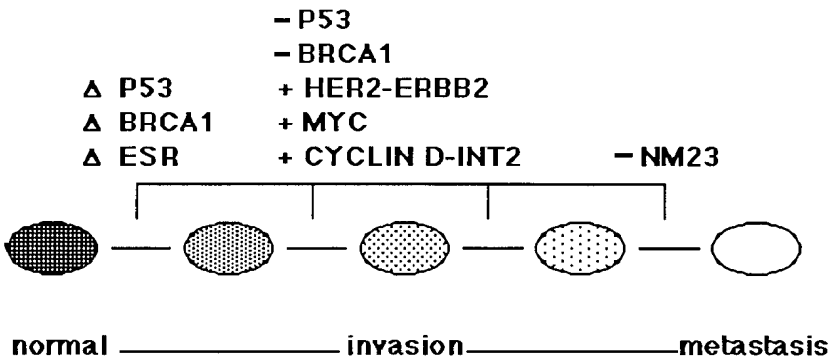

Figure 1. Some of the genetic changes that may be involved in the progression of a breast ductal epithelial cell from normal to malignant growth and metastasis. The hypothesis is that genetic alterations in p53, BRCA1, or the estrogen receptor (ESR) may be either inherited in families or acquired only in the target cells. Other alterations appear to be purely somatic.

Inherited mutations in p53 may account for about 1% of breast cancer among patients diagnosed before age 40 [8,9].

The BRCA1 (*br*east *ca*ncer) gene is still identified only by its chromosomal position; its sequence is not yet known. The role of BRCA1 in inherited breast and ovarian cancer has been determined by genetic analysis of families with multiple cases of these cancers [5,10,11], as described in the sections that follow. BRCA1 is being isolated by the approach of positional cloning [12]. The gene is known to lie within a region of a few million nucleotides on chromosome 17q21 [13], and at worst, could eventually be identified by scrutinizing every gene in that region. In all likelihood, it will not be necessary to screen every gene in the critical region, but to focus on genes that are both in the right place and have relevant functions, and on genes that are altered in breast and ovarian tumors. The chromosomal region including *normal* alleles of BRCA1 is frequently lost in breast and ovarian tumors [14]. These tumor deletions are somatic, not inherited, and may occur in tumors of patients with or without inherited breast cancer. This suggests that alterations in BRCA1 may lead to breast cancer either by inheritance or by somatic alteration [7].

The possibility of inherited alterations in the estrogen receptor gene ESR is very tentative. A few families with breast cancer linked to the estrogen receptor locus have been described [15]. However, no alterations in the estrogen receptor gene have yet been identified in these families. Breast cancer in these few families was generally diagnosed after menopause.

Somatic alterations

The genes shown in the center of figure 1 are the sites of genetic changes frequently observed in breast tumors [16]. Somatic alterations of p53 may

be either deletions or point mutations, which may lead to either loss of expression of the p53 protein or overexpression of abnormal p53 protein [17]. Amplifications of oncogenes HER2/erbB-2 and MYC, and of the cyclin D/int-2 region, have been observed in breast tumors at both early and late stages of progression [18]. Genetic amplification of these regions is frequently, although not always, associated with overexpression of the protein encoded by the gene [19,20]. Overexpression of HER2/erbB-2 and of cyclin D in breast tumors is associated with poor prognosis [18,21,22]. Finally, the gene NM23, on the right of figure 1, may encode a factor inhibiting metastasis of breast tumors, and loss of expression of this gene may be critical as a late step in tumor progression [23]. There are certainly other genes whose alterations are critical to progression of some breast tumors [24]. Not every breast tumor will undergo all these alterations, however. An as-yet-unanswered question is what combinations of these changes, and in what order, are rate limiting in tumor progression.

Why are breast cancer rates increasing?

In the past few years, the number of women developing breast cancer, particularly young women, has risen dramatically. Much of this increase is the result of demography and the fact that the risk of breast cancer increases with age. The number of women now entering the ages of breast cancer risk is very high, as the generation of women born after World War II passes age 40. The number of elderly women is also higher than ever before. However, it is also true that age-specific breast cancer incidence rates (i.e., risks of developing breast cancer) have increased 25% in the past 20 years [25], reaching one woman in ten by age 80 today. Meanwhile, mortality rates have not increased, because survival of breast cancer patients has improved [25].

Breast cancer risk in the twentieth century has probably increased as the result of several gradual changes in the lives of women with respect to established risk factors for breast cancer [26]. At the beginning of this century, the average age at menarche was 12 to 13 years and the average maternal age at first birth 21 to 22 years [27]. The interval of intense breast ductal cell growth and hormone production between menarche and first full-term pregnancy was thus about nine years. Among young women in the United States now, the average age at menarche is 11 to 12 years and childbearing frequently begins in the late 20s or 30s. The comparable interval of exposure of dividing breast ductal cells to hormonal stimulation is thus about 20 years, twice as long as a century ago. Epidemiologic studies of women exposed to radiation as teenagers indicate that the first mutations leading to breast cancer probably occur in this period [28,29]. An association between increased breast cancer risk and an extended interval of menstruation before pregnancy is biologically plausible, because all breast ductal

cells, whether normal or mutant, are dividing rapidly during this period, so abnormal cells would have every opportunity for clonal growth [26]. The biological basis of breast tumor development suggests that creating molecular tools for much earlier diagnosis and developing ways to reverse the first steps of tumor development may be the most effective means of breast cancer control. Social intervention will be necessary to ensure universal availability of diagnostic and preventive tools.

Who has inherited susceptibility to breast cancer?

Approximately 1 in 200 women — 600,000 women in the United States today–have inherited susceptibility to breast cancer [2,3]. These women often, but not always, come from families in which breast and/or ovarian cancer have occurred multiple times, particularly among younger women. In general, inherited breast cancer appears earlier than purely somatic breast cancer, because among women with inherited susceptibility, one of the cancer-causing mutations is present in all cells from birth, so fewer somatic mutations specific to breast ductal cells need occur [30]. Susceptibility can be inherited from either a mother or father, although fathers virtually never develop breast cancer. (Inherited male breast cancer exists, but is not associated with BRCA1 or p53 [5].) Susceptibility is dominantly inherited, with each child of a carrier having a 50% risk of inheriting the susceptibility allele.

When the BRCA1 gene is cloned and the sequence known, it will be possible at this locus to type women and determine who carries an allele that confers high risk. Meanwhile, a woman's inherited risk can only be determined if her family includes multiple women with breast or ovarian cancer. An asymptomatic woman's risk can be evaluated by genotyping the breast and ovarian cancer patients in her family, determining whether the disease is linked to BRCA1 in the family, and then determining whether the woman at risk shares the genetic markers associated with BRCA1 in the family.

Figure 2 illustrates a family in which breast and ovarian cancer are inherited. Susceptibility is linked to the BRCA1 gene on chromosome 17q21 in this family, as indicated by the coinheritance of these cancers with the same alleles (in boxes) of the three genetic markers THRA1, D17S855, and D17S579 [13]. These markers are from a larger set that maps to the chromosomal reigion of the BRCA1 gene [31]. BRCA1 is between THRA1 and D17S579, and within a few million nucleotides of D17S855 (figure 3). In families such as this one, with several affected relatives in three generations, these markers can be used to determine whether breast and ovarian cancer susceptibility is inherited with BRCA1. Furthermore, if in the family these markers and cancer are inherited as a set (i.e., without recombination),

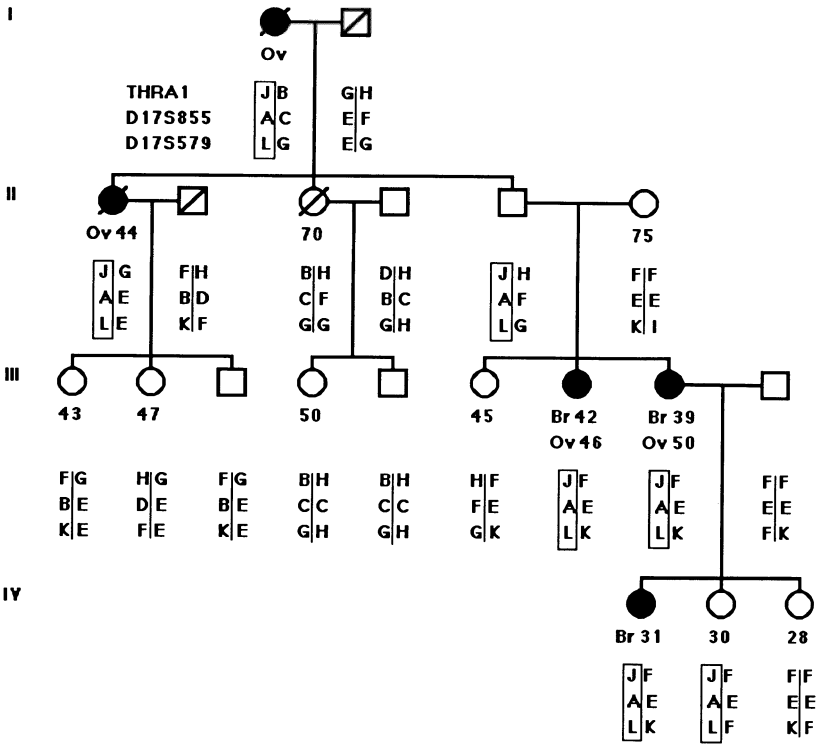

Figure 2. A family with inherited susceptibility to breast (Br) and ovarian (Ov) cancer. Filled circles represent women with these cancers; diagonal lines represent deceased individuals; ages shown for each woman are her age at diagnosis (if a patient), age at death (if deceased), or current age (if cancer free). Symbols in boxes are alleles of the genetic markers THRA1, D17S855, and D17S579 on chromosome 17q, which are coinherited with breast and ovarian cancer. Genetic typing in the extended family indicated that IV-2 inherited risk of breast and ovarian cancer and that IV-3 did not.

then it is also possible to determine whether as-yet-unaffected women have inherited susceptibility.

The cancer-free women in this family were concerned about their risk of breast and ovarian cancer. The three sisters IV-1, IV-2, and IV-3 met with us to learn the results of our genetic analysis. IV-3 has not inherited susceptibility, but IV-2 has. IV-2 is now considering her choices, which we discuss below. We were able to tell all four unaffected women in generation III that they had not inherited susceptibility. Therefore, neither they nor their daughters (who are not shown on this pedigree) are at increased risk.

Figure 4 illustrates another family with inherited breast and ovarian cancer [31]. In this family, breast cancer occurs both in women with inherited susceptibility and in two women (II-1 and III-5) who have *not* inherited

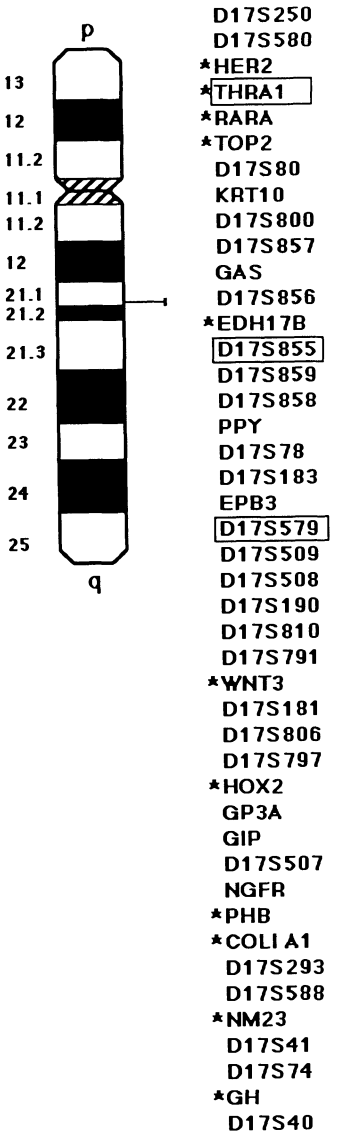

Figure 3. Genetic markers in the BRCA1 region of chromosome [31].

susceptibility to the disease. The lifetime risk of breast cancer of 10% applies to women without inherited susceptibility in these families, just as to women in the general population of the U.S. As one would expect, breast cancers in women without inherited susceptilibity were diagnosed at older ages (71 and 45 years) than inherited breast cancer in the family. Patient IV-2 in this family was diagnosed as the result of being identified as high risk.

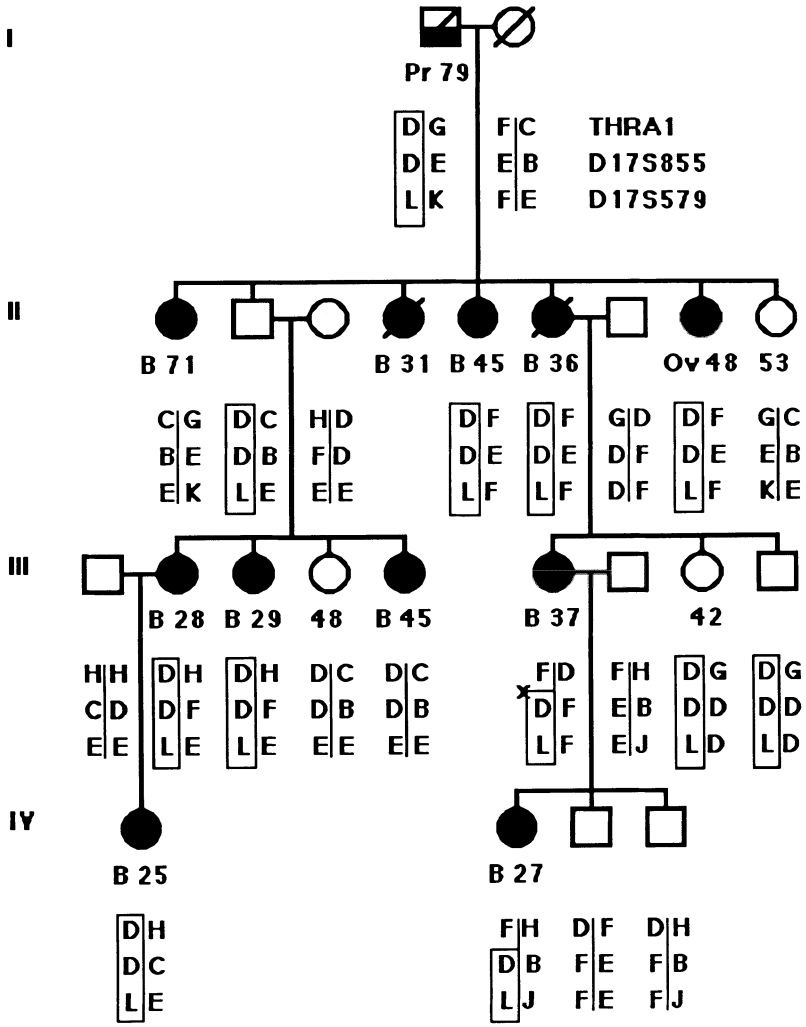

Figure 4. A family with inherited breast and ovarian cancer illustrating a recombination event that places BRCA1 below THRA1. In addition to the cancers in this family linked to inherited susceptibility, II-1 and III-5 developed breast cancer (at ages 45 and 71, respectively) for reasons apparently unrelated to inherited susceptibility. The breast cancer of IV-2 was diagnosed at age 27 by her first mammogram, which she undertook when her risk was identified. Symbols are the same as in figure 2.

When she was identified as inheriting susceptibility, IV-2 underwent a breast exam and a mammogram, despite her young age (27 years). The mammogram was positive, and subsequent biopsy revealed a poorly differentiated, infiltrating adenocarcinoma. No nodes were involved. IV-2 has undergone bilateral total mastectomy and is doing well. Her experience dramatically

illustrates the importance of surveillance beginning at young ages for women in these families.

In these families, women with inherited risk of breast and ovarian cancer could be identified because they had multiple affected relatives who could be typed. Genotypes of markers linked to BRCA1 were determined for breast and ovarian cancer patients and for unaffected women in each family. These linked markers identified the chromosome 17 homolog carrying the altered BRCA1 gene in each family. However, without an extended family, all of whom have been tested for chromosome 17 markers linked to BRCA1, it is not yet possible to distinguish women with the susceptibility gene from women without it. In order to identify women with inherited risk in the general population, we must know the sequence of BRCA1.

What risk is associated with inherited susceptibility?

Breast cancer

Risks of breast cancer by age to women inheriting BRCA1 are shown in table 1 for the more than 200 families studied so far [11]. These risks are extremely high, exceeding 50% before age 50 and reaching 80% by age 65. However, the families who have participated in these genetic studies have been selected because multiple relatives developed breast cancer. It may be that there exist other families with breast cancer linked to less severe mutations in BRCA1. Women carrying those BRCA1 mutations might be at increased, but not such extraordinarily high, risk. When the BRCA1 gene is cloned and its normal and variant sequences known, it will be straightforward to identify women carrying variant alleles and to determine the risk associated with each sequence. Meanwhile, we use the risks of table 1 to advise women in the severely affected families with whom we work.

Table 1. Cumulative risk of breast cancer to women in the United States generally [25] and to women with BRCA1 [11]

Age in years	General population	Inherited BRCA1
40	0.005	0.16
45	0.01	0.42
50	0.02	0.59
55	0.03	0.72
60	0.04	0.77
65	0.06	0.80
70	0.07	0.82
75	0.09	0.84
80	0.10	0.86

The risk of ovarian cancer to women in these families is certainly elevated, but how much is not certain. Epidemiologic analysis suggests that the risk of ovarian cancer to (all) sisters of patients with inherited breast cancer is about 5% by age 60 [32]. Therefore, among the 50% of sisters with the inherited susceptibility gene, the risk of ovarian cancer by age 60 is probably about 10%. A better estimate of ovarian cancer risk will be obtainable when the critical susceptibility genes are cloned.

It is completely unclear why some women in a family develop breast cancer while other women in the same family, who must have inherited the same susceptibility allele, develop ovarian cancer. The consequences of this uncertainty are illustrated by the family shown in figure 5. The proband in this family is III-2 (arrow in the figure). Her mother and two maternal aunts (II-1, II-2, and II-3) had developed breast cancer in their 30s, and her identical twin sister (III-1) had undergone bilateral mastectomy following diagnosis of a stage I breast tumor. Confronting an 86% lifetime risk of breast cancer, III-2 also decided on prophylactic mastectomy, although she was symptom free herself. The surgery was uneventful. Three months later, III-2 was diagnosed with stage III ovarian cancer. There was no history of ovarian cancer in the family and nothing in her history that would have presaged the disease.

When the variant sequences of BRCA1 are known, it will be possible to determine whether some alleles predispose only to breast cancer and others

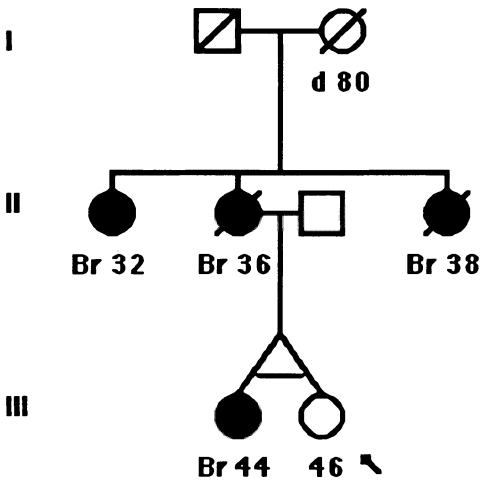

Figure 5. A family illustrating the risk of ovarian cancer in a family with inherited breast cancer. III-2 (arrow) underwent bilateral mastectomy at age 46 because of her high risk of breast cancer. She was subsequently diagnosed with ovarian cancer. Symbols are the same as in figure 2.

to both breast and ovarian cancer. However, knowing the sequence of BRCA1 will not explain why patient III-2 in this family developed ovarian cancer. III-2 carries the same BRCA1 sequence as the women in her family who developed breast cancer. Only III-2 developed ovarian cancer, for reasons that remain unknown.

What next?

At present, the choices available to women with inherited risk are surveillance (screening by mammography and physical exam) and surgery (prophylactic mastectomy, oophorectomy, and/or hysterectomy) [33]. At this time, participation in the Tamoxifen Prevention Trial is also a possibility for some high-risk women older than age 35. None of these options are proven preventive strategies. There is probably no one right answer for all women with inherited risk. Every woman at inherited risk should be provided with a thorough discussion of our current level of information and adequate counseling regarding her particular situation before she chooses to act. A woman's decision will be highly individual and may change over time. That is, a woman choosing surveillance without surgery at one time in her life retains the option of surgery at a later date. Her decision, with its enormous consequences, should never be rushed, and all providers need to respect both the process and her conclusion.

A registry of women at risk

Given that we are almost totally ignorant of the effectiveness of any preventive strategies for women at high risk, we believe it is essential to develop a registry of women who are identified with inherited susceptibility, to record their treatment choices, and to follow them for the indefinite future. Apart from a randomized trial of each intervention in this high-risk group, the next best way to determine the effectiveness of surveillance, surgery, chemoprevention, or combinations of these strategies is by long-term follow-up of the cohort of women with inherited risk. The registry would be used to assess which interventions have been most effective for which women.

The point of basic research

The goal in identifying genes responsible for breast cancer is to learn biological features of this disease that can be harnessed for the development of preventive strategies. Screening could be revolutionized by combining molecular diagnostic methods with mammography and physical examination. The approach could be to iedentify the product of a mutant gene on the surface of breast ductal cells, when the mutant clone had undergone only a

few cell divisions. The abnormal cells could be removed by needle biopsy, curing the patient. Ultimately, knowing the biology of the first steps of tumor development could enable these steps to be reversed, either by replacing the products of the genes, or replacing the aberrant genes themselves in the breast ductal cells. These molecular diagnosis and prevention strategies could be useful both for women with inherited risk and for women with breast cancer caused entirely by somatic mutations. The goal is that the next generation of women not face death from breast cancer.

Acknowledgments

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4. The p53 tumor-suppressor gene in human breast cancer

D. Craig Allred, Richard Elledge, Gary M. Clark, and Suzanne A.W. Fuqua

P53 is a 20-kilobase gene located on human chromosome 17p13 that encodes a 53-kilodalton (kDa) nuclear phosphoprotein (reviewed in [1–5]). Homologues of p53 are found in all animal species. The human gene has 11 exons, four of which (5 through 8) have been highly conserved during evolution, suggesting that they are critical for p53 function [6]. Rodents and humans, for example, show over 90% sequence homology in the conserved exons. The brief, intense, and still incomplete history of investigation into the function of the p53 gene and its protein product has evolved from initial thinking that it was a tumor antigen [7,8], to later speculation that it was a protooncogene [8–13], to the currently favored hypothesis that it is a tumor-suppressor gene involved in regulation (suppression) of the cell cycle.

Several converging lines of evidence now strongly suggest that p53 has tumor-suppressor activity, including 1) the realization that earlier transfection experiments initially interpreted as demonstrating transforming activity actually involved mutant forms of p53, suggesting that loss of function was oncogenic [14]; 2) the fact that transfection and overexpression of mutant p53 into cell lines with wild-type p53 are associated with transformation and/or increased cell proliferation [15,16]; 3) the finding that transfection of wild-type p53 suppresses oncogene-induced transformation, growth, and in vivo tumorigenic capacity of cell lines [15,17–21]; and 4) the observations that mutations, deletions, and losses of heterozygosity involving p53 are very common in diverse types of malignant human neoplasms [1,22–32]. Current data indicate that 50% or more of cancers involving colon, lung, breast, and several other tissues contain mutations of the p53 gene [23,30,33–35].

Model of p53 function

Although our understanding of the molecular mechanisms of p53 tumor suppression and cell cycle inhibition is incomplete, a model outlining important aspects of p53 function is emerging (figure 1). It is known, for example, that wild-type p53 forms oligomers of uncertain size that bind, and

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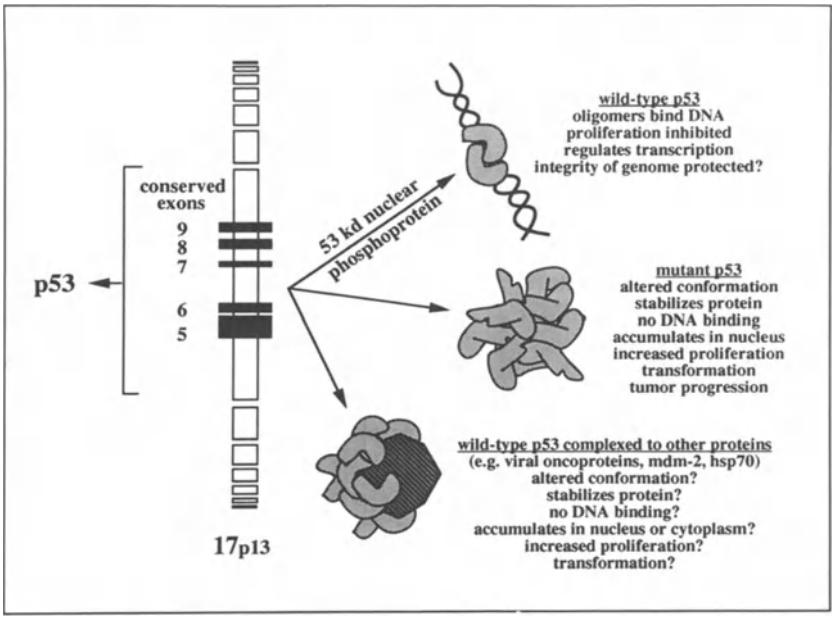

Figure 1. Model of p53 function. Refer to text (section 1.1) for discussion.

presumably regulate, other genes. A potent DNA binding domain has been localized to the 47 carboxy-terminal amino acids of the p53 protein [36,37]. Wild-type p53 has been shown to repress the transcription of several cellular and viral promoters [38,39]. Growth suppression of transformed cell lines mediated by wild-type p53 transfections is accompanied by decreased expression of late G1 genes that are essential for cell division, including proliferating cell nuclear antigen and DNA polymerase alpha [40,41]. These data suggest that p53 may indirectly suppress cell proliferation by binding and inhibiting the transcription of genes that promote proliferation. An alternative possibility is that p53 binds and activates the transcription of genes that suppress proliferation, although data supporting this idea in human tumors are lacking. Whatever the mechanism, cell cycle inhibition is certainly consistent with tumor-suppressor function.

Observations that wild-type p53 may be transiently overexpressed in response to various types of environmental nuclear damage (e.g., ultraviolet radiation) have led to the speculation that p53 may also be acting as a molecular 'guardian' of the cell, allowing time for DNA repair to occur in nondividing cells and, thus, helping to maintain the integrity of the genome [3].

P53 mutations are one of the most common (perhaps the most common) genetic defects in human cancer, and sequence analysis of over 300 tumors of various types studied so far show that most are missense/point mutations

in conserved exons 5 through 8 [1,42]. Transcriptional splicing errors and deletions also occur, but appear to be much less common. The majority of p53 mutations result in a conformationally altered protein with a significantly prolonged half-life that is unable to bind DNA or to suppress cell proliferation [5,36,43–45]. Large amounts of nonfunctional protein accumulate in the nuclei of transformed cell lines and tumors with p53 mutations [43,44].

Whether a mutant p53 gene alone is sufficient to transform cells is controversial, but there is no doubt that it can at least cooperate with certain oncogenes (e.g., v-ras) to induce transformation [14,34]. Several viral proteins, such as the SV40 T antigen, adenovirus E1b, and human papilloma virus E6, are known to bind the wild-type p53 protein, prolonging its otherwise short half-life, inhibiting its function much the same as a mutation, and perhaps enabling the oncogenic properties of these viruses to be manifested [2,8,46]. Wild-type p53 also binds several cellular proteins, including the mdm-2 oncoprotein [47] and the heat shock cognate protein 70 [48], and the formation of these complexes may stabilize p53 and inhibit its functions. Studies showing increasing rates of p53 mutations with increasing tumor grade and/or stage suggest that p53 mutations, in addition to their role in transformation, may also be involved in tumor progression [49–52].

With this general background in mind, the remainder of this chapter will review the implications of p53 mutations in clinical breast cancer.

p53 in human breast cancer

Incidence of mutations

The sensitivity of detecting p53 mutations varies a great deal with the method being used, and there have been many, including direct sequencing of genomic DNA or cDNA (usually PCR amplified and restricted to conserved exons 5 through 8), single-strand conformation polymorphism (SSCP) analysis of DNA (again, usually PCR amplified and confined to the conserved exons), and measuring the nuclear accumulation or overexpression of stabilized but nonfunctional mutant protein by assays such as immunohistochemistry (IHC).

The composition of the specimen being analyzed for p53 mutations also influences the quality of results. For example, direct sequencing methods theoretically provide the most accurate information, but only if the sample is composed primarily of tumor cells. A mutation rate of 50% was observed in a study of 20 breast cancer cell lines by direct sequencing of genomic DNA [53]. An innovative study of 11 clinical breast cancers using very small samples of 'pure' tumor cells manually dissected from cytological touch preparations observed a mutation rate of 36% by sequencing PCR-amplified genomic DNA [54]. A mutation rate of 33% was observed in a similar

sequencing study of 44 clinical breast cancers sampled by fine-needle aspiration, which often contain a relatively large proportion of tumor cells [55]. In contrast, a sequencing study of 26 invasive breast cancers obtained by standard open surgical procedures showed only a 12% mutation rate [56], emphasizing the nearly insurmountable limitations of sensitivity imposed by nontumor cell contamination when attempting to sequence solid tumors composed of variable mixtures of normal and malignant cells.

By far, most studies of p53 in clinical breast cancer have used SSCP or IHC to detect mutations. SSCP is an electrophoretic method that indicates the presence of a mutation by a gel band shift (i.e., conformational alteration) of tumor relative to wild-type p53 DNA (figure 2). Unlike direct sequencing, SSCP does not identify the exact type or location of the mutation, but nonetheless has become a popular screening method because of its relative ease, economy, and ability to detect mutations in surgical specimens with tumor cellularity as low as 20%. Ease and economy have also made IHC a popular screening method for detecting p53 mutations. Positive nuclear immunostaining (figure 3) is interpreted as the accumulation of conformationally altered and stabilized protein that is thought to result from most p53 mutations. Because tumors are evaluated microscopically, the ability to detect mutations by IHC is not significantly influenced by the proportion of tumor cells in the specimen. A large number of antibodies specific for various domains of the p53 protein are available that perform

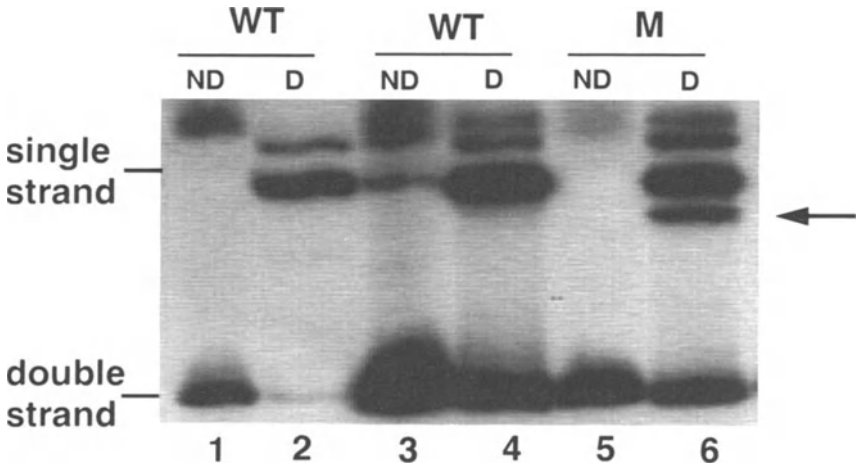

Figure 2. Example of single-strand conformation polymorphism (SSCP) analysis of PCR-amplified tumor DNA for conserved exons 8 and 9 run under nondenaturing (ND) and denaturing (D) conditions. Lanes 1 to 4 show wild-type (WT) DNA migration patterns of two breast cancers. Lanes 5 and 6 are from a breast cancer with a p53 mutation (M) that is manifested as an aberrant migration pattern (i.e., an extra band) seen under denaturing conditions for single-strand DNA (arrow).

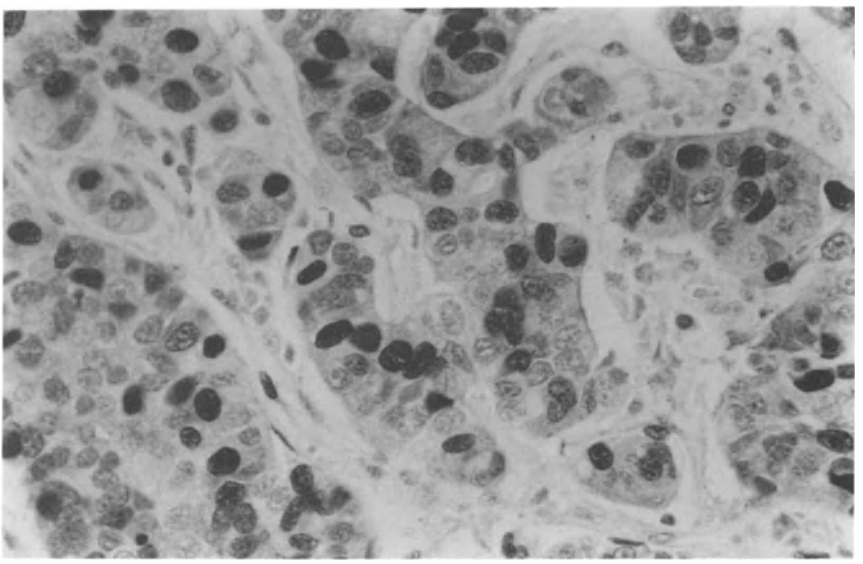

Figure 3. Example of frozen-section immunohistochemistry of a breast cancer with a p53 mutation. The strong dark nuclear immunostaining in tumor cells indicates the presence of accumulated nonfunctional mutant protein.

Table 1. Incidence of p53 mutations measured by SSCP[a] or IHC[b] in studies of clinical invasive breast cancers

Reference no.	Assay	No. tumors	Mutation rate
53, 56, 68, 76, 77	SSCP	494	24%[c]/(14%–42%)[d]
22, 30, 32, 60, 64, 65, 72, 74	FS-IHC	1456	47%/(27%–58%)
50, 57, 66, 67, 72, 73, 75, 78, 79	PS-IHC	1339	27%/(15%–50%)

[a] SSCP = single-strand conformation polymorphism.
[b] IHC = immunohistochemistry. The table lists both frozen-section (FS-IHC) and permanent-section (PS-IHC) results. Positive nuclear immunostaining was interpreted as evidence of a mutation.
[c] Average mutation rate of studies.
[d] Range of mutation rates in studies.

well in frozen-section and, more recently, permanent-section immunostaining [45,57–59].

Table 1 summarizes results from a recent personal review of the literature of studies measuring p53 mutations in clinical invasive breast cancers using SSCP or IHC. It appears, on average, that frozen-section IHC is more sensitive than either permanent-section IHC or SSCP. Studies using IHC have employed a variety of antibodies, immunostaining techniques, and methods of specimen preparation, leading to a wide range of results. Simi-

larly, differences in primers, specimen preparation, and various other technical factors have lead to a wide range of SSCP results. However, assuming that false-positive results by any of these tests are relatively rare, then at least a few studies using each method have measured mutation rates in the range of 50%, which agrees with direct DNA sequencing of breast cancer cell lines [53], and is generally considered to be a close approximation of the overall p53 mutation rate in clinical invasive breast cancer by most investigators.

There is, however, controversy regarding the specificity of IHC in detecting p53 mutations based on studies showing discordant results when the same tumors were evaluated by both SSCP and IHC [60,61]. We have studied this issue by evaluating 176 node-negative breast cancers by SSCP (for exons 5 through 9) and frozen-section IHC (using a cocktail of antibodies PAb1801 and PAb240) [62]. The various combinations of SSCP and IHC results were then compared to biological and clinical tumor behavior (i.e., p53 protein expression, tumor proliferation rate, and five-year disease-free survival), enabling us to test the hypothesis that p53 mutations will be associated with unfavorable behavior, regardless of how they are measured. As shown in table 2, tumors negative or positive for mutations by both assays were associated with highly favorable (i.e., wild-type) or unfavorable (i.e., mutant) characteristics, respectively. Discordant tumors were common (63/176), and most (93%) were IHC-positive/SSCP-negative. Although not conclusive, the intermediate levels of p53 expression, tumor proliferation

Table 2. Biological and clinical associations of combined IHC and SSCP phenotypes in a study of 176 node-negative breast cancers

IHC/SSCP[a]	No. tumors	IHC score[b]	S-phase[c]	DFS (5 yrs)[d]
negative[e]/negative[f]	88	0.0	6.6%	85%
positive[g]/negative	63	4.0	9.6%	78%
negative/positive[h]	5	nd[i]	nd	nd
positive/positive	20	6.0	15.4%	54%

[a] IHC/SSCP = immunohistochemistry/single strand conformation polymorphism. The four possible combined IHC and SSCP tumor phenotypes are listed, and their associated biological and clinical characteristics are shown in the following columns.

[b] IHC Score = median IHC score representing the proportion and intensity of positive immunostaining tumor cells (range 0–8; $p < 0.03$ for all pairwise comparisons in the column).

[c] %S-phase = median % S-phase determined by flow cytometry (only 6.6% vs. 15.4% and 9.6% vs. 15.4% were significant ($p = 0.0008$ and $p = 0.03$, respectively)).

[d] DFS (5 yrs) = percent disease-free survival at 5 years (only 85% vs. 54% was significant ($p = 0.003$)).

[e] negative ⇒ negative for mutation (i.e., IHC score = 0).

[f] negative ⇒ negative for mutation (i.e., wild-type gel migration pattern).

[g] positive ⇒ positive for mutation (i.e., IHC score > 0).

[h] positive ⇒ positive for mutation (i.e., aberrant gel migration pattern relative to wild-type).

[i] nd = not done (because of such small numbers).

rate, and clinical outcome associated with the IHC-positive/SSCP-negative tumors suggests that IHC measures accumulation of mutant or otherwise nonfunctional and stabilized p53 protein.

Mutations and tumor proliferation rate

There are convincing data from in vitro transfection experiments showing that wild-type p53 suppresses [15,19,20,63] and mutant p53 enhances [15,16] cellular proliferation. However, the few studies that have examined the relationship between p53 mutations and tumor proliferation rate in vivo, in clinical breast cancers, have yielded conflicting results [30,62,64–67] (table 3).

We have studied this issue in node-negative breast cancer by measuring and comparing accumulation of mutant p53 protein by frozen-section IHC and tumor proliferation rate by flow cytometry in the same large series of tumors [62] (figure 4). Fifty-two percent of tumors showed positive nuclear immunostaining (IHC score > 0). Negative tumors (IHC score = 0) had significantly lower proliferation rates than positive tumors (4.1% vs. 7.1% median %S-phase, respectively ($p = 0.0001$)), and there was a strong direct correlation between the amount of mutant protein present and tumor proliferation rate. Tumors with the highest amounts of nuclear p53 present

Table 3. Summary of studies (chronological) evaluating the relationship between p53 mutations and tumor proliferation rate in clinical invasive breast cancers

Ref. no.	No. tumors	p53 assay[a]	Mutation rate[b]	Prolif. assay	Assoc. between mut. and prolif.
30	88	FS-IHC	46%	Ki67 IHC[c]	mut. ⇒ high prolif. ($p = 0.001$)
30	200	FS-IHC	16%	Ki67 IHC	mut. ⇒ high prolif. ($p = 0.01$)
64	70	FS-IHC	53%	Ki67 IHC	ns[d]
65	184	FS-IHC	27%	%S-phase	ns
66	289	PS-IHC	28%	MI[e]	mut. ⇒ high prolif. ($p = 0.0001$)
66	289	PS-IHC	28%	%S-phase	mut. ⇒ high prolif. ($p < 0.0001$)
67	106	PS-IHC	26%	Ki67-IHC	mut. ⇒ high prolif. ($p = 0.001$)
67	106	PS-IHC	26%	PCNA-IHC[f]	ns
62	316	FS-IHC	52%	%S-phase	mut. ⇒ high prolif. ($p < 0.0001$)

[a] p53 Assay. All studies used either frozen-section immunohistochemistry (FS-IHC) or permanent-section immunohistochemistry (PS-IHC), and positive nuclear immunostaining was interpreted as evidence of a mutation.

[b] Mutation rate. The various studies used many different antibodies and immunostaining techniques, resulting in diverse sensitivities for detecting the accumulation of mutant p53 protein.

[c] Ki67 IHC ⇒ frozen-section immunohistochemistry using antibodies against the proliferation-associated antigen Ki67.

[d] ns = not significant.

[e] MI = mitotic index (semiquantitative microscopic estimate of mitotic count).

[f] PCNA-IHC = proliferating cell nuclear antigen immunohistochemistry.

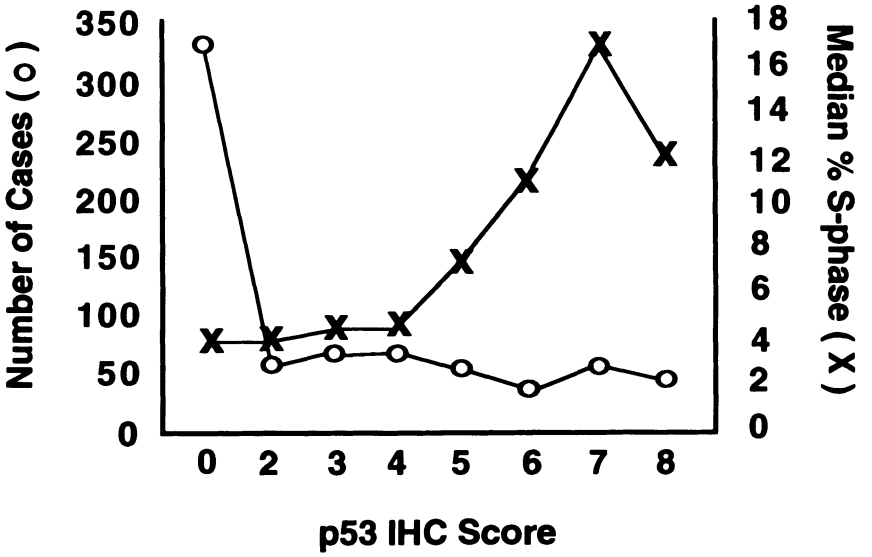

Figure 4. A double-plot showing the accumulation of mutant p53 protein (IHC score) relative to number of tumors (left axis), and tumor proliferation rate (median % S-phase (right axis)) relative to accumulation of mutant p53 protein. Fifty-two percent of tumors had IHC scores >0 (consistent with accumulation of mutant protein). There was an obvious direct correlation between the amount of mutant p53 present and proliferation rate.

showed 18% median S-phase values. These results are consistent with the hypothesis that wild-type p53 is involved in suppression of the cell cycle.

Preliminary data by Merlo et al. [68] suggest that the protein domain encoded by conserved exons 5–6 may be primarily responsible for the cell cycle regulatory functions of p53.

Mutations and tumor evolution

One of the least understood issues in breast cancer involves the early evolution of the disease. Our current knowledge relies almost entirely on a few insightful epidemiological studies showing an increased risk of later developing cancer in breasts diagnosed with certain morphologically defined hyperplastic and dysplastic lesions [69–71].

There is a growing momentum to identify biological, rather than morphological, markers associated with potential precursor lesions in breast cancer, and a few studies have evaluated p53 in this context. Using IHC to detect p53 abnormalities, Bartek [72] found a 0% rate of p53 accumulation in a series of 66 typical and atypical ductal hyperplasias, 22% in 9 pure in situ carcinomas, and 54% in 170 invasive carcinomas. A similar study by Davidoff [50] found 13% overexpression in 15 pure in situ carcinomas, 17% in 35 lesions composed of combined in situ and invasive disease, and 50% in

advanced node-positive cancers. Walker [64] and Iwaya [73] also found an association between increased rates of p53 mutations and advanced clinical stage in small series of breast cancers. These studies suggest that p53 plays a relatively small role in early breast cancer development, but appears to be more important later during tumor progression.

Mutations and prognosis

Evidence suggesting that p53 mutations may be important in tumor progression has stimulated interest in studying their prognostic implications. To obtain an indirect indication of potential prognostic significance, several studies have evaluated the associations between p53 and other factors known to influence the outcome of patients with breast cancer. In general, these studies have found strong correlations between p53 mutations and the poor-risk phenotypes of several factors including large tumor size, negative hormone receptors, high histological/nuclear grade, high tumor proliferation

Table 4. Summary of studies (chronological) evaluating the relationships between p53 mutations and clinical outcome in patients with invasive breast cancer

Ref. no.	No. patients	p53 assay (mutation rate)	DFS and/or OS[a]
73	31	PS-IHC[b] (23%)	decreased[c]
75	295	PS-IHC (23%)	decreased[d]
66	289	PS-IHC (28%)	decreased[e]
77	200	SSCP[f] (14%)	decreased[g]
62	700	FS-IHC[h] (52%)	decreased[i]

[a] DFS and/or OS = disease-free survival and/or overall survival. This column shows the relative clinical outcome of patients with tumors containing mutant p53 compared to wild-type p53.
[b] PS-IHC = permanent-section immunohistochemistry. Positive immunostaining was interpreted as evidence of a p53 mutation.
[c] decreased ⇒ positive p53 immunostaining associated with decreased OS (univariate $p < 0.01$) in this series of mixed clinical-stage patients.
[d] decreased ⇒ positive p53 immunostaining associated with decreased DFS and OS in all study patients (univariate $p = 0.003$ and $p = 0.0008$, respectively), in the subset of node-negative patients (univariate $p = 0.02$ and $p = 0.008$, respectively), and OS in the subset of node-positive patients (univariate $p = 0.0005$). Accumulation of mutant p53 retained prognostic significance in a multivariate analysis.
[e] decreased ⇒ positive p53 immunostaining associated with decreased OS (univariate $p < 0.0001$) in this series of mixed clinical-stage patients. In a multivariate analysis including %S-phase, p53 was not independently significant.
[f] SSCP = single-strand conformation polymorphism.
[g] decreased ⇒ p53 mutations were associated with reduced DFS (univariate $p = 0.01$) in this series of node-negative patients. p53 remained an independent predictor of reduced DFS in a multivariate analysis ($p = 0.02$; relative risk = 2.1).
[h] FS-IHC = frozen-section immunohistochemistry.
[i] decreased ⇒ p53 mutations were associated with reduced DFS and OS (univariate $p < 0.05$ for both) in this series of node-negative patients. p53 was the most powerful independent predictor of reduced DFS in a multivariate analysis ($p = 0.0008$; relative risk = 2.5).

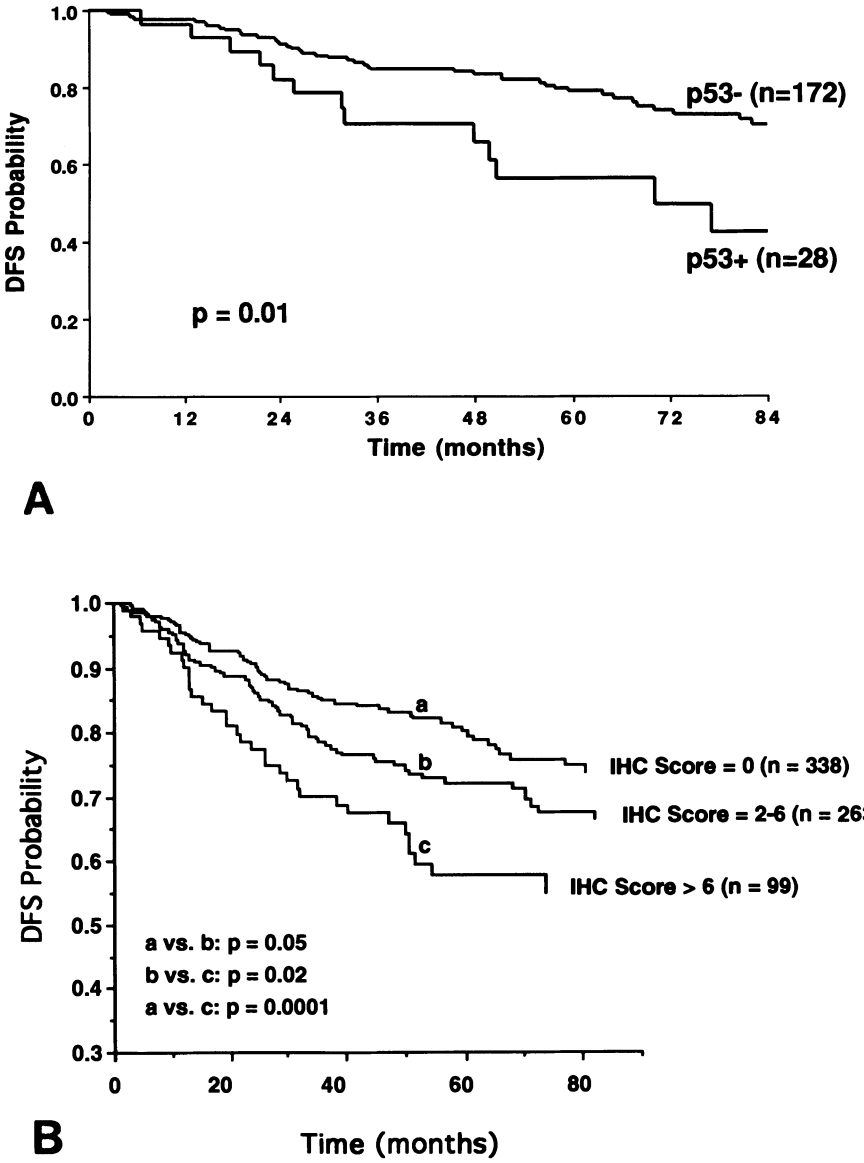

Figure 5. (A) Disease-free survival (DFS) curves for 176 node-negative breast cancers evaluated for p53 mutations in exons 5 through 9 by SSCP; (B) DFS curves for 700 node-negative breast cancers evaluated for accumulation of mutant p53 protein by frozen-section IHC. A univariate cutpoint analysis showed three ranges of scores with significantly different outcomes.

rate, aneuploidy, overexpression of epidermal growth factor receptor, and overexpression of the c-erbB-2 oncogene [30,60,62,64–66,73–76].

Only a few studies have directly examined the relationship between p53 and prognosis in clinical breast cancer, but all have shown that mutations are associated with significantly reduced disease-free and/or overall survival [62,66,73,75,77] (table 4).

Two studies from our laboratory evaluating p53 by SSCP [77] (figure 5A) and frozen-section IHC [62] (figure 5B) showed strong correlations between p53 mutations and reduced disease-free survival in patients with node-negative breast cancer. In a multivariate analysis of the frozen-section IHC study, both accumulation of mutant p53 and high %S-phase were independently associated with reduced DFS ($p = 0.0008$/relative-risk = 2.5 and $p = 0.01$/relative-risk = 1.8, respectively), suggesting that p53 may have other important biological functions in addition to cell cycle regulation.

Conclusions and future directions

P53 is a tumor-suppressor gene involved in regulation (suppression) of the cell cycle. Mutations of p53 appear to be common (approximately 50% incidence) in clinical invasive breast cancer. These mutations are associated with high tumor proliferation rates and independently predict for poor clinical outcome. More extensive and accurate sequencing of p53 mutations in clinical breast cancers may allow us to associate specific mutations with specific carcinogens, histological tumor subtypes, clinical outcomes, and responses to therapy. In addition, identification of the genes transcriptionally regulated by p53, and the specific cellular proteins interacting with p53, may provide new insights into normal p53 function and the involvement of p53 in carcinogenesis and, hopefully, may suggest new and more effective strategies for breast cancer prevention and therapy.

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5. Tumor-suppressor genes in breast cancer progression

Helene S. Smith

Elegant studies have characterized the stepwise accumulation of genetic lesions responsible for colon cancer (for a review, see [1]). In the case of breast cancer, modifications of this model are necessary to account for differences in malignant progression between colon and breast cancers. Unlike colon cancer, there is much evidence suggesting that breast cancer is many different diseases. Supporting this hypothesis are the observations that molecular lesions causing aberrations of gene expression for oncogenes, tumor-suppressor genes, growth factors, proteases, angiogenesis factors, and stromal components all have been seen in a varying, and sometimes small, proportion of breast cancers.

In contrast to colon cancer, where the premalignant stages are represented by increasingly atypical polyps, malignant progression in breast cancer is poorly understood. It is generally agreed that ductal carcinoma in situ usually becomes invasive with time; however, it is not certain that all breast cancers proceed through a clearly delineated in situ phase [2–6]. Furthermore, it is not clear which, if any, nonmalignant lesions found in breast tissue other than ductal carcinoma in situ are precursors of either invasive or in situ carcinoma. It is thought that the stimuli promoting the development of clinically evident breast carcinoma have a field effect on the surrounding breast tissue. This field effect confounds studies to define specific premalignant lesions temporally involved in progression to invasive cancer. Breast lesions that themselves may progress to malignancy if left in the breast ('premalignant') must be distinguished from lesions that do not themselves progress to malignancy but result from the same stimuli that also cause malignancies.

Breast cancers are also unusual in that tumors of similar stage and histology can have very different clinical outcomes. At one extreme are the patients who succumb with metastatic disease within a year of diagnosis, while at the other extreme are patients with cancer that appears similar but who can have 20 years of disease-free survival. It is likely that this biologic heterogeneity among breast cancers at least partially results from the combination of genetic lesions present in a given tumor. Figure 1 schematically illustrates a model of breast cancer progression that accounts for this extreme

R. Dickson and M. Lippman (eds.), Mammary Tumorigenesis and Malignant Progression.

Increasing Aggressiveness	Phenotypic Change Characteristic of Malignancy: Dysregulated Proliferation	Invasion of Basement Membrane	Migration	Angiogenesis
↓	1	1	1	1
↓	2	2	2	2
↓	3	3	3	3
↓	4	4	4	4

Figure 1. Stochastic model of breast cancer progression. Each number represents a gene(s) whose aberrant expression result in acquisition of the indicated malignant phenotype. A cell progressing to malignancy stochastically acquires these lesions; invasive carcinoma occurs with the acquisition of at least one aberration in each phenotypic change required for malignancy. Depending on which gene becomes abnormal, the resulting tumor can be more or less successful in accomplishing its indicated phenotype. Carcinomas can acquire additional lesions, resulting in increasingly aggressive behavior.

heterogeneity [7]. It is assumed that each step required for malignant growth (i.e., dysregulated proliferation, degradation of basement membrane, invasion, angiogensis, etc.) can be accomplished by more than one genetic lesion, with some of the lesions causing a more aggressive phenotype than others. Thus, a cell progressing to malignancy stochastically acquires at least one aberrant gene for each phenotype required for malignancy. If a breast cancer cell is incapable of completing any crucial step, it will not successfully metastasize. For example, mammary cells capable of extensive and dysregulated cell proliferation still need other changes to become malignant. Likewise, a cell that has gained the capacity for degrading basement membrane will not be detected as cancer if it cannot continue proliferating. The biologic heterogeneity of breast cancers may arise from the many possible molecular changes accomplishing a given step with variable efficiency. Biologic heterogeneity may also result from various possible orders of acquiring the sum of steps necessary for malignant growth. Even after the invasive cancer phenotype has been acquired, the process may continue, with additional lesions resulting in increasingly aggressive behavior.

Within this complex and heterogeneous biologic framework, many studies have been undertaken to understand the molecular basis of breast cancer progression. Four different approaches implicate various recessive oncogenes in this process (for reviews, see [8–16]). First, breast cancers have been

characterized for loss of heterozygosity at various chromosomal loci. The assumption in this approach is that mutations in the remaining allele of a putative recessive oncogene impart a growth advantage to the tumor cells with such losses. A second approach has been to characterize breast cancers for loss or mutation of suppressor genes known to be relevant in other systems (i.e., the p53 and retinoblastoma (rb) genes). A third type of study uses various methods to screen cDNAs for genes expressed in normal cells but not in tumor cells. Finally, genetic linkage to familial breast cancer is used to identify recessive genes involved in sporadic breast cancer. Presumable, breast cancer risk is elevated in cancer-prone families because one allele of a recessive oncogene is mutated in all the somatic cells of affected family members. Only one additional event (i.e., mutation or deletion) is necessary to completely inactivate the gene, while two events in the same gene are necessary for sporadic breast cancer [17].

Loss of heterozygosity

Loss of restriction fragment polymorphism is one of the most frequent genetic aberrations reported in breast cancers (for reviews, see [18–20]). Classically, it has been measured by comparing tumor DNA with normal DNA from the same patient cleaved with specific restriction endonucleases and run on Southern blots. In cases where the normal DNA is polymorphic, diminution of one of the polymorphic bands is called loss of heterozygosity (LOH). Usually the loss of one allele is incomplete because of contaminating normal cells in the tumor tissue. LOH is commonly interpreted to be the result of either a physical loss of one allele or a recombinational event that results in two copies of one allele. The length of the homozygous region varies among individual tumors, but is usually a substantial portion of the chromosome arm in question. Presumably, the remaining allele harbors an inactivated gene (by mutation, small deletion, hypermethylation, etc.) within the region that is deleted in common among all affected tumors.

Interpretation of LOH studies is confounded by the fact that breast cancers are heterogeneous for the number of copies of each chromosome [21]. Increased copy number of one allele can result in its appearing diminished relative to the other allele and therefore in its being misinterpreted as LOH [18]. For this reason, a more rigorous term, *numerical imbalance* (for example, see [22]), has been used to describe the phenomenon, because it does not have mechanistic implications. Also, it will be important to distinguish which areas of numerical imbalance represent true losses of heterozygosity and are therefore locations for putative suppressor genes.

LOH or numerical imbalance has been described in a varying proportion of breast cancers at chromosomal regions 1p, 1q, 3p, 6q, 7p, 11p, 13q, 16q,

17p, 17q, 18p, 18q, and 22q [22–46]. The incidence of loss varies for the different markers, with the more frequently lost regions being 3p, 6q, 7p, 16q, 17p (40%–60%) and the less frequently lost regions being 1p, 1q, 11p, 13q, 18p, 18q, 22q (15%–20%). The baseline level of LOH for any randomly selected probe is approximately 5% [44]. Among the different studies, there is some variability in incidences at the same locus. For example, one study [25] found LOH at 11p to be approximately 20% of cases, while a second study found it to be not significantly different than background level of 5% [44]. These discrepancies probably represent selection biases of the different tumor banks. The selection biases may be trivial; for example, some banks may contain a larger proportion of higher-stage tumors. Alternately, they may reflect unique etiologies among different populations. For example, Sato et al. [46] reported a high incidence of LOH at chromosome 3p14 with few, if any losses at 3p24–26 in breast cancers from Japanese women. In contrast, among breast cancers from French and American patients, there are higher incidences of loss at 3p24–26 and fewer at 3p14 [39,46,47].

Fluorescence in situ hybridization (FISH) on interphase nuclei has recently been used to further characterize the chromosomal regions with frequent LOH in breast cancer. Using two-color fluorescence with labeled centromeric and cosmid probes, a comparison is made between the number of copies of a given centromere and a specific gene on that chromosome. For chromosome 17p, the LOHs represent an actual physical deletion of portions of the p arm [48], while LOHs on 13q are probably the result of recombinational events, since physical deletions are not found by FISH [49]. In neither of these cases is there evidence that increased copy number rather than allelic loss is responsible for the numerical imbalance.

In specific subsets of tumors, there seems to be coordinate occurrence of LOH at various chromosomal loci, suggesting an etiologic association. For example, LOH on chromosomes 11p, 17p, and 18q frequently occurs in the same tumor [20,32]. In all of these studies, the statistical significance of the observation is limited by the large number of correlations made. The studies are also limited because of small sample size, since it is difficult to find the same tumor polymorphic for all the loci of interest. This inherent limitation of LOH, namely, its requirement for informative (i.e., polymorphic) cases, can be circumvented by FISH, which does not require normal tissue for comparison and is informative in all cases where LOH is caused by a physical deletion.

For three of the regions of deletion, namely, 3p [39,46,47], 13q [41], and 17p [31,32,34], more than one common region of deletion has been identified, suggesting that more than one gene in that region may be selected for and therefore is important in disease progression. However, it is possible that LOH might be merely incidental and reflect overall genomic instability of the aneuploid, rapidly proliferating tumor cells in which it is most frequently found, raising the question of cause or effect [7,50]. Arguing against this hypothesis are reports that aneuploidy and rapid proliferation correlate with

LOH at specific sites (i.e., 17p) rather than with overall incidence of LOH [34].

Technical limitations have prevented LOH from being utilized extensively in studies of prognostic significance. Most tissue banks with long-term follow-up are in formalin-fixed, paraffin-embedded blocks. This fixation results in DNA being too small for LOH studies using Southern blotting. Only recently have PCR techniques been developed that permit evaluation of LOH in paraffin-embedded material [51]. These techniques suffer even more than standard Southern blots in being inadequate to distinguish allelic losses from chromosome gains. Hence, their usefulness will increase as other methods (i.e., FISH) define which numerical imbalances are truly losses.

There have been a number of studies correlating LOH with various pathobiological parameters. For example, LOH on chromosome 17q correlated with estrogen receptor-negative cancers. Similarly, LOH on chromosome 18q was associated with grade III lesions [20]. However, other studies did not find this correlation [52]. Two studies showed correlation between overall number of allelic losses and higher tumor grade [32,46], but one other did not [34]. LOH at chromosome 17p correlated with high proliferative index and overall aneuploidy [34,48]. The criticism of all these studies is that the small sample size and large number of measurements can result in statistical biases. Nevertheless, the correlations are useful for creating hypotheses that can be independently validated using larger data sets.

LOH has not been evaluated systematically on either in situ carcinomas or metastases because these lesions are usually only available as paraffin-embedded materials. Thus, it is unclear whether particular LOHs are acquired early or late in malignant progression. We indirectly approached this question by comparing the completeness of the LOH at more than one locus in a given DNA preparation. If a particular DNA sample had no residual band on LOH, then the sample had little if any normal cell contamination. Using this DNA, a residual band identified with a probe located at a different site would indicate that fewer tumor cells had acquired a loss at the second location. At each region examined, we found that a proportion of tumors acquired a given LOH later than another one. Hence, we proposed that the LOHs are acquired stochastically, with any one sometimes being acquired earlier in progression [44].

The p53 gene

A number of recent articles have reviewed the rapidly expanding literature on the involvement of the p53 gene in human cancers and breast cancer specifically [16,53–55]. P53 was discovered as a normal cellular protein bound to the viral transforming oncogene, large T-antigen [56,57]. Mutant p53 transforms cells like a dominant oncogene [58,59]; however, absence of p53 is also associated with transformation. The explanation for this paradox

is that mutant p53 is a dominant suppressor gene that inactivates wildtype p53 by binding in a tetrameric configuration. The aminoterminus of the p53 molecule contains a transcription-activating sequence [60,61] while the carboxyterminus contains a cluster of nuclear localization signals [62,63]. There are 11 exons in p53 with 5 domains (in exons 2, 5, 7, 8) that are highly conserved in evolution, indicating regions of important function in the molecule. Most of the p53 mutations in breast cancers are missence mutations distributed in all of the highly conserved regions of the molecule [54].

The p53 gene product is thought to be involved in a number of different cellular functions, including cell cycle regulation [64–68], transcriptional regulation [60,61,69–72], differentiation [73–75], and apoptosis [76]. Most recently, in fibroblast model systems, wild-type p53 has been found to suppress entry into the S-phase of the cell cycle in cells with DNA amplifications associated with generation of drug resistance [77,78]. Thus, it has been suggested that wild-type p53 is a 'watchdog' type of molecule that inhibits replication of cells with DNA damage (i.e., potential malignantly transformed cells). In these studies, gene amplification only occurred when both p53 alleles were either mutated or lost. Whether a similar mechanism also holds for breast cancers remains to be established, particularly since breast cancers do not show a strong correlation between p53 mutations and LOH of the other p53 allele [34,79].

Wild-type p53 gene codes for a nuclear protein characterized by a short intracellular half-life which is increased by mutation [16]. Mutant p53 is known to bind a number of different molecules, including heat-shock protein 70 or the rb gene product, that apparently stabilize the molecule, thus accounting for the protein's increased stability.

The increased protein stability of mutant p53 forms the basis of immunohistochemical assays for its detection (for reviews, see [53,80]). In normal cells, the concentration of wild-type p53 is so low that it is undetectable by immunostaining. Increased molecular stability of the mutant protein enables it to be visualized under identical staining conditions [81]. Three different antibodies have been used to detect nuclear immunopositivity. An epitope on the aminoterminal end of the molecule is recognized by Pab1801, at the middle of the molecule by Pab240, and at the carboxyterminal end by Pab421. The Pab421 antibody is also known to cross-react with cytokeratin, thus confounding any cytoplasmic staining [53]. There is some controversy regarding whether these antibodies effectively stain paraffin-embedded material as opposed to frozen tissues [53,82–84]. The discrepancies may be related to the type of fixative used rather than to the paraffin embedding. The ability to stain paraffin-embedded material is particularly important, since it allows access to archival banks of breast cancers with long-term patient follow-up so that the prognostic significance of the immunopositivity can be assessed. All studies agree that there is heterogeneity in the percent of positive cells within a given tumor, with some tumors containing only a few scattered immunopositive cells.

Among various studies, the percentage of breast cancers with nuclear immunopositivity varies from approximately 25% to 50% [53,82–86]. Incidences of 15%–35% were reported by analyzing breast cancers for p53 mutations in the conserved regions of the gene [87–92]. Differing sensitivity of immunoassays may account for this variability. Whenever nuclear immunopositivity has been detected, mutation of the p53 gene has been confirmed [83,86]. However, immunopositivity may only detect a portion of p53 mutations. Obviously, it cannot detect nonsense mutations that prevent the synthesis of any p53 protein. Depending on the immunoassay, it may not detect all the missense mutations as well.

Inappropriate cytoplasmic localization of normal p53 protein has been suggested as one mechanism of inactivation in breast cancers [93]; however, only inflammatory breast cancers were evaluated. In other studies, few breast cancers have been detected with cytoplasmic staining under conditions where cytokeratin cross-reactivity can be excluded [53]. Among over 500 breast cancers that we have examined [83,94], only one case with strong cytoplasmic staining was detected.

In a number of reports, p53 mutations are associated with tumors that are negative for estrogen receptors (ER), have a high histologic grade, and overexpress epidermal growth factor receptor (EGFR) [53,83,85,92,95–98]. In some reports, p53 immunopositivity also correlated with high S-phase fraction [95]. It is unclear what might be the biological significance of these associations. Since p53 is known to be a nuclear binding protein, perhaps it is involved in regulation of ER transcription. Association with high histologic grade and EGFR and possibly high S-phase fraction may be a consequence of negative ER. In fact, when ER status was accounted for, p53 immunopositivity no longer correlated with high S-phase [94]. The net result of high EGFR, ER negativity, and inactivation of p53 may be synergistic dysregulation of various growth-stimulatory pathways leading to rapid proliferation and/or other manifestations of aggressive behavior by the tumor. Unlike other tumors, for breast cancer, there was no correlation between LOH at the p53 locus and p53 mutations [98]. Thus, only one inactivated allele is sufficient for breast cancer malignancy. At least one other report [91] did find a correlation between mutation and LOH, although the number of cases is small.

Immunopositivity for p53 has also been detected in 13% to 25% of in situ carcinomas, suggesting that it can be acquired early in malignant progression [83,86]. Like invasive breast cancers, p53 immunopositivity strongly correlated with the presence of mutations, although additional mutations were detected in immunonegative tumors [79]. Immunopositivity was seen predominantly in the comido type of in situ lesion, which is thought to be the most aggressive form of in situ breast cancer [83]. To determine whether p53 mutations can also be acquired later in progression, the presence of p53 immunopositivity was compared in primary breast cancers and lymph node metastases, subsequent recurrences, or distal metastases from the same

patient. In all cases, primary breast cancers and concomitant lymph nodes [99] or subsequent local recurrences (unpublished observation) were identical. We have found a few cases where distant metastases were positive when the primary was negative, suggesting that p53 mutations can occasionally occur after invasive disease, perhaps conferring increasingly aggressive behavior to already existing breast cancers.

The retinoblastoma gene (RB)

The first tumor-suppressor gene to be molecularly cloned was RB, a complex gene with 27 exons dispersed over more than 200 kb of the genome. Its 4.7-kb mRNA codes for Mr 105,000 nuclear protein (pRB) [13,100,101]. One allele is known to be mutated in the somatic cells of patients with familial retinoblastoma. In the retinoblastomas of these patients, the second allele of the RB gene is also inactivated by deletion, mutation, or rearrangement. In sporadic retinoblastomas, both alleles of the RB gene are either deleted or inactivated, verifying the classic hypothesis proposed by Knudson [17] that both sporadic and familial forms of retinoblastoma arise due to lesions of a common gene, altered by a 'two-hit' mechanism. pRB is presumed to be crucially involved in normal cell growth regulation, with its phosphorylation status being highly cell cycle dependent. The unphosphorylated form of pRB is found in quiescent and G1-phase cells, restricting cell cycle progression in G1 by interacting with the E2F transcription factor [102,103].

Patients with hereditary retinoblastoma are at increased risk of developing second primary tumors, particularly osteosarcomas and other soft tissue sarcomas [104]. The RB gene is also frequently altered in other tumor types that have not been associated with familial retinoblastoma, including bladder, prostate, and small cell lung cancer [105].

The involvement of RB in breast cancer is much less clear. If RB were involved in the induction of breast cancer, one would expect an increased incidence of breast cancer in retinoblastoma families; however, no such relationship exists [106]. Nevertheless, there is circumstantial evidence implicating RB in some breast cancers. Structural changes in the gene have been found in approximately 25% of breast cancer cell lines, although they have been seen in less than 10% of primary breast cancers [107–109]. Varley et al. [109] detected gene abnormalities or deletion in 19% of primary breast cancers, and immunohistochemical analysis of pRB showed reduced expression in all but one of these tumors. Additionally, LOH, which is though to define the locations of putative recessive oncogenes, has been seen in approximately 25% of breast cancers at the RB locus, chromosome 13q14 [28,41].

The controversy regarding the role of RB in breast cancer comes from two types of data. First, Borg et al. [110] did not detect a correlation between LOH at 13q14 and lack of expression of the pRB gene measured

immunohistochemically. Hence, the classic mechanism of unmasking a recessive mutation by allelic loss did not occur in these breast cancers. Similar to the studies implicating loss of a gene in addition to p53 on chromosome 17p in breast cancer [31,32,34,48], Borg et al. [110] hypothesize a second gene on chromosome 13q. All the studies of LOH on 13q in breast cancer are consistent with this hypothesis. In the study by Lundberg et al. [28], the entire q arm of chromosome 13 was deleted. One other study mapped the deletion in various regions of chromosome 13q and reported that the common region of deletion was not at the RB locus but was proximal to the centromere [41].

Other putative suppressor genes involved in breast cancer

Nm23

The *Nm23* gene was originally identified by differential screening of a murine melanoma cell line cDNA library with RNA from cell lines of differing metastatic potentials [111]. *Nm23* gene expression was inversely related to metastatic potential, with tenfold more RNA in the cells with low relative to high metastatic potential. The human *Nm23* protein has 78% homology with a protein encoded by the developmentally regulated *awd* gene in Drosophila; mutation of the *awd* gene causes aberrant differentiation [112,113]. It is also strongly homologous to nucleoside diphosphate (NDP) kinase [114,115]. Two closely related genes have been identified, namely, *Nm23*-H1 and *Nm23*-H2, and these are located on chromosomes 17q22 and 16q, respectively [116]. LOH at the *Nm23*-H1 locus indicated that over 60% of breast cancers had an allelic imbalance at this locus [117].

A number of studies suggest that decreased expression of *Nm23*-H1 correlates with increased aggressiveness of breast cancers. In a small panel of breast cancers (for review, see [118]), *Nm23* mRNA levels were measured by both Northern blot and in situ hybridization techniques [119]. The in situ technique permitted evaluation of expression in tumor cells relative to normal stromal cells in the same specimen. Most of the tumors from patients with positive lymph nodes were low in *Nm23* mRNA content, while adenomas and breast carcinomas from most node-negative patients exhibited higher *Nm23* mRNA levels. Two other studies using antibodies to *Nm23* also reported an inverse correlation between level of expression and metastatic ability [120,121]. However, each of these studies examined relatively small patient data sets. More recently, Sastre-Garau et al. [122] examined expression of the product of the *Nm23*-H1 gene, NDP kinase A, finding increased expression of NDP kinase A in malignant breast tumors compared with normal tissues and no correlation with lymph-node metastases. The reasons for these discrepancies have not yet been elucidated. Additionally, breast and colon cancer differ in that the *Nm23* gene does not predict prognosis of colon cancer [123].

Prohibitin

The rat prohibitin gene was isolated for its ability to negatively regulate cell proliferation [124]. Its mRNA is more frequently expressed in normal, quiescent liver than in regenerating liver [125]. The human prohibitin gene is located at chromosome 17q12–21, a region near, but proximal to the *Nm23* gene. Sato et al. [126] found that this gene, like its neighboring *Nm23* gene, is located in a common region of allelic imbalance in breast cancers. When two highly conserved exons were examined, 4 of 23 sporadic breast cancers that showed LOH at 17q12–21 had somatic mutations at these loci.

Other putative recessive genes detected by differential screening techniques

A number of genes that are overexpressed in normal mammary cells relative to breast cancer cell lines have been isolated. Included among these are keratins [127,128], glutathione-S transferase, the gap-junction protein, connexin 26, and a small calcium-binding protein related to calmodulin, NB-1 [129]. The role of these genes in breast cancer has not yet been defined. At least some of them have reduced mRNA expression without concomitant gene mutations. This fact led Lee et al. [127] to hypothesize that there are two mechanisms for generating recessive oncogenes. Classically, loss of tumor-suppressor gene expression has been viewed as the consequence of mutations or rearrangements in the gene itself. However, loss of expression of a particular gene may also result from altered regulatory events in which the gene maintains its integrity. This latter type of lesion may be important clinically because such genes are candidates for up-regulation by therapeutic interventions.

Familial breast cancer

Familial clustering of some breast cancers has been observed for a long time. Epidemiological studies have tended to support the view that an autosomal dominant gene, with high but incomplete penetrance, accounts for most breast cancer families. It is likely that several different predisposing genes are responsible for the familial breast cancer clusters (for reviews, see [130–132]). An important breakthrough in this field occurred with the identification of linkage with chromosome 17q21 in some early-onset breast cancer and breast/ovarian cancer families [132,133]. Another early-onset breast cancer family did not show linkage at 17q21, indicating genetic heterogeneity even among early-onset families [134]. Currently, a major effort in the field is involved with identifying the gene on 17q. Although both prohibitin and *Nm23* are located on 17q, both of these genes have been excluded as the target gene.

The p53 gene has been identified in the germ cells of patients with the Li–Fraumeni syndrome, a rare cancer-prone familial disorder [135] that

includes soft tissue sarcomas and breast cancer [136–138]. However, p53 germline mutations have been observed only rarely in other breast cancer families or in early-onset sporadic breast cancer cases [139].

Both the putative 17q gene and the p53 gene are thought to be classic recessive oncogenes in that elevated risk in cancer-prone families occurs because one allele of a recessive oncogene is mutated in all the somatic cells of affected family members. High risk for cancer occurs because only one additional event is necessary to completely inactivate the gene. However, familial disease is not always the result of recessive oncogene mutations. It is possible that some families may inherit a propensity for breast cancer because they inherit a susceptibility to a premalignant state that itself carries only an increased probability of conversion to a malignant lesion. Additional genes involved in breast cancer susceptibility have been hypothesized, including the gene for ataxia telangiectasia, which results in decreased repair of X-irradiation-induced damage, and a gene that results in increased proliferative breast disease, a premalignant lesion [140].

Conclusion

It is clear that breast cancer procession is associated with inactivation of a number of different recessive oncogenes. The most widely evaluated gene is the tumor-suppressor gene, p53. Somatic mutation of p53 is responsible for the rare familial disease Li–Fraumeni syndrome, but is not linked to other types of familial breast cancer. Mutations of p53 are found in approximately 30% to 50% of sporatic breast cancers. They usually occur early in progression and are associated with poor prognosis. The role, if any, of the rb tumor-suppressor gene is much less clearly understood. Additional recessive genes that have been associated with breast cancer include prohibitin, a cell proliferation gene, Nm23, a nucloside diphosphate kinase gene, NB-1, a calmodulin-related gene, and connexin, a gap-junction protein. Additional evidence for recessive oncogenes in breast cancer come from evaluation of familial breast cancer where linkage with chromosome 17q21 has been seen in some but not all families. Finally, loss of restriction fragment polymorphism (LOH) is one of the most frequent genetic aberrations seen in breast cancers. LOH at has been detected in a proportion of breast cancers at 13 different chromosomal regions. Defining the target genes for these LOHs and their role in breast cancer progression are important challenges for the future.

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6. Estrogen receptor variants in breast cancer

Carlos A. Encarnacion and Suzanne A.W. Fuqua

The relationship between estrogen (E2) and carcinoma of the breast has been appreciated for nearly 100 years. In the late nineteenth century, Beatson reported that patients with inoperable breast tumors frequently responded to surgical castration [1], still a first-line therapeutic modality in premenopausal patients. Other manipulations designed to lower the concentrations of circulating E2 or to block its effects are also as effective, inducing remissions in about 30%–40% of patients [2]. Since alternative treatments are available for breast cancer patients, it is important for clinicians to consider all available biological factors that can predict which of these women are likely to respond to hormonal treatments. Among these variables, the estrogen receptor (ER) is the most important in predicting response to hormonal treatments. Multiple clinical studies show that those patients whose tumors express ER have a 70%–80% chance of response, compared to only 10% in those that do not [3–5].

Multiple studies published over the last few years have enhanced our knowledge of the structure and function of the ER. This knowledge is of utmost importance in the understanding of certain clinical characteristics shown by patients treated with hormonal therapies. For example, the development of resistance to antiestrogens could be induced by several possible alterations in the ER. Thus, it is important to review the normal function of the receptor in order to understand its relevance in the development of antiestrogen resistance.

Structure and function of the estrogen receptor

The presence of an intracellular estrogen-binding protein in E2 target tissues was first identified in the late 1960s, after radiolabeled estrogens became available for research. It soon became evident that this protein, initially called estrophillin, was associated with the intracellular accumulation of E2 and the modulation of its cellular effects [6,7]. It is now known that once ER binds to estrogen, it dimerizes and, through a complex tridimensional alteration, acquires a transcriptional-activating function with specificity

R. Dickson and M. Lippman (eds.), Mammary Tumorigenesis and Malignant Progression.

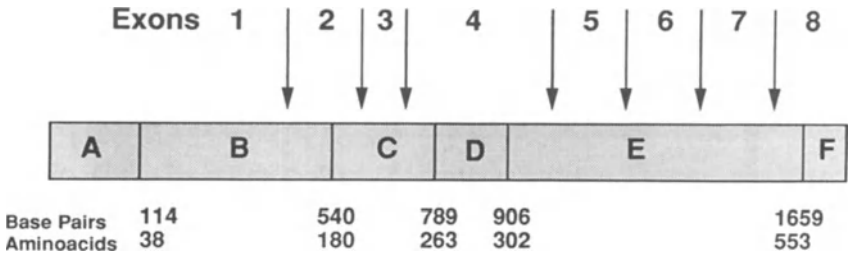

Figure 1. Genomic sequence of the human estrogen receptor.

toward certain DNA sequences of estrogen-responsive genes. In recent years, the genomic sequence of the human estrogen receptor (hER) was determined by cloning experiments using MCF-7 human breast cancer cells. It consists of 6322 nucleotides, encoding a 595-amino-acid protein with a molecular weight of 66 kilodaltons [8,9]. The ER belongs to a superfamily of steroid hormone receptors, which includes progesterone, mineralocorticoid, glucocorticoid, thyroid hormone, vitamin D, and retinoic acid receptors [10]. It has six distinct functional domains, labeled A thru F, some of which show marked interspecies homology (figure 1). For example, domains A, C, and E of the hER have a respective homolgy of 87%, 100%, and 94% with their counterparts in the chicken ER [11]. Furthermore, comparison of the mentioned hER domains with those from the mouse, rat, xenopus, and trout also discloses considerable homology [12]. These different ER domains confer the ability to recognize and bind its ligand, identify specific DNA segments of estrogen-regulated genes (the estrogen response elements or EREs), and finally to stimulate transcription, probably by interacting with other transcriptional proteins. The details of these functional regions will be discussed below.

The A/B region

This domain has intrinsic transcriptional activation function. This activity, known as TAF-1, does not depend on the presence of estrogen, and is much more active in certain cell types. Studies introducing an ER lacking the hormone-binding domain (where the principal hormone-dependent activation function resides) into HeLa and CV1 cells showed only a 1%–5% of the transcriptional activity as compared to wild-type receptor [13]. Similar studies showed 2%–10% of wild-type activity when NIH-3T3 D4 cells were used [14]. In contrast, a 60%–70% wild-type activity was seen in chicken embryo fibroblasts using a vitellogenin ERE and a similar TAF-1 construct [15], while a Saccharomyces system showed 100% wild-type activity [16]. These data suggest that TAF-1 may be more important for the regulation of certain E2-responsive genes than for others. In another series of experi-

ments, and ER construct lacking the A/B domain also showed variable results. In a HeLa cell system, this A/B-deficient construct had wild-type transcription activating function with a vitellogenin-CATERE, whereas its activity was only 17% of wild-type when a pS2-CATERE was used [13]. Similar results were also obtained in experiments using an A/B-mutated murine ER with a vitellogenin ERE reporter [14]. Thus, the relative importance of TAF-1 appears to be both promoter- and cell-type specific. As we shall see later, its independence from estrogen activation could relate to the phenomena of clinical estrogen-independence and/or resistance to antiestrogenic drugs.

The C region

This 66-amino-acid domain is the most highly conserved between species [17,18]. It is thought to fold into two zinc-binding structures (CI and CII) that interact with the DNA in a similar fashion to the zinc 'fingers' of the ribosomal protein TFIIIA [17]. Each DNA-binding 'finger' binds a zinc atom with four cysteine residues and is encoded by a different exon [19]. There are potentially functional differences between the two 'fingers,' since CI has several hydrophylic amino acids and four cysteines, whereas CII has five cysteine residues and is richer in basic amino acids. These zinc 'fingers' are important for the recognition of specific ERE sequences, as shown by experiments in which a hER chymera containing the C region of the glucocorticoid receptor activated glucocorticoid-responsive genes in the presence of estrogen [20]. Furthermore, experiments in which either CI or CII were substituted for by their glucocorticoid counterparts showed that it is CI that largely determines the receptor's target specificity [17]. There are a number of specific amino acids in the base of this zinc 'finger' that appear to play a key role in target recognition [21–23]. It has been theorized that CI could be responsible for the recognition of specific nucleotide bases in the major groove of the DNA, while CII stabilizes this bond by associating to the phosphate backbone of the DNA chain [24].

The two zinc 'fingers' are not the only regions required for DNA binding. Some experiments using ERs with deletion of amino acids 250–264, or ERs with truncation after amino acid 261 (both abnormalities distal to the zinc fingers), showed that these receptors do not bind to their ERE sequences [25], suggesting that these additional regions have a role in further stabilizing the ER–DNA bond, either directly or by enhancing receptor dimerization.

Two additional functions are also thought to be mediated by this region: collaboration with the E region for binding to heat-shock protein 90 (hsp90) and a signal for the proper localization of the ER in the nucleus. In 1990, Chambraud et al. [26] first showed that ERs with deletion of amino acids 250–274 were unable to interact with hsp90. In addition, these experiments also showed that receptors with deletions in this region were localized exclusively in the cytoplasm, whereas smaller deletions in the same area

generated ERs that were distributed between the cytoplasmic and nuclear compartments.

The D region

This segment of the ER is considered a molecular 'hinge' that allows the hormone-mediated conformational change. In fact, it has been reported that mutations of this region induce no effects on estrogen binding or transcriptional activation capability of the ER in HeLa cells [13]. On the other hand, v-erbA species (an oncoprotein with analogy to thyroid and estrogen receptors) with mutations in this region were inactive [27]. We also have cloned an ER from a clinical breast tumor with an amino acid substitution at residue #887 (S.A.W. Fuqua, unpublished data). This variant hER has a reduced transcriptional ability as compared to wild-type receptor in vitro assays. Thus, at the present time, it is not possible to disregard this region as one with only a purely allosteric function.

The E region

Domain E is a very large and complex region, composed of 250 amino acids encoded by different exons [19]. It encompasses subdomains implicated in hormone binding, transcriptional activation, dimerization, and estrogen-relieved transcriptional repression. It is commonly known as the hormone-binding domain, since it forms a hydrophobic pocket into which estrogen binds [28]. It is believed that binding of ligand induces a conformational change in the receptor that rearranges important amino acid residues into the proper tridimensional position, inducing ER's main transcriptional-activating function (known as TAF-2). Most experts agree that TAF-2 is estrogen-dependent, and thus requires the presence of ligand for activity [13,29]. Others propose that this segment has some transactivating function in the unbound state, although this point is currently debated [30]. Interesting new data suggest that not only the binding of E2 but also the association to DNA itself induces a conformational change in the ER. This change, detected due to a decrease in the E2 binding affinity, could account for part of the conformational changes required to activate ER [31].

The fact that the unoccupied ER is incapable of binding DNA suggests that its C domain is protected, either by the ER itself or by other proteins. The E domain appears to play a major role in this phenomenon, since ER mutants lacking the E region will constitutively bind ERE sequences [13]. It has also been suggested that the E region folds itself over the DNA-binding region, thus preventing an association between unbound ER and DNA [32]. Alternatively, ER's inability to bind DNA while unoccupied may be secondary to its association with other proteins, like hsp90. The presence of this protein was first detected by a change in the sucrose gradient migration

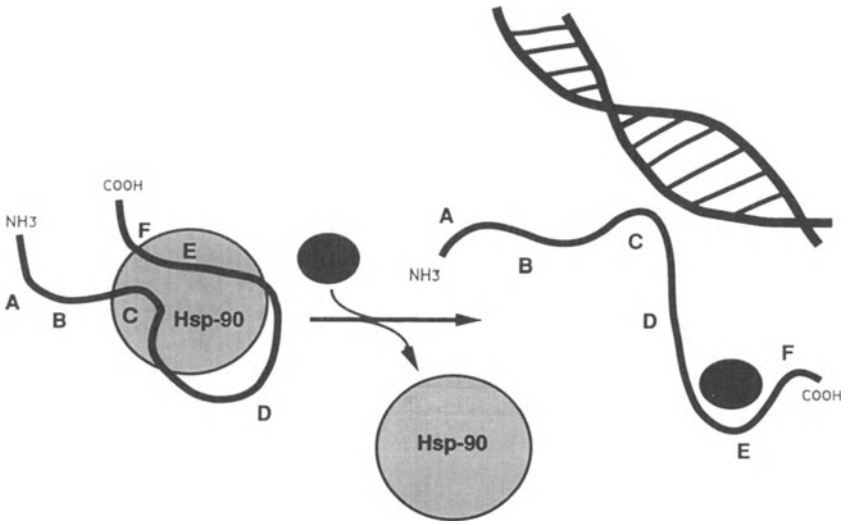

Figure 2. The interaction between estrogen receptor and hsp 90.

pattern of occupied vs. unoccupied ER [33], and it is possible that it could physically obstruct the C region and prevent the association of ER with DNA [34]. Both the E region and certain specific amino acids of the C region are necessary for the interaction with hsp90 [26]. Once the ligand binds to the ER, hsp90 dissociates from the receptor, exposing the DNA-binding domian as illustrated in figure 2.

Finally, results from deletion and point mutagenesis analyses indicate that the E domain is important in the dimerization process, which occurs after hormone binding. This dimerization, in turn, may be necessary for DNA binding. Deletion mutants that lack the C-terminal part of the dimerization subdomain do not dimerize or bind to DNA [35]. On the other hand, the E domain is not absolutely required for DNA binding, since an ER with a deletion of the entire E region can bind to the ERE, although with less affinity [13].

As this brief review demonstrates, the ER is a very complex protein with multiple functions, all of which interact to initiate transcription of certain genes, which ultimately result in cellular proliferation, and which are potentially candidate functions that may be disrupted in breast tumors.

The use of antiestrogens in breast cancer

Since the binding of E2 to the ER activates certain pathways that lead to tumor growth, it is logical to think that blocking of these steps would decrease or prevent this process. Therefore, one of the most frequently used

methods to achieve this purpose is the administration of competitive antagonists such as tamoxifen. This medication has become the mainstay hormonal treatment for postmenopausal women with carcinoma of the breast. A recent review by the Early Breast Cancer Trialist Group showed that the use of postoperative tamoxifen for a period of more than two years decreased the mortality of women with early-stage disease by 25% [36]. In addition, tamoxifen induces durable remissions in a significant number of women with ER-positive, metastatic breast cancer [37,38].

Tamoxifen is the transisomer of a triphenylethylene derivative with antifertility properties in laboratory animals [39,40]. It has a species-specific pharmacology, being purely antiestrogenic in chickens [41], partially estrogenic in rats [42], and in short-term studies, potently estrogenic in mice [39,43]. With prolonged treatment, tamoxifen inhibits the stimulatory effects of E2 in the target tissues of these animals. Interestingly, tamoxifen is also tissue specific, being antiestrogenic in some tissues, while estrogenic in others [44,45]. This differential activity permits its long-term use in breast cancer patients without adverse effects on the bones or cholesterol profiles [46–48]. Tamoxifen works mainly by binding to the ER and inhibiting its action [49] without preventing receptor dimerization or interaction to DNA. Many studies have demonstrated that the recognition of the ERE by a tamoxifen-bound ER complex is similar to that of E2 ER [14,25]. These tamoxifen–ER and E2–ER complexes, however, have a different migration pattern in gel-retardation assays, suggesting that tamoxifen induces an alternate tridimensional conformation that fails to activate TAF-2 and makes it migrate aberrantly in this electrophoretic assay system.

A major problem related to the use of tamoxifen is the development of drug resistance. This phenomenon, which occurs invariably in all patients after different time courses [50], dictates the use of alternative treatments, some of which are considerably more toxic. Since a possible cause for this phenomenon is the selection of cells that no longer express ER, and thus are E2-independent, many researchers have evaluated the pattern of expression of both estrogen and progesterone receptors (PgR) in patients treated with tamoxifen for different periods of time, while others have used in vitro cell culture models to study this problem. Most of these studies show inconsistent results, in part because of the different approaches and methods utilized. To clarify this issue, we directly analyzed tumors from patients who had been treated with tamoxifen and recurred while taking the medication. ER and progesterone receptors (PgR) were assayed in these patients both by ligand-binding assays (LBA) and by immunohistochemical (IHC) methods, in order to eliminate potential false-negative LBA results caused by receptor occupancy by endogenous or exogenous ligands. Our results, shown in Table 1, show that a large percentage of patients remained ER positive, either by both LBA and IHC or by IHC alone (Encarnacion et al., unpublished results). Furthermore, another group of patients were PgR positive, although ER negative, suggesting that these tumors had a func-

Table 1. Receptor phenotypes in tamoxifentreated patients

ER (LBA)	ER (IHC)	PgR	No. patients
+	+	+	3
−	+	+	6
−	−	+	5
−	−	−	8

Note: ER, Estrogen receptor; PgR, progesterone receptor; LBA, ligand-binding assay; IHC, immunohistochemistry.

Table 2. Estrogen receptor variants at the different functional domains

Domain	Reference	Source	RNA alteration	Protein alteration	ER activity
A/B	[59,60]	Human tumors	C-to-T substitution at nucleotide 257	Substitution of alanine to valine	Decreased E2 binding
C	[56]	Human tumors	Exon 3/intron variant (4.5 kb)	Truncated protein	Non-functional
			Exon 2 deletion + Exon 3/intron variant (3.8 kb)	Truncated protein	Unknown
			Exon 2/intron variant (2.5 kb)	Truncated protein	Non-functional
	[62]	T47D cells	Exon 3 deletion	Truncated protein	Dominant negative
			Exon 2 deletion	Truncated protein	Unknown
	[5]	Human tumors	6 bp insertion at exon 2/intron	Insertion of aspartic acid and arginine	Decreased activity
	[63]	Tamoxifen-resistant tumors	Exon 2 variants	Unknown	Unknown
E	[64]	ER−/PgR+ human tumors	Exon 5 deletion	Truncated protein	Constitutive function
	[58]	ER+/PgR− human tumors	Exon 7 deletion	Truncated protein	Dominant negative

tional, even if unmeasurable, ER. The receptors in these patients could have been down-regulated by the continued exposure to ligand, in this case, tamoxifen. Thus, the development of tamoxifen resistance in most of our cases appears related to mechanisms other than a total loss of ER and estrogen dependence. This is also supported by the fact that patients who fail tamoxifen after an initial response frequently do well with second- or third-line hormonal manipulations.

Our group has recently begun to study some of the possible mechanisms causing tamoxifen resistance in these patients. We have identified that tamoxifen-resistant tumors have a lower intracellular concentration of drug when compared to tamoxifen-sensitive tumors [51]. Furthermore, resistant tumors have higher concentrations of the cis-isomer of tamoxifen as well as other metabolites that are known to be estrogenic [52,53]. This suggests that resistant tumor cells are able to eliminate the drug, either by exclusion or metabolism to noninhibitory or even stimulatory substances. However, in the remainder of this review, we will concentrate on some of the ER variants identified from breast tumor and will speculate on their potential role in the development of clinical tamoxifen resistance. These variants are summarized in table 2.

ER variants in breast cancer

Three important things must be mentioned before detailing specific ER variants. First, even though the data are suggestive, none of these variants has been clearly linked to the development of clinical antiestrogen resistance. Second, these variants frequently involve changes at the exon/intron boundary, which suggests splicing variants, rather than mutations of the DNA itself. Finally, in a large number of cases, wild-type ER is present together with the variants [5,54–56], which makes it even more difficult to predict their contribution to clinical tamoxifen resistance.

Mutations or variations at the A/B domain could be of clinical importance, since this domain has ligand-independent transcriptional activity. Tamoxifen-bound ER can interact with DNA; thus, it is possible that an ER with a mutationally induced enhancement of TAF-1 could initiate transcription even when bound by an antiestrogen. Through the use of RNase protection assays, a mutated variant was detected with a substitution of a C for T at nucleotide 257. This creates an amino acid substitution at position 86, resulting in an ER with reduced E2-binding affinity [57,58]. The clinical importance of this variant in the development of E2 independence or tamoxifen resistance is unknown. There are presently no additional data on A/B domain mutations that correlate with clinical antiestrogen resistance. In fact, preliminary results in our laboratory suggest that mutations in this domain are relatively infrequent (S.A.W. Fuqua, unpublished results).

The C domain is obviously an important region where mutations may have clinical importance. Point mutations in the zinc fingers could directly affect the ability of ER to recognize specific EREs. Furthermore, nonsense mutations or splicing abnormalities in this area could result in ERs truncated in the downstream E domain and thus able to bind freely to DNA and initiate transcription through the TAF-1 region. One study using gel retardation assays to indirectly evaluate the C domain found that more than 60% of the tumors expressing >100 fmol/mg of ER did bind correctly, whereas

the majority of those with <100 fmol/mg did not bind to ERE [59]. DNA binding capacity also correlated with the expression of PgR by these tumors, suggesting that this differential DNA binding has functional relevance. Whether this binding is clinically relevant in tamoxifen resistance, however, is unknown at present.

Several ER variants have been described in which deletions and insertions are found at exon/intron boundaries. One study with T47D human breast cancer cells found two variant ERs in which either exon 2 or 3 was deleted. These abnormal receptors, when transfected into normal cells, acted in a dominant-negative fashion, inhibiting transcription of wild-type ER [60]. Again, the clinical relevance of these variants in unclear, since similar mutations isolated from human breast cancer biopsies did not act in a similar fashion in other transcription assays. There are now several reports of C region changes detected in breast cancer biopsies [5]. Using Northern analysis, three variant mRNAs of 4.5, 3.8, and 2.5 kb, respectively, were found in tumors [54]. A cDNA library produced from tumors showed that abnormalities at intron/exon boundaries are the predominant change in all three variant mRNAs [61]. One of them, clone 24, has a stop codon following an abnormal sequence of amino acids at the exon 3/intron boundary. This clone can bind to DNA and, since it lacks the E domain, theoretically could be able to activate transcription via TAF-1. In vitro transfection experiments with this clone using COS cells, however, show that it is nonfuctional, at least in this system. Future experiments should test whether it is capable of functioning in other model systems. The cDNA of the smallest species (2.5 kb) also showed a divergence at this intron/exon boundary [61]. Again, in vitro experiments suggest that this clone is nonfunctional. More recently, ER mutations at exon 2 were detected by single-strand conformational polymorphism in two tumor samples from patients with tamoxifen resistance. The functional value of these mutants remains to be explored [62]. Thus, presently there is no conclusive evidence that mutations in the C region are associated with tamoxifen resistance, but since TAF-1 is tissue and promoter specific, it is possible that these described C domain ER variants in vitro may be clinically active and importnt in vivo.

We have approached our search for alterations in the E domain by analyzing tumors that show a discordance in ER and PgR expression [5]. Since PgR expression correlates well with ER activity and function, we hypothesize that those tumors that were ER positive, yet PgR negative, or conversely ER negative but PgR positive, were potential candidates in which to search for ER variants of clinical importance.

We first reported an ER variant with a delection of exon 5 in ER-negative, PgR-positive tumors [63]. This deletion results in the truncation of ER and the production of a 40-kDa protein that lacks a functional hormone-binding domain, and that is thus unable to bind ligand. This explains why tumors containing this variant are ER negative, since LBA used routinely to measure ER would fail to detect these receptors. We also have shown that

this receptor variant has constitutive transcriptional activity, which did not require or is enhanced by E2. Furthermore, stable transfectants of the exon 5 deletion into MCF-7 cells confers resistance to tamoxifen treatment (S.A.W. Fuqua, unpublished data). Therefore, overexpression of this variant may potentially be involved in clinical tamoxifen resistance.

Finally, while studying ER-positive, PgR-negative tumors, we discovered another truncated ER. This ER variant, which lacks exon 7, was capable of DNA binding, as determined by gel retardation assays, and also acted as a dominant-negative regulator of wild-type ER function when co-expressed in yeast cells [56]. It has to be mentioned, though, that a similar variant identified in T470 breast cancer cells did not exhibit a dominant-negative phenotype, and this discrepancy is not understood at present. However, it is tempting to speculate that dominant-negative variants, such as the exon 7 deletion described by us, might be potential modulators of ER fraction that could be clinically exploited. This hypothesis is further substantiated by such preliminary studies where we have introduced and overexpressed the exon 7 variant with MCF-7 cells with wild-type ER. The overexpression of the exon 7 variant in these cells shuts down proliferation of these cells (S.A.W. Fuqua, data unpublished). This underscores the potential for using recombinant ER variants as potential therapeutic tools [60].

Conclusions and future directions

The acquisition of new insights into the structure and function of the ER has made a major impact in understanding the role of steroid hormones in the modulation of growth of breast carcinoma. This structural information also contributes to a theoretical explanation of the phenomena of E2 independence and antiestrogen resistance. The presence of ER variants in both cell lines and human tumor samples suggests that these ERs may be important in the clinical behavior of these tumors. Spontaneous mutations may confer cells with a growth advantage as well as drug resistance. Furthermore, some of these variations at the exon/intron boundary may represent alternative splicing rather than mutations, perhaps directed by the tumors through some unknown mechanism.

As we have seen, the studies of receptor function done on in vitro models has several limitations and can be frequently misleading. ERs with no activity in a cell line may be extremely active in others. By the same token, ERs with abnormal function in vitro may be completely unimportant in vivo. The additional confounding factor of the coexpression of both variant and wild-type ERs poses many questions on the significance of these variant receptors.

The data are still insufficient to definitely ascribe to these ER alterations any relevance in clinical syndromes such as tamoxifen resistance. These tantalizing in vitro data, however, are sufficient to warrant further study of

ER variants. Hopefully, as assays are developed to measure these variants in large numbers of tumors, their true significance will be elucidated.

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7. Hormone 'resistance' in breast cancer: The role of normal and mutant steroid receptors

Kathryn B. Horwitz

Faithful expression of genetic information is lost in tumor cells due to the formation of spontaneous cell variants. In breast cancer, this evolution is marked by progression of tumors from hormone-dependent, through hormone-responsive, to hormone-resistant states. Many resistant tumors no longer express estrogen receptors (ERs) and progesterone receptors (PRs), and this may be the basis for their hormone resistance. However, half of all advanced breast cancers are receptor positive, yet they too fail to respond to antiestrogen therapy. Both the cellular heterogeneity that mark progression of the disease and the hormone resistance that characterize the end stages of the disease have been longstanding clinical problems that are slowly yielding to basic research focused both on solid tumors taken directly from patients and on breast cancer cell lines derived from such tumors. This chapter discusses how mutant ERs serve as one mechanism for development of resistance. A suggestion is made that subpopulations of tumor cells can be stimulated, rather than inhibited, by antiestrogens like tamoxifen. Our recent work with normal PRs, showing conditions in which progesterone antagonists, too, can have inappropriate, agonist-like effects, is also described. These PR models represent additional mechanisms that may explain the hormone-resistant state. It is suggested that many 'resistant' tumors are not simply ignoring the hormone antagonist treatment; instead, in these tumors, the hormone antagonist has become stimulatory rather than inhibitory.

Estrogen receptors

The molecular biology of estrogen receptors has been extensively explored in recent years. Their cDNA was independently cloned and sequenced from MCF-7 breast cancer cells by Green et al. [1] and Greene et al. [2], and the ER gene was cloned and analyzed two years later [3]. The protein is comprised of 595 amino acids within which Kumar et al. [4] distinguished six functional domains identified by the letters A through F. The A/B domains contain regions that regulate the transcriptional function of the proteins.

R. Dickson and M. Lippman (eds.), Mammary Tumorigenesis and Malignant Progression.

The C domain contains two DNA-binding zinc fingers and is the region of the protein that binds to the estrogen response element (ERE). Mutations in this portion of the protein change its affinity for DNA, resulting in suboptimal or complete loss of DNA binding. The hormone-binding properties of the receptors map to region E by mutagenesis analysis. Since these two functions, DNA binding and hormone binding, are carried out by separate parts of the protein, they are to some extent independent. Thus, it is possible to have variant receptors that can bind to DNA with limited affinity without first binding hormone, and vice versa [4,5]. Three additional specialized regions of steroid receptors have also been identified: a nuclear localization signal, a heat-shock-protein (hsp 90) binding region, and a dimerization domain. The nuclear localization signal, located downstream of the DNA-binding domain, is a region of the protein that must be present for the receptor to remain within the nucleus in the absence of ligand [6]. It has been identified in PRs and is presumed to be similar in ERs. The hsp 90 appears to bind to regions in the hormone-binding domain of some steroid receptors when ligand is absent, and its binding is believed to prevent receptor dimerization and DNA binding [7]. Ligand activation leads to hsp 90 dissociation and monomer dimerization in solution [5]. The dimerization domain that mediates this interaction between two ER molecules has been localized to the carboxy-terminal end of the hormone binding domain [8]. A weak dimerization domain may also be present in the second zinc finger of the DNA-binding domain [5]. Additional sites for heterologous protein–protein interactions may also be located in the hormone-binding domain [9], and covalent modifications by phosphorylation [10] further enhance the complexity of these protein molecules.

Molecular heterogeneity: Estrogen receptors

Several reports of naturally occurring mutant or variant ER forms have recently appeared [11]. In addition, polymorphic forms of the ER gene have been described [12–14]. The majority of these genetic changes are found in introns, which do not directly encode the mRNA, or in turn, the protein. Of interest is the recent report by Keaveney et al. [15] identifying an alternative estrogen receptor mRNA that appears to be the primary transcript present in the human uterus, as opposed to the breast cancer line MCF-7. This transcript is alternatively spliced in the 5′-untranslated region, and has an additional exon with two small open reading frames upstream of the alternative splice site. Although the receptor proteins encoded by these two types of messages are identical, the nucleotide sequences that flank the translated regions are differet, and are likely to lead to differential regulation of the protein, depending upon which type of message predominantes in the tissue in question. Equally interesting is a truncated ER message specific to pituitary cells [16]. This deletion involves the translated region

and presumably encodes a variant receptor, although expression of the protein has not yet been documented. Thus, in normal cells, the regulation of ER gene transcription, and even ER protein structure, may be tissue specific.

Mutant estrogen receptors in solid tumors

Turning to malignant cells, there is now mounting evidence to show that in addition to silent mutations and regulatory heterogeneity, mutations in ER exons exist that would influence protein structure and protein function. Garcia et al. [17,18] identified a polymorphic variant in the B region of ER mRNA in some human breast cancer biopsies. This variant has since been correlated with lower than normal levels of hormone-binding activity, and preliminary evidence suggests that women who are heterozygous for this variant have a higher proportion of spontaneous abortions than those who are homozygous at the same locus [19].

Wild-type ER mRNAs from several normal and malignant tissues and species are reported to be approximately 6.2 kb in size. However, Dotzlaw et al. [20] have identified truncated ER-like mRNAs is human breast cancer biopsy samples by Northern blotting. These messages appear to lack significant portions of the 3′ region including the hormone-binding domain. By polymerase chain reaction amplification of mRNA from breast tumor specimens, Fuqua et al. [21] have also identified mutant forms of ERs missing part of the hormone-binding domain due to deletion of exon 5 and exon 7. These mutants are an alternatively spliced form, capable of constitutively activating transcription of an ER-dependent gene, or of dominantly inhibiting the activity of wild-type ER. PCR amplification was also used to identify a mutation in the D domain of ER mRNA expressed in a murine transformed Leydig cell line, B-1 F [22]. The functional significance of these mutations has yet to be fully explored, but they clearly suggest mechanisms by which mutant receptor forms can subvert the activity of wild-type forms, when both are co-expressed in the same tumor cell.

The weakness in all these analyses is the assumption that message variants reflect protein variants. While this may indeed be the case, until recently, given the immunologic tools currently available, no mutant proteins had been detected. This may have been rectified by two studies in which gel shift assays were used to examine the ability of tumor ERs to bind an ERE [23,24]. The studies show that some tumors containing abundant immunoreactive ER failed to demonstrate DNA-binding ER, or the DNA-binding ER forms appeared to be truncated, or they were immunologically ER negative but positive by the mobility shift assay. Based on these preliminary data, the prevalence of non-DNA binding ER forms or of truncated ER forms among ER-positive or PR-positive tumors may exceed 50% — a significant number whose structural analysis may become a critically important prognostic tool.

Estrogen receptors play a critical role in the development, progression, and hormone responsiveness of breast cancers. Their structural analysis, by methods like those described above, can be used to generate functional predictions. Alternatively, a product of ER action can be monitored, and PRs have served this role for many years [25]. In all estrogen/progesterone target tissues, estradiol is required for PR induction. This relationship holds true for breast cancers [26] and led us to propose that the presence of PR could be used as a tool to predict the hormone dependence of human breast tumors. Thus, a tumor that contains PRs would, of necessity, have a functional ERs. This idea has in general been borne out by studies showing that ER-positive tumors that also have PRs are much more likely (75%) to respond to hormone treatment than tumors that are ER positive but PR negative (35%) [25]. These studies also identify a small group of puzzling tumors that are ER negative but PR positive and have a higher response rate than is usually expected of ER-negative tumors. These tumors are puzzling because, according to dogma, such tumors should not exist. Thus, either PR synthesis in these tumors is entirely independent of ER, or a variant or other unmeasured form of ER is stimulating PR synthesis.

In 1978, while measuring the steroid receptor content of a series of cultured human breast cancer cells, we found one cell line, T47D, that had no soluble ER by sucrose density gradient analysis, yet had the highest PR levels of any cell line surveyed [27,28]. These cells seemed to be ideally suited to study this ER-negative but PR-positive paradox.

We subsequently found that a subline, which we called $T47D_{co}$, did have ERs, but they were in a permanently activated state in the nucleus. The ERs were not sensitive to the action of estrogens, suggesting that the estrogen regulatory mechanism was defective at a step beyond the initial inter-action of the steroid–receptor complex with DNA. The PR levels were also insensitive to estradiol or to antiestrogens but were synthesized in extraordinary amounts and were functional. Additional studies suggested that the PRs retained characteristics of inducible proteins. Thus, we suggested that persistent nuclear ERs were constitutively stimulating PRs, even in the absence of exogenous estradiol [29,30]. Recently, the tools became available to test this conjecture. Two cDNA libraries were constructed from $T47D_{co}$ cells, that yielded clones consistent with wild-type ER, plus several mutant ER cDNA clones [31]. One cDNA would encode a putative mutant protein lacking the nuclear localization signal and hormone-binding domains of ER. Another ER cDNA clone appears to be an RNA-processing intermediate or splicing error and contains ~1 kb of intron 5 linked upstream of exon 6. Three clones were found with insertions in exon 5. The inserts contain at least two blocks of direct repeats of ~130 nucleotides terminating in A residues that are 70%–85% homologous to the human *alu* family. One clone has a point deletion in the hormone-binding domain just upstream of

the end of exon 5. This leads to a frame shift and a translation termination seven codons later. This mutant cDNA would encode an ER truncated in the middle of the hormone-binding domain at aa 417, with a unique 7 aa COOH-terminal end. Such a protein could be constitutively active. Two independent clones were isolated that had an identical in-frame deletion. These cDNAs would encode a mutant ER of 442 aa instead of the normal 595 aa, having a 153-aa deletion from the end of the DNA-binding domain C, through the hinge region D, to the mid-hormone-binding domain E. The deletion originates in the sequence encoding the putative nuclear localization signal (aa 256–263; R-K-D-R-R-G-G-R). However, the aa sequence encoded by the deletion mutant (R-K-D-R-N-Q-G-K) preserves 4 of the 5 basic aa residues of the wild-type sequence.

We do not know whether the abnormal proteins are expressed. Gel mobility shift analyses of $T47D_{co}$ nuclear extracts show considerable amounts of specific ERE-binding proteins that neither co-migrate with wild-type receptors, nor are supershifted by anti-ER antibodies. The identity of these proteins is still under investigation. However, based on deletion mutagenesis analyses [5], we can begin to predict the consequence to the cells of mutant ERs. Especially in $T47D_{co}$ sublines with hypertetraploid subpopulations (see further below) that contain 4 to 5 alleles of the ER gene [32], cells having a mixture of wild-type and mutant receptors could co-exist. Heterodimers of the wild-type and mutant monomers, having dominant positive or dominant negative activity [33], could override the estrogen requirement of the wild-type receptors. This would result in ER-positive but estrogen-resistant cells — a phenotype that describes 50% of hormone-resistant breast cancers.

Consequences of mutant estrogen receptors: Cellular heterogeneity?

The consequences of this molecular diversity in ERs may reach beyond issues of hormone dependence to the broader problems of tumor progression and cellular heterogeneity that also characterize advanced breast cancer. Cellular heterogeneity has usually been assumed to exist within tumors, but has been difficult to demonstrate. The concept is, however, important, since it means that in practice, the clinician must treat not just one tumor, but a variety of possibly heterogenous subtumors. Is it possible that heterogeneity of ERs among cells can lead to heterogeneity of cells among tumors? While the analyses of ERs described above have led to the discovery of variant receptor forms, the methods cannot answer a fundamental question: Do all or only some of the cells carry the variants? Moreover, wild-type ER are always present together with the variants. Are wild-type ER present alone in some cells of the tumor or are they always co-expressed with the variants in any one cell? We postulated that the genetic diversity of ER would be reflected in heterogeneity of other molecular markers and set out to develop

an assay that could simultaneously measure DNA content and PR heterogeneity in subpopulations of tumor cells [32,34]. We have used this immunologic, dual-parameter flow cytometry (FCM)-based assay to demonstrate and quantitate a remarkable heterogeneity in PR content, DNA ploidy, and mitotic indices among subpopulations of breast cancer cells [35].

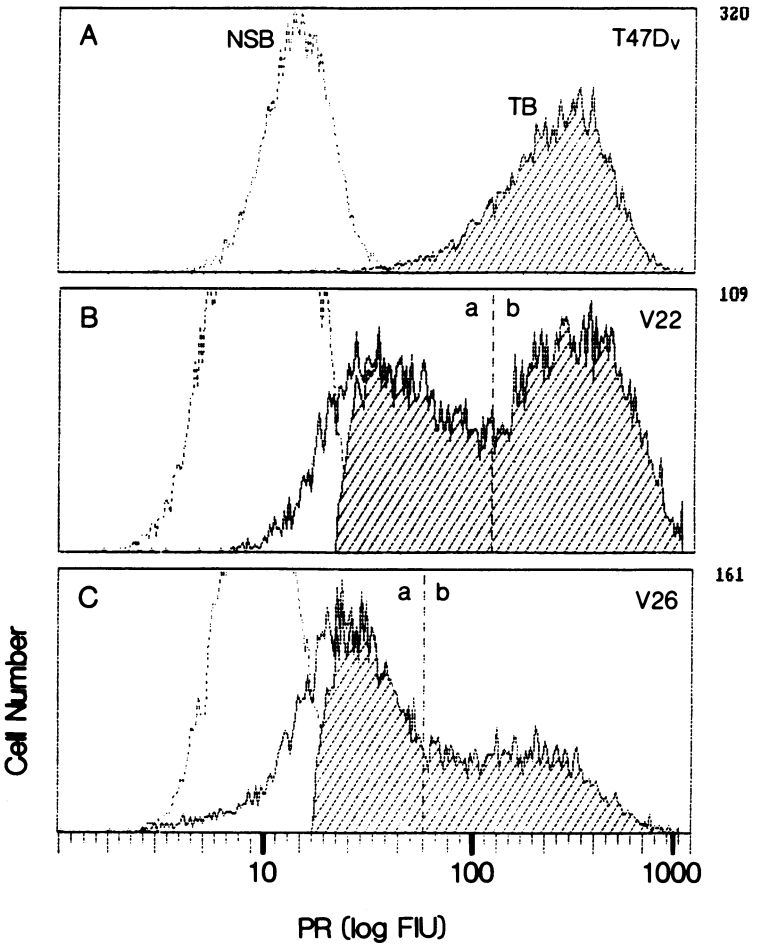

Figure 1. Heterogeneity of PR levels in three T47D cell lines. Total (TB) and nonspecific (NSB) PR were measured by dual parameter flow cytometry in (A) $T47D_v$ cells and in two clonal sublines, (B) V22 and (C) V26. Fluorescence intensity unit (FIU) levels in 10,000 cells of each set were analyzed and plotted. Cells with TB levels falling under the NSB curve are considered to be PR negative. Using the 1-Par program, subpopulations with low ('a') and high ('b') PR levels were gated and quantitated. PR levels are plotted on a log scale. Originally published in Graham et al. [35]. Reproduced with permission.

Heterogeneity of progesterone receptor distribution

The heterogeneity of PR distribution is illustrated in figure 1 by three cell lines derived from $T47D_{co}$, in which only the first, $T47D_v$ (figure 1A), has the PR phenotype that most current receptor measurement methods assume, namely, that all cells are PR positive at a level greater than measured background. However, even $T47D_v$ cells have PR levels that range extensively, as shown by the width of the receptor peak on the log scale. Figures 1B and 1C illustrate entirely different PR-positive patterns, namely, two cell lines (V22 and V26) that have more than one PR-positive population, despite the fact that they were derived as single cell clones from $T47D_v$.

To quantitate PR in the subpopulations, we have developed a computer program entitled *1-par*. Calculations using this solftware show that 12.8% of cells in V22 and 23.2% of cells in V26 are PR negative, and that in addition each cell line also contains two distinctly different PR-positive subpopulations. Starting with a cell line having a PR-negative subpopulation and cloning by limiting dilution plus FCM analysis, we have selected new T47D cell lines, in which 100% of the cells are PR negative by FCM and by enhanced chemiluminescence immunoblotting, and in which a progesterone response element linked to a reporter does not activate transcription after progestin treatment (unpublished).

Does a 'PR' population model adequately describe cells such as those depicted in panels B and C of figure 1? Probably not. Bimodality of a single variable like PR hints at still greater numbers of subpopulations when a second variable is analyzed simultaneously. The simultaneous analysis of PR and DNA indices shows that V26 is a mixture of 47.2% hyperdiploid (HD) cells and 52.8% hypertetraploid (HT) cells (figure 2, showing the univariate DNA data on the X-axis and the univariate PR data on the 4-axis). The HD cells, with 24.3% of cells in S and G_2M, grow slightly faster than the HT cells, which have 17.0% of cells in the proliferating fraction [35]. Combining the PR and DNA data (figure 2) shows that there are two distinct HD subpopulations: one has cells with low PR levels (a), and the other has cells with high PR levels (b). In addition, the HT cells also contain subpopulations with low (a′) and high (b′) PR levels. Thus, there are at least four subpopulations in this cell line, each having a different combination of PR, DNA content, and mitotic indices. V22 cells are similarly heterogenous.

Tumor cell 'remodeling' by tamoxifen

The practical consequence of this PR heterogeneity in breast cancer cells is illustrated by an experiment in which the $T47D_v$ cell line was treated of eight weeks with or without 1 μM tamoxifen. Tamoxifen at 1 μM generally suppresses growth and PR in estrogen target tissues [26] that carry a normal ER, and is the major endocrine therapeutic drug used in breast cancer [36].

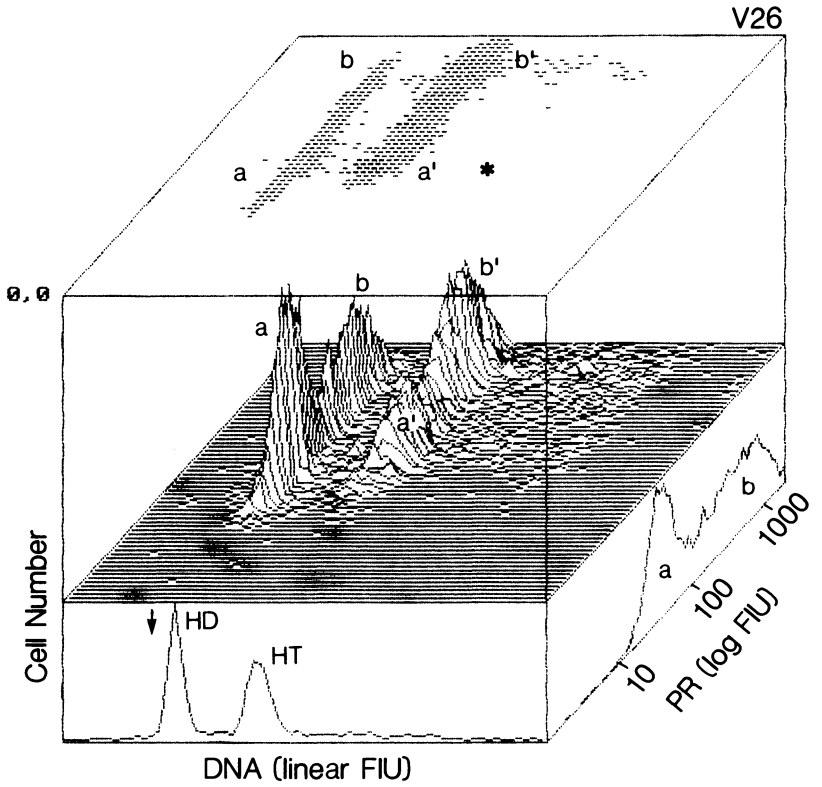

Figure 2. Multiple cell subpopulations in one cell line demonstrated by simultaneous FCM for PR and DNA. Cells from line V-26 were harvested and analyzed for PR and DNA by dual parameter FCM; 10,000 cells were analyzed. Cube and bivariate plots of simultaneous DNA (linear scale on x-axis) versus PR (log scale on y-axis). PR levels are plotted on the log scale to reflect the nearly 100-fold range of values. Cell number is on the z-axis, and peak heights represent the frequency of the component subpopulation. The DNA peaks are HD or HT; arrow, diploid DNA content. PR peaks correspond to populations containing low ('a, a'') or high ('b, b'') PR levels. The upper surface of the cube shows the bivariate DNA content and receptor distribution at a plane that intersects the proliferating cells. *, area free of proliferating cells derived from population 'a''.

But what is the effect of tamoxifen in cells that carry not only normal but also variant ER?

Figure 3 shows a univariate PR analysis of control cells (−TAM) and 1 μM tamoxifen-treated cells (+TAM), with the two curves superimposed to demonstrate the shifts in PR patterns after eight weeks under the influence of the drug. Cell growth was suppressed by 40% (not shown), and there was a marked shift in the PR pattern — mostly to the left, reflecting a complete loss or decrease in PR, as shown by the large shadowed area. However, there was an unexpected small subpopulation shifted to the right, in which

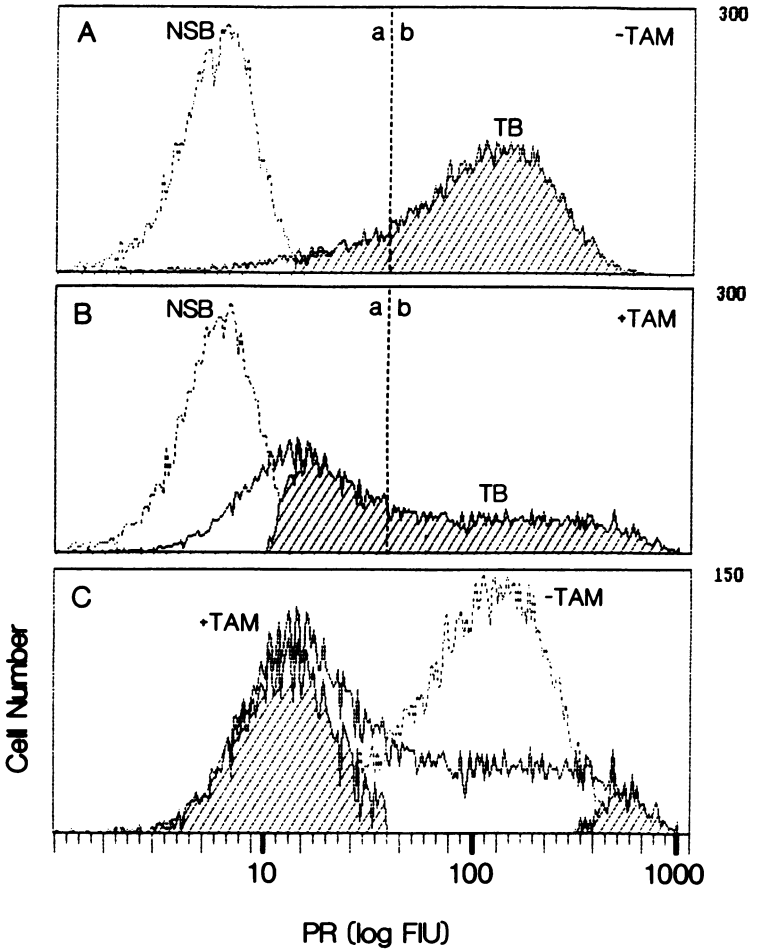

Figure 3. Tamoxifen remodeling of PR subpopulations in the T47D$_v$ cell line. Total (TB) and nonspecific (NSB) PR signals and simultaneous DNA levels were measured by flow cytometry in T47D$_v$ after eight weeks of growth in control med (−TAM) or in 1 μM tamoxifen (+TAM). PR FIU levels in 10,000 cells of each set were analyzed and are shown. In (A) and (B), specific binding was calculated by curve subtraction and is shown by the shaded area. TB curves from control (−TAM) and tamoxifen-treated (+TAM) cells were superimposed, and compared to the nonspecific binding peak (NSB). In (C), PR subpopulations present in +TAM, which are excluded in −TAM, were calculated by curve subtraction and are shaded. Similar data were originally published in Graham et al. [35]. Reproduced with permission.

PR levels have apparently been induced by tamoxifen. This subpopulation represents 5.2% of the cells in this experiment, and contains an average PR of 571.6 fluorescence intensity units (FIUs), or greater than one million PR molecules/cell — levels that none of the untreated cells attain. Thus, tamoxifen, while decreasing PR levels in a majority of cells, appears

paradoxically to *increase* PR levels in a selected subset of cells. The ominous consequence of tumor cell populations that may be stimulated by tamoxifen requires little comment.

In addition, analysis of the DNA indices [35] demonstrates that tamoxifen has a dual effect on proliferation. First, for the same number of cells, fewer tamoxifen-treated cells are in mitosis, and second, the populations that are in mitosis under tamoxifen differ from the controls. Thus, while the overall growth of the tamoxifen-treated cells lags behind that of the control cells, the DNA data show what we term the *remodeling* influence of the drug, that is, the growth and emergence of at least two new subpopulations of cells that are not present in controls: a PR-negative or low-PR, HD subset; and an ultra-high PR, HT subset. If the biologic behavior of this cell line mimics the pattern seen in patients with metastatic breast cancer who have an initial growth-inhibitory response to tamoxifen but then relapse, it may be these emerging subpopulations that lead to later tumor progression and our present impression of recurrent breast cancer as an incurable disease.

A variety of mechanisms have been proposed for development of the acquired resistance to tamoxifen that arises in animal model systems [37] and in virtually all patients [38,39] undergoing hormone therapy. Genetic mechanisms include the variant and mutant forms of ER described above, which may exert dominant controls over estrogen- and antiestrogen-regulated growth. Additionally, heterogeneity of ERs and mutant ERs may in part explain the extreme PR heterogeneity documented here. Epigenetic mechanisms center on pharmacokinetic issues related to drug absorption, distribution and metabolism. While some of the metabolites of tamoxifen are more potent antiestrogens than the parent compound [36], other metabolites may be estrogenic [40]. Recent data indicate that tamoxifen and its antiestrogenic metabolite, *trans*-4-hydroxyta-moxifen, may be selectively excluded from tamoxifen-resistant breast cancers, or be further metabolized to relatively inactive forms [41].

Although in different tumors and different cells, one or both general mechanisms of resistance may become operative, we propose that tumor progression to the resistant state includes the selection and expansion of cell subpopulations, some of which remain strongly influenced by tamoxifen. That hormone treatment may itself provide the selective remodeling pressure is suggested by the studies described here, and by studies showing that human breast cancer cells change significantly in response to hormone deprivation [40,42,43] or stimulation [44].

Our data suggest that subsets of cells may actually be stimulated by tamoxifen. Little is known about the mechanisms underlying these 'agonist' actions of some antiestrogens (but see progestin resistance, discussed below). It is possible that binding of tamoxifen to specific types of ER mutants establishes a transcriptionally productive receptor complex. The agonist activities of tamoxifen are usually expressed at low doses [26], but they may also be tissue specific [45]. While tamoxifen at high doses suppresses PR, it

induces PR at low doses [26]. The tumor 'flare' that occurs during initiation of tamoxifen therapy in patients [46], and the withdrawal response that occurs when the drug is stopped after tumors become resistant [47], may also be explained by this property. Additionally, we have previously shown that pretreatment of cells with an antiestrogen can sensitize them to a subsequent challenge with estrogens. In this state, cells respond more rapidly and more extensively to estrogens; for example, superinduction of PRs is observed [48]. It is possible that antiestrogen pretreatment can sensitize tumor cells to low levels of estrogens, or to weak estrogens, to which, in other settings, they would be unresponsive. The molecular mechanisms underlying the phenomenon of superinduction remain unknown.

Progestin resistance

The emergence of hormone-resistant cells eventually reduce the effectiveness of all therapies in advanced breast cancer, and progestin agonists or antagonists are unlikely to be exceptions. This is essentially an unexplored field. To address possible mechanisms of progestin resistance, Murphy et al. [49] generated a subline of T47D cells that are resistant to the growth-inhibitory effects of progestins. This was done by sequential selection in medium containing 1 μM MPA. The cells remained PR positive, but receptor levels were halved. Transforming growth factor-α and EGF receptor mRNA levels were both increased. The investigators suggest that increased growth-factor expression and action, and decreased PR levels, may be involved in the development of progestin resistance. Also, as shown above, it is likely that extensive heterogeneity exists in the PR content within cell subpopulations of tumors that are PR positive. Factors or treatments that lead to the selection and expansion of PR-poor or PR-negative populations would, in the long run, produce progestin resistance. Additionally, as reviewed briefly below, novel mechanisms involving normal PR may produce inappropriate responses to progestins, and especially to progesterone antagonists.

Progestin resistance and the two natural PR isoforms

Complementary DNAs for chicken PR were cloned by Jeltsch et al. [50] and Conneely et al. [51] and for human PR by Misrahi et al. [52]. The single-copy human PR gene encodes at least nine messenger RNA species ranging in size from 2.5 to 11.4 kilobases. The nine messages direct the synthesis of at least two, and possibly three, structurally related receptor proteins. The two major protein species, the B- and A-receptors, were originally described in the chick oviduct. Subsequent studies using breast cancer cells showed that human PR also exist as two isoforms, namely, the 116 kilodalton (kDa) B-receptors and N-terminally truncated 94 kDa A-receptors. While A-receptors were originally thought to be produced by a proteolytic artifact,

it is now clear that these amino-terminal truncated receptors, at least in chickens and humans, are a naturally synthesized form. In human endometrial carcinoma and breast cancer cell lines, the two receptor isoforms are expressed in approximately equimolar amounts. It is not known whether this quantitative relationship between the two isoforms is maintained in all human target tissues and tumors, and the mechanisms for their differential regulation are not known, but at least 2 of the 9 mRNA species lack the translation initiation site for B-receptors and can therefore encode only A-receptors. These messages arise by transcription from an internal promoter in the human PR gene. Five other message species can potentially encode both receptor isoforms, by alternate translation initiation from two in-frame AUG codons. In theory, use of the upstream codon would generate the B-receptors and use of the downstream codon generates the A-receptors, but it is not known whether initiation at the downstream site actually occurs in intact cells (reviewed in [53] and references therein).

PRs are unique among steroid receptors in having two naturally occurring hormone-binding forms, and this structural feature may have important functional implications with respect to receptor function. Since both homo- and heterodimers can form between the A- and B-isoforms, three possible classes of receptor dimers (A:A, A:B, B:B) can bind DNA at a progesterone response element, each having a potentially different transcription regulatory capacity.

That this molecular heterogeneity is indeed translated into functional heterogeneity was first demonstrated by the study of Tora et al. [54] that assessed the cell-specific transcriptional activation of two different target genes by the chicken A- and B-receptors. Depending on the gene being modulated and the cell being analyzed, A-receptors can be stimulatory in a setting where B-receptors are inactive or are inhibitory.

Recent molecular analyses of hPRs are beginning to address mechanisms of resistance to progesterone antagonists. These studies, like those for ER discussed above, also suggest that the term *resistance* may be inappropriate. *Resistance* implies that the tumor stops responding to the drug, and ignores it instead. This may be an oversimplification, since under appropriate conditions, progesterone antagonists can behave like agonists. Rather than ignoring the drug, the cell alters its transcriptional response to the drug. How is that possible? One explanation focuses on mutant PR. Unlike the case for other members of the steroid-receptor family, no examples of natural PR mutants have yet been reported. The explanation for this may be that, unlike mutations in androgen receptors, systemic mutations in PRs are imcompatible with life. However, theoretically, acquired receptor mutations could develop in tumors as one mechanism for the development of resistance, and a systematic search might demonstrate them. In view of this, Vegeto et al. [55] recently showed that a synthetic hPR mutant with a 42-amino-acid truncation at the C-terminus of the 933 amino acid hPR B-receptors loses its progesterone-binding ability but retains RU486-binding

ability. This synthetic receptor mutant, when occupied by RU486, has agonist transcriptional activity.

Additional models of resistance associated with functional reversion have emerged from our recent studies of progesterone antagonists as transcriptional inhibitors [56,57]. These studies provide two scenarios in which antagonists can have inappropriate agonist-like effects on normal PR. We believe that the mechanisms underlying these functional switches may be analogous to mechanisms by which tumor cells become hormone resistant. The first case in which an antagonist behaves like an agonist involves studies with the human breast cancer cell line T47D, which expresses high natural levels of PR and is stably transfected with the progestin-responsive MMTV promoter linked to the CAT reporter. In this model, PR-antagonist complexes are transcriptionally silent, and as expected, the antagonists inhibit the effects of agonists. However, if cAMP levels are elevated in these cells, the antagonists become strong transcriptional stimulators — they behave like agonists. This functional reversal occurs only if the antagonist-occupied receptors are bound to DNA, and it does not involve hPR phosphorylation by cAMP. The model we propose involves transcriptional synergism, in which a promoter that is independently regulated by cAMP-responsive proteins, and by hPR, is selected for positive or negative transcriptional regulation through cooperative interactions between the two DNA-bound proteins [56].

The second case in which a progesterone antagonist behaves like an agonist involves the functional difference between progesterone A- and B-receptors [58]. A-receptors occupied by progesterone antagonists are transcriptionally silent on a progesterone response element (PRE) thymidine kinase promoter-CAT reporter. By contrast, in the same cells and with the same promoter-reporter, antagonist-occupied B-receptors strongly stimulate transcription. We have shown that, interestingly, this unusual property of antagonist-occupied B-receptors does not require the presence of the PRE. Furthermore, the agonist-like effects of antagonist-occupied B-receptors can be eliminated in the presence of A-receptors. Thus, antagonist-occupied A-receptors are *trans*-dominant repressors of B-receptors [57]. Our working model is that transcriptional stimulation by antagonist-occupied B-receptors proceeds through a mechanism in which the receptors do not bind to DNA themselves, but are tethered to a DNA-bound protein partner at the promoter. Antagonist-occupied A-receptors cannot bind to this protein. These data further suggest that antagonist-occupied B-receptors may be able to transcriptionally activate a gene that, lacking a PRE, is not a normal target for PR regulation.

Each of these recent experimental models suggests that resistance can be a condition in which tumors respond inappropriately to hormone antagonists. We have observed this with tamoxifen and with progesterone antagonists. These studies may also explain why, in some normal target cells, antagonists have tissue-specific, agonist-like activity. We propose that these agonist-like

effects occur on genes that are regulated by at least two signaling pathways. A steroid receptor antagonist complex, which is inhibitory by itself, may in the presence of other DNA-bound factors be switched to stimulatory activity through cooperative protein–protein interactions.

Summary

In summary, we propose that the molecular heterogeneity of ER in breast tumor cells characterized by the presence of mutant receptor forms generates the cellular heterogeneity evident when PR or DNA ploidy are analyzed in cell subpopulations. Furthermore, it is likely that cellular heterogeneity leads to the lack of uniformity in response to tamoxifen that we have described. We find that heterogeneity of PR distribution and DNA ploidy reflects existence of mixed subpopulations of breast cancer cells that are substantially remodeled under the influence of tamoxifen. It appears likely that rather than being resistant, different subsets of cells can be inhibited or stimulated by tamoxifen, and their suppression or outgrowth alters the phenotype of the tumor. PR heterogeneity in solid tumors of patients may predict for such a mixed, and potentially dangerous, response to antiestrogen treatment. Similarly, the molecular heterogeneity resulting from the presence of two normal PR isotypes can lead to inappropriate responses to progesterone antagonists in certain genes or cell types. These agonist-like responses are due to cooperative interactions between the receptors and other transcription factors. As we learn more about the heterogeneity of PR, ER, and other proteins in tumors, we may be able to recognize such lethal cell subpopulations, or combinations of regulatory factors. Specifically, with respect to tamoxifen, our data suggest that its use as a chemopreventant in women at high risk of developing breast cancer [59] should be viewed with caution, since in the presence of tamoxifen subpopulations of cells may arise that are stimulated, rather than inhibited, by the drug.

Acknowledgments

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III

Growth Factors, Receptors, and Polyamines

8. The epidermal growth factor family in the mammary gland and other target organs for ovarian steroids

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The influence of ovarian steroids on the growth and development of the normal mammary gland and mammary tumors in laboratory rodents is well known to the research investigator, and has emphasized the need to understand the pathways by which these compounds exert their effects on target organs. The clinical significance of the ovaries to breast cancer was evident around the turn of the century, when ovariectomy was shown to ameliorate the course of this disease in some women [1]. The high incidence of breast cancer in women in the United States and the marked reduction in incidence of this disease in women ovariectomized an early age suggest a permissive or promotional role for ovarian steroids [2–4]. Similarly, the influence of age at menarche or menopause on breast cancer risk may be explained by the duration or extent of exposure to ovarian steroids [5]. The long-term use of estrogens to relieve postmenopausal symptoms in women has already been linked with an increased incidence of endometrial carcinoma [6]. This exogenous use of natural or synthetic ovarian steroids and their antagonists for the purposes of estrogen replacement therapy, contraception, or cancer treatment and prevention has become a major health issue and dilemma, especially when we consider the millions of women treated with this category of drugs.

Over the past three decades, the discovery of numerous polypeptide factors, or growth factors, that could influence the growth and differentiation of various cell types in vitro and in vivo by interaction with specific receptors has led to the notion that such factors might mediate some of the actions of ovarian estrogens or progestins. It was reasonable to consider epidermal growth factor (EGF) as the elusive 'estromedin,' since this polypeptide stimulated in vitro proliferation of epithelial cells derived from organs such as the mammary gland [7], uterus [8], and vagina [9]. This notion is encouraged by the fact that levels of EGF in some organs could be influenced by sex steroids [10–12]. However, compelling evidence for a role of EGF — or another member of this family — as a mediator for steroid action in normal cells is still elusive. One impediment has been the limited progress in developing in vitro systems that mimic the biological actions of sex steroids in vivo. Normal epithelial cells from the mammary gland and uterus, for

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example, are generally refractory to the proliferative action of ovarian steroids when cultured in vitro [8,9,13]. Another impediment has been the absence of selective inhibitors of the EGF receptor or other steps in growth factor signaling pathways. The physiological role(s) of members of the ever expanding EGF family has not been forthcoming in the way observed for cytokines, such as the interleukins. The multiple sites of synthesis of EGF and transforming growth factor-α (TGF-α) preclude the classic organ ablation experiments. In spite of the high availability of EGF to researchers and the numerous reports of the effects of either EGF or TGF-α on various cells, we have yet to place these polypeptides in a functional biological circuit in vivo. Such a circuit would be composed of 1) a cell population that is a source for the growth factor; 2) a stimulus that regulates growth factor bioavailability; and 3) target cells that have specific receptors for the growth factor and are functionally regulated by ligand-receptor interactions. This circuit could be 'closed' by a single cell (autocrine) or by adjacent or proximate cells (paracrine). As will be discussed below, there is no strong evidence that indicates that EGF, per se, provides a positive in vivo signal for cell proliferation in normal or neoplastic cells; however, the expansion of EGF family members and corresponding receptors suggests that multiple aspects of cell behavior, including proliferation, will be regulated by one or more of these polypeptides.

The present review focuses on the structural and functional aspects of the EGF family of ligands and their properties in selected ovarian steroid target organs. Since similar functional aspects of EGF or other family members probably exist in ovarian steroid-responsive organs, the author felt compelled to include relevant studies of the uterus and vagina with those of the mammary gland. Within this context, emphasis will be placed on aspects of EGF that are distinct from those of TGF-α, since much more information has accumulated on these polypeptides than the more recently discovered members of this family.

Epidermal growth factor

Since its discovery, EGF has been shown to stimulate growth and influence other functional properties of numerous types of cells. On account of the thousands of publications related to EGF and the limited scope of this chapter, the reader is referred to more comprehensive reviews of this growth factor and its receptor [14–17]. EGF is a 53-amino-acid polypeptide originally isolated from the male mouse submaxillary gland [18,19]. Subsequent studies revealed that EGF and EGF transcripts occurred in other organs as well, such as the kidney [20], mammary gland [20,21], and uterus [12]. Human EGF, or urogastrone, was originally isolated from urine [22]; the sequence of this polypeptide is shown in figure 1. In both the mouse and human, the polypeptide originates from a much larger linear precursor of

Figure 1. Comparison of sequences of bioactive members of the human EGF family of growth factors. The sequences have been aligned to provide overlap of the cysteine residues. Shaded boxes depict conserved residues. Dashes were used to maximize homology where there were differences in the number of residues between overlapping cysteines. EGF and TGF-α are shown as complete mature forms, whereas only the EGF-like domains are shown for amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF) and heregulin-α (HRG-α). The numbers give the residue assignment according to the sequences reported for the proposed mature forms. The actual sequences of the mature forms for some EGF-like growth factors, such as HB-EGF, are either undetermined or variable.

~1200 amino acids, which exhibits ~66% overall sequence homology in these species [23–25]. The genes encoding the mouse and human EGF precursors occur on chromosomes 3 and 4(q25→27), respectively [26,27]. The EGF sequence (residues 971–1023 in the human precursor) is located near the C-terminus of the precursor (see domain 9 of the EGF precursor in figure 2). Several notable structural features of the EGF precursor might help in understanding the functions of this protein and, perhaps, EGF. A hydrophobic stretch (residues 1033–1057) of amino acids exists just C-terminal to the EGF domain and, as in other precursors of the EGF family, apparently serves as a membrane-spanning region. Experimental evidence supports that the EGF precursor does occur as a transmembrane protein in epithelial cells of the kidney [12,28] and lactating mammary gland [21]. This suggests that the sorting and trafficking pathways for the precursor are tissue specific, since in organs such as the mouse submaxillary gland, the precursor is apparently rapidly processed intracellularly following translation and mature EGF packaged in secretory granules by cells of the granular convoluted tubules [29–31]. Transfection experiments with a human EGF precursor cDNA showed that the translation product spans the cell membrane with the N-terminal aspect, including the EGF sequence, oriented on the exterior face of the cell membrane [25]. Compatible with the precursor as a transmembrane protein is the finding that release of the protein from purified kidney membranes does not occur with high salt or alkaline buffers but requires detergent [32]. As with many transmembrane proteins, the mouse and human precursors have canonical sites for glycosylation in the extracellular domain [20,24]. The solubilized mouse kidney precursor can bind to wheat germ and lentil lectin columns [32]. Labeling of the precursor with [^{3}H] mannose and [3H] glucosamine was demonstrated in NIH 3T3 cells transfected with prepro EGF cDNA under a metallothionein promoter [33]. Perhaps the most notable feature of the EGF precursor — at least by comparison to the precursors of other members of this family — is the presence of multiple EGF-like[1] domains (nine in the human precursor); each of these domains (~40 residues) has six cysteines that are assumed to form the disulfide motif characteristic of EGF [23–25,34]. As shown in figure 2, these EGF-like domains are organized in two major groups that are separated by approximately 270 amino acids; together, these domains constitute over one fourth of the linear sequence of the precursor. It is not known whether any of these other EGF-like domains are specifically elaborated during proteolytic processing or can react with the EGF receptor. The detergent-solubilized intact precursor can compete with ^{125}I-EGF for binding to intact fibroblasts, activate the intrinsic tyrosine kinase activity of the EGF-receptor, and stimulate cell growth in vitro [32,33]. This activity could be conferred by EGF tethered to the precursor backbone, since most of the repeat EGF-like domains have at least two substitutions at invariant residues present in sequences known to bind and activate the EGF receptor. In accord with this notion, an EGF-like domain (residues 361–406 of the

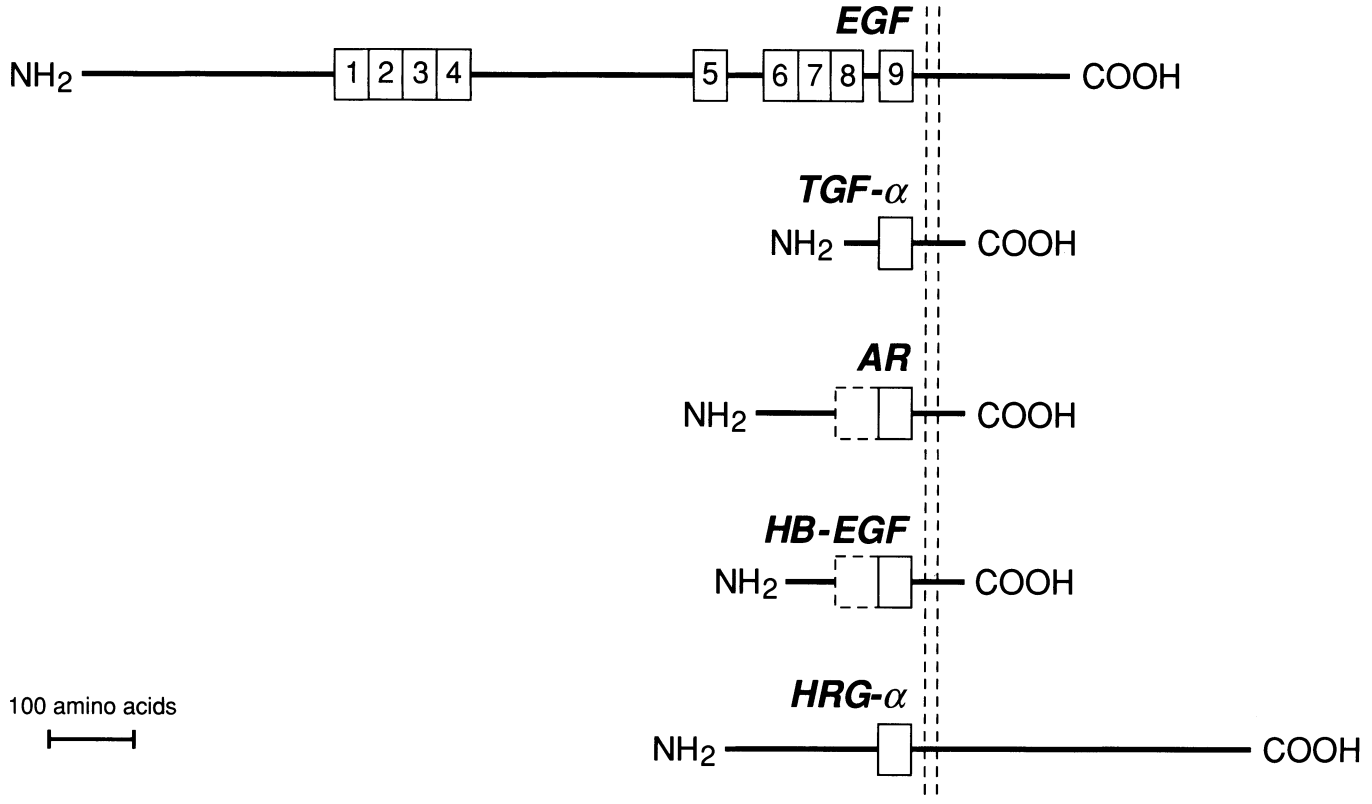

Figure 2. Comparison of the structural aspects of the human EGF family members. The solid-line boxes represent EGF-like domains (first to sixth Cys residue) for each precursor. The corresponding sequence for EGF occurs in box 9 of the EGF precursor. The broken-line boxes next to the EGF-like domains for AR and HB-EGF represent N-terminal extensions present in the apparent mature polypeptides that were isolated. The vertical parallel broken lines depict a membrane intersecting with the reported transmembrane domain for each precursor. Each precursor is assumed to have an orientation such that the N- and C-terminal region constitutes the ectodomain and cytoplasmic domain, respectively.

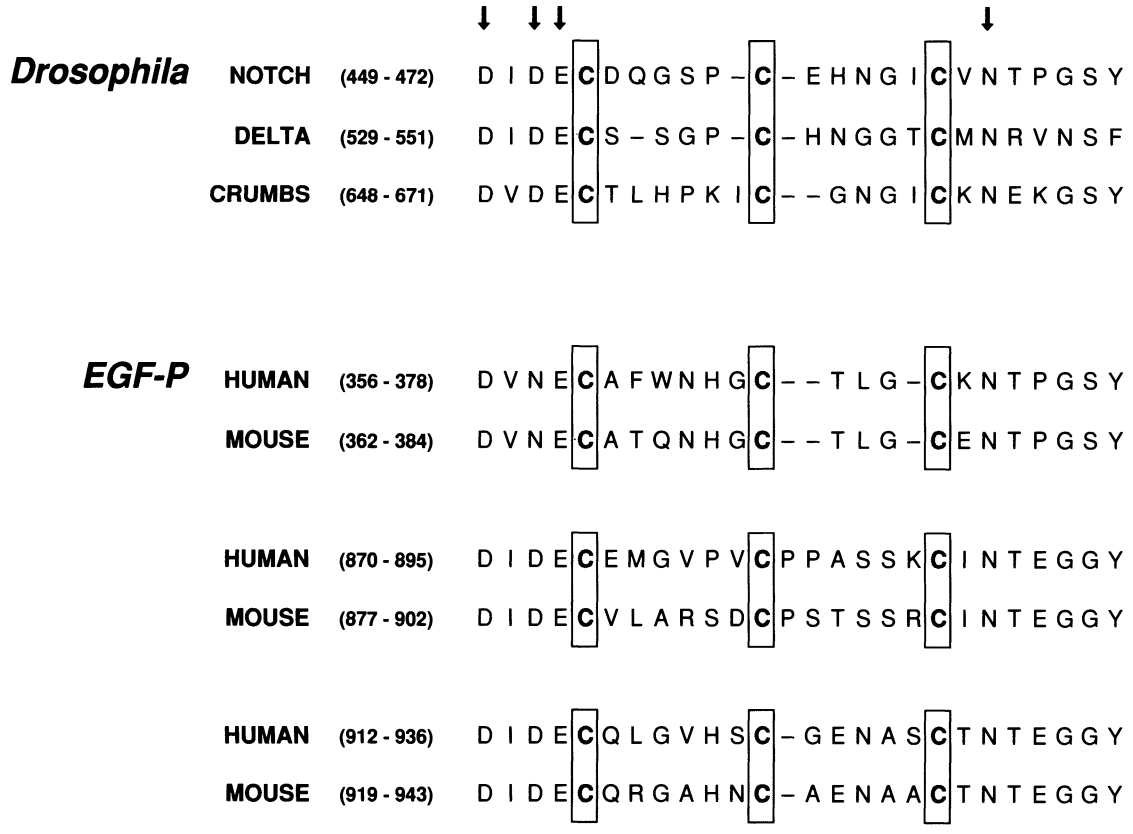

Figure 3. Regions in EGF-like domains of the human and mouse EGF precursors (EGF-P) that have putative calcium-binding consensus residues. Arrows denote residues that are considered to participate in forming the calcium-binding motif. A comparison is made with the proposed calcium-binding motifs found in EGF-like domains of Drosophila *Notch*, *Delta*, and *crumbs*.

mouse precursor) that was synthesized and renatured did not compete with binding of ^{125}I-EGF to mouse liver membranes (RP DiAugustine, unpublished observation).

Nonmitogenic proteins that are known to contain EGF-like domains compose a functionally diverse group of proteins that include serine protease coagulation factors, thrombospondin, entactin, LDL receptor, laminin, TGF-β1 binding protein, *lin-12* gene product of *C. elegans* and the *Delta*, *Notch*, and *crumbs* gene products of *Drosophila* [17,35]. These domains could provide specific ligand or recognition sites for cell surface or extracellular matrix proteins. Since the EGF precursor appears on the luminal aspect of epithelial cells of the kidney [28] and lactating mammary gland [21], it is unlikely that this protein has a role in cell–cell interactions in these organs. Some EGF-like domains in the aforementioned proteins can bind Ca^{2+}, and a consensus sequence has been proposed within an EGF-like structure that apparently confers this property [36,37]. Three of these putative Ca^{2+}-binding motifs exist within the EGF precursor: the sequences that form the putative Ca^{2+}-binding pockets for the mouse and human EGF precursors are shown in figure 3. One such EGF-like domain occurs among the 'N-terminal' group of EGF-like domains; the other two occur in tandem and precede the EGF sequence (domains 2, 7, and 8 of the hEGF precursor in figure 2). These Ca^{2+}-binding consensus sequences are not found in precursors of 'receptor reactive' family members that include TGF-α, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, or the heregulins that are described below (figure 1). Site-directed mutagenesis experiments with factor IX have led to the proposal that the EGF-like Ca^{2+} binding domain is essential in the interaction with other components of the coagulation cascade [36]. A β-hydroxy group has been reported on a conserved aspartic/asparagine residue following the third cysteine residue of the domain for some of the coagulation factors [38], the LDL receptor [39], thrombomodulin [39], and the TGF-β1 binding protein [35]. However, it is not known whether such a posttranslational modification occurs in the EGF precursor. The sequestration of Ca^{2+} by the membrane-bound EGF precursor might influence interactions with extracellular or membrane-associated proteins, such as enzymes that function in proteolytic processing. Alternatively, this property could be one of a series of related steps by a pleiotropic precursor protein that can sequester and facilitate delivery of Ca^{2+} and also trigger influx of Ca^{2+} by activation of EGF receptors [40] (see figure 4).

The deduced EGF precursor sequences obtained for several species reveal Arg-X cleavage sites at both the N-and C-terminal bonds that join EGF to its precursor, which suggests that a serine protease functions in the elaboration of mature EGF. Earlier studies demonstrated that an EGF-arginine esteropeptidase ('2+2') complex could be obtained from the mouse submaxillary gland when nondissociative conditions were used for isolation [41]. Binding of the enzyme to EGF apparently requires a C-terminal arginine,

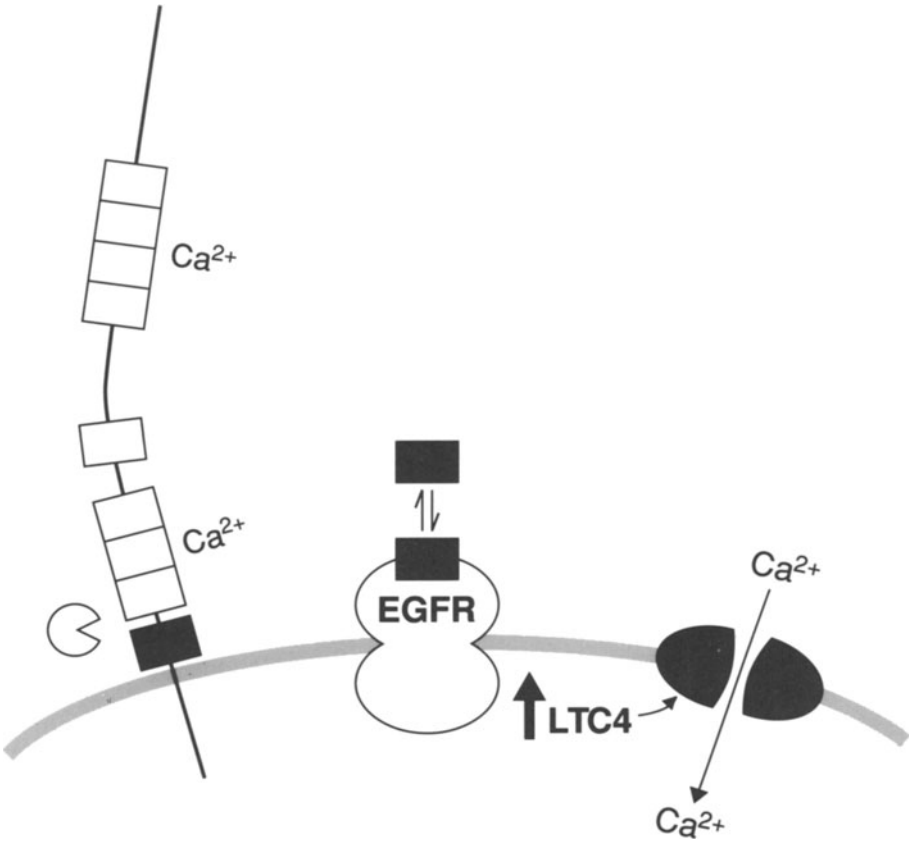

Figure 4. Proposed functions of the EGF precursor in the sequestration and transport of Ca^{2+}. The EGF precursor shown on the left might sequester Ca^{2+} through specific motifs in some of the EGF-like domains (open rectangles) in the extracellular (ectodomain) of the protein. Processing enzymes (Pac-man symbol) elaborate EGF (closed rectangles), which interacts with the EGF receptor (EGFR). This interaction stimulates the formation of a leukotriene (LTC4) through activation of a phospholipase (A_2). The leukotriene can act as a second messenger to directly stimulate Ca^{2+} channels (see [155]).

since EGF 1–48 and 1–51 do not form the complex [42]. Binding is EGF specific, since the enzyme does not bind to nerve growth factor [43]. More recent studies have identified a member of the kallikrein serine protease family, mGK-9, that can bind to EGF [44]. This enzyme localizes along with other kallikreins in the granular convoluted tubule cells of the submaxillary gland, where EGF is synthesized, and can hydrolyze the Arg-His bond of a mouse EGF precursor C-terminal cognate cleavage site peptide [45]. The mouse submaxillary gland is unlike most organs in this animal in that it synthesizes many members of the kallikrein gene family [46]. By contrast,

the mouse (BALB/c) kidney, where EGF occurs predominantly as the membrane-bound precursor, does not appear to express mGK-9. Instead, this organ expresses an mGK-6-type kallikrein [47], which was shown by in situ hybridization experiments to occur in the distal convoluted tubule and the thick ascending limb of Henle [48]. This is the same distribution shown for sites of EGF synthesis in the mouse kidney [20]. A kallikrein-like enzyme was found on the external surface of rat kidney cells [49], and incubation of isolated rat kidney membranes was shown to elaborate mature EGF, which was inhibited by aprotinin [50]. Recent studies reveal that transcripts for mGK-6, but not mGK-9, occur in the mouse lactating mammary gland [51], where processing of the alveolar cell membrane-bound EGF precursor is assumed to provide the mature EGF found in milk [21]. Kallikrein-like immunoreactivity has also been found in the lumen and along the lumenal aspect of the alveolar cell (GD Jahnke, unpublished observation). These cumulative findings suggest that a kallikrein participates in the processing of the EGF precursor, but the specific kallikrein member utilized — at least in the mouse — might vary with cell type or sorting pathway of the precursor. It is speculated that cleavage of EGF from the membrane-bound precursor occurs on the external face of the cell following secretion of a protease. Such 'ectoprocessing' has been proposed for other integral membrane proteins such as the polymeric immunoglobulin receptor [52] and other transmembrane precursors of EGF-like ligands, such as TGF-α [53]. For the present, more direct evidence is required to ascertain whether a kallikrein cleaves EGF from the precursor in vivo. The amino acid sequences bracketing other EGF-like domains of this protein would require different candidate proteases for cleavage. The processing reactions that provide mature EGF or other mature growth factors might be a critical juncture or rate-limiting step in the sequential events involved in a signaling pathway.

Transforming Growth Factor-α

TGF-α was first isolated from culture fluids of retrovirally transformed cells and tumor-derived cell lines on the basis of its capacity to compete with ^{125}I-EGF in binding to the EGF receptor and to induce anchorage-independent growth of normal rat kidney fibrolasts in soft agar in the presence of TGF-β [54–56]. The sufficient reviews [17,53,57–59] that cover various aspects of this polypeptide only require emphasis here of its salient structural and biological properties, especially those that set this factor apart from EGF. The sequences reported for TGF-α among various species reveal relatively high homology, unlike those reported for EGF. For instance, rat and human TGF-α are 50 amino acids in length, have a single EGF-like disulfide motif, and exhibit over 90% homology. The homology between human TGF-α and human EGF is about 35%, which accounts for the low cross-reactivity between these polypeptides in immunoassays. In spite of the limited

homology to EGF, TGF-α interacts with the EGF receptor and can mimic the biological actions of EGF qualitatively, if not quantitatively [57]. These actions are all assumed to occur by interaction of TGF-α with the EGF receptor, since no other TGF-α-specific receptors have been identified. The gene encoding TFG-α occurs on human chromosome 2(p11→p13), and a transcript size of ~4.8 kb is commonly observed in Northern blots of RNA obtained from various organs [17]. The mature form derives from a 159- or 160-amino-acid precursor, which is glycosylated (N-terminal of TGF-α) and has a transmembrane domain (C-terminal to TGF-α) (figure 2). As with the mature TGF-α, the overall precursor sequences of human and rat exhibit high homology (~93%); this homology is especially high (~99%) in the short C-terminal stretch that follows the TGF-α sequences. The comparable regions of the mouse and human EGF precursors exhibit about half this homology. The C-terminal cytoplasmic domain (~39 residues) of the hTGF-α precursor is about one fourth that of the hEGF precursor. One or more of the cysteines in this region may have a covalently attached palmitate, but the significance of this modification is not known [60]. The cumulative data from several studies of cells transfected with a TGF-α expression vector have given insight into the sequential aspects of sorting and processing of the TGF-α precursor. These studies indicate that the generation of soluble TGF-α occurs after the precursor is glycosylated and translocated to the cell membrane [53]. Some cell lines release a partially processed TGF-α precursor (17–19 kDa) into the medium that consists of an N-terminal extended and glycosylated form of TGF-α [61]. This form retains reactivity with antibodies to TGF-α (but not with antibodies to the pro-TGF-α cytoplasmic domain) and has the bioactivity of the mature 6-kDa polypeptide. On this basis, it was assumed that cleavage of the membrane-bound precursor occurred at a site between the C-terminus of the TGF-α sequence and the membrane-spanning domain. In other cell lines, the membrane-bound precursor first undergoes removal of an N-terminal portion without loss of the TGF-α epitope; the next step results in the appearance of mature TGF-α in the medium [62]. The hydrolysis required to form the fully mature TGF-α occurs at Ala-Val bonds at N- and C-terminal ends of the polypeptide, which suggests that an elastase-type protease functions in the processing of pro-TGF. Whether this is preceded by cleavage of a Lys–Lys bond between the TGF-α and the membrane-spanning domain is not known. Exogenous elastase can convert the high molecular weight forms of TGF-α to mature TGF-α [63]. Addition of leukocytic or pancreatic elastase to cells expressing the precursor at the cell surface releases mature, bioactive TGF-α into the medium [64]. More recent studies have shown that when mutated TGF-α precursor cDNAs designed to eliminate the Ala–Val cleavage sites are cloned into baby hamster kidney (BHK) cells, TGF-α is not detected in the conditioned media even though TGF-α immunoreactivity localizes at the cell membrane [53,65]. The importance of ectoprocessing in the biological function of TGF-α remains uncertain, especially in light of studies of the stimu-

latory properties of the membrane-bound TGF-α precursor [65–67]. When BHK cells (EGF receptor negative) expressing the 'cleavage resistant' pro-TGF-α were coincubated with EGF receptor-rich A431 cells, subsequent analysis revealed enhancement of phosphorylation of the A431 EGF receptor. BHK cells that were not induced to express pro-TGF-α failed to activate the EGF receptor. Expression of cleavage-resistant mutant forms of TGF-α in normal rat kidney (NRK) cells did not prevent formation of foci in culture or colonies in soft agar; colonies expressing the wild-type or mutated pro-TGF-α also formed tumors, whereas the uninfected cells, or cells transfected with the parental vector, never formed tumors [65,67]. Thus, a membrane-anchored pro-TGF-α could predispose adjacent cells to increased growth and neoplasia through activation of the EGF receptor.

To gain further insight into the function of TGF-α in normal tissues or in pathogenesis, several laboratories generated transgenic mice bearing rat or human TGF-α cDNA expressed under the control of various promoters [68–70]. The effects of TGF-α overproduction or constitutive expression in nearly all cases were tissue specific and presented a full spectrum of morphologic abnormalities that included increased DNA synthesis, hyperplasias, and carcinomas. The cumulative results support an autocrine/paracrine function for TGF-α in vivo related to growth regulation and the likelihood that enhanced expression of this polypeptide can predispose some cells to abnormal growth and neoplasia, as was demonstrated by earlier in vitro studies [71,72]. Transgenic mice generated with TGF-α cDNA under a metallothionein-1 promoter developed either an increased amount of liver parenchyma [68] or well-differentiated hepatocellular carcinomas [69]. The pancreas of the TGF-α transgenic mouse also revealed acinar metaplasia and interstitial fibrosis [68]. It is interesting that the metaplasia occurred in the absence of fibroplasia in those transgenics generated with the cleavage-resistant pro-TGF-α construct driven by an elastase promoter [68]. In transgenic mice generated with a TGF-α cDNA construct under an MMTV enhancer/promoter, the females developed mammary histologic abnormalities that ranged from hyperplasia of terminal ducts and alveolar glands to adenocarcinomas [70]. Hyperplasia was not observed until some time after four weeks of age, which suggests that an additional hormonal stimulus is required to increase expression through the MMTV enhancer/promoter. That the local effects were due to overproduction of TGF-α in the mammary epithelium was confirmed by in situ hybridization and immunohistochemistry and the observation of local up-regulation of EGF receptor mRNA. Overexpression, per se, was insufficient for induction of hyperplastic or neoplastic changes, since a number of organs, such as the kidney and salivary glands, exhibited markedly increased levels of transcript over controls, but did not reveal any pronounced morphological changes [68,70]. It is likely that the occurrence of neoplastic changes in the TGF-α transgenics requires an EGF receptor linked to a mitogenic pathway.

It is quite apparent from many studies that the expression of TGF-α is not

limited to only transformed cells, but can also occur in normal developing or adult tissues. For instance, transcripts for TGF-α have been detected in the preimplantation embryo [73], keratinocytes [74], activated macrophages [75], and in cells derived from the mammary gland [76–78], anterior pituitary [79], and trachea [80] (see [58] for review). It is likely that other new sites of TGF-α synthesis will be identified in addition to those already reported. An emerging view is that even though both EGF and TGF-α originate at multiple sites in vivo, TGF-α appears to have a more widespread distribution than that of EGF. The relatively low abundance of TGF-α has probably made it difficult to fully determine the distribution and properties of TGF-α in vivo. It is not known, for instance, whether multiple (cell-specific) systems exist in vivo for sorting the TGF-α precursor, as has been discussed above for the EGF precursor. Under the conditions of cell culture, especially those that favor growth or dedifferentiation, cells such as keratinocytes [74] or mammary carcinoma cells [81] generally express TGF-α but not EGF. There has been a tendency, then, to associate the autocrine/paracrine role of TGF-α with cellular self-renewal and transformation and that of EGF to regulation of a nonproliferative cell population. These differences will be more apparent in the ovarian-steroid regulated systems discussed in more detail below.

Other EGF-like bioactive polypeptides

Several novel polypeptides that possess an EGF-like cysteine motif (see figure 1) have recently been isolated from human or rodent cells on the basis of either their mitogenic properties or their capacity to interact with the EGF receptor or the structurally related $p185^{erbB2}$ protein. There are only a limited number of reports on these factors, and it is not known at the time of this writing whether the expression of any of these newer members are influenced by ovarian steroids in target organs such as the mammary gland. The expansion of both the EGF family of ligands and receptors, e.g., erbB2 [82] and erbB3 [83], clearly emphasizes the large number of regulatory signals that are transmitted at the surface of cells through interactions of polypeptides and their cognate receptors.

Amphiregulin (AR) and heparin-binding EGF (HB-EGF) were originally isolated from 12-O-tetradeconoylphorbol-13-acetate-treated MCF-7 human breast carcinoma and U-937 human histiocytic lymphoma cell lines, respectively [84,85]. Alignment of the EGF-like domains in AR and HB-EGF with EGF and TGF-α is shown in figure 1. AR and HB-EGF each exhibit about 40% homology to human EGF between the first and sixth Cys; when AR is compared to HB-EGF, this homology rises to 52%. These newer members of the EGF family can compete with ^{125}I-EGF binding to the EGF receptor and mimic some, but not all, of the actions of EGF in vitro. It is possible that the in vivo functional roles for AR and HB-EGF are exerted through

receptors that are different from, but structurally similar to, the EGF receptor. AR transcripts (1.4 kb) were found in a variety of human tissues and tumor cell lines; relatively high abundance was found in the ovary and placenta. Transcripts were also detected in normal human breast tissue. The highest levels of AR expression were observed in breast carcinoma cell lines that were estrogen-receptor positive and contained low levels of the EGF receptor [86]. Transcripts (~2.5 kb) for HB-EGF have been reported in preparations of poly(A)$^+$ RNA from human macrophages, skeletal muscle, lung, placenta, brain, and heart [85,87].

The *neu* proto-oncogene erbB2, or HER-2, encodes a transmembrane tyrosine kinase ($p185^{erbB2}$) that is structurally related to the EGF receptor [82,88]. Amplification and overexpression of *neu* in breast and ovarian cancers has been associated with poor prognosis [89]. Neither EGF nor TGF-α binds to or activates $p185^{erbB2}$ [90,91], which prompted a search for a polypeptide that could specifically interact with this putative receptor. This search was encouraged by studies that showed that unidentified polypeptides in conditioned media of some cells could influence $p185^{erbB2}$ function [90,92, 93]. Recent studies report the isolation of a novel group of proteins from conditioned media of human breast carcinoma cells (heregulins) or ras-transformed rat fibroblasts (Neu-differentation factor) that apparently bind to and induce phosphorylation of $p185^{erbB2}$ [94,95]. The Neu-differentiation factor (NDF) appears to be the rat homolog of the HRG-α form of the human heregulins (HRG-α, -B1, -B2 or -B3). HRG transcripts (6.6, 2.2, and 1.8 kb) were found in many different human tissues that included the breast, ovary, testis, and prostate [95]. The biological function of an HRG/$p185^{erbB2}$ autocrine loop is not known. Members of this group of polypeptides have been reported to induce growth or differentiation of various human breast cancer cells [94–96]. The mature (~45 kDa) form of the HRGs does not compete with ^{125}I-EGF binding to the EGF receptor [95]. Between the first and sixth Cys residue in the EGF-like domain, the HRGs exhibit only about 27% homology to hEGF; a greater homology of 35% and 45%, respectively, is observed with the same region of AR and HB-EGF (see figure 1). The precursors of AR, HB-EGF, and the HRGs all reveal a single EGF-like motif (figure 2), a transmembrane domain C-terminal to the EGF-like motif, and apparently undergo proteolysis in the ectodomain to elaborate the mature polypeptide.

Several members of the Pox virus family have been shown on the basis of polypeptide characterization or gene sequencing to encode a bioactive EGF-like growth factor. These factors may provide the virus with the capacity to stimulate proliferation of infected cells or uninfected proximate cell populations. The structural and biological properties of these factors have been previously reviewed [17]. The deduced amino acid sequence of the 13762 rat mammary adenocarcinoma sialomucin ASGP-2 reveals a membrane-spanning region and also two EGF-like sequences in the extracellular domain that have the consensus residues for bioactive members of the EGF family;

the ASGP-2 protein can apparently stimulate phosphorylation of the EGF receptor in A431 cell membranes [97].

Mammary gland

The mammary gland undergoes profound changes postnatally with regard to growth and differentiation. Hormones from several endocrine organs, such as the ovaries, pituitary gland, and adrenals, contribute to the morphological and functional changes observed during ductal morphogenesis, lobuloalveolar growth, and lactation [98–101]. The importance of ovarian estrogens, for instance, in the growth of the normal mammary gland and certain breast neoplasias has stimulated research to determine whether there are convergent aspects in sex steroid action and growth factor regulatory pathways. Several growth-regulating polypeptides have been found in the mammary organ or in cells derived from breast carcinomas [102]. The capacity for EGF to specifically bind to and stimulate growth of mammary epithelial cells in culture is well established [7,103], but a local role for EGF, per se, in mammary gland growth has not been demonstrated. There are, however, emerging findings that suggest that an EGF-receptor pathway is operative during ductal morphogenesis. The onset of ovarian secretion of estrogens triggers ductal formation in the mammary gland, and ovariectomy during this phase of development drastically reduces cell proliferation and formation of terminal end buds, which are the growth points for elongation and branching of the ducts within the fatty stroma of the mammary organ [104]. Local end-bud proliferation can be restored in the 'regressed gland' of the mouse by implanting a slow-release plastic pellet containing of 17β-estradiol into the mammary organ [105]. These findings and those demonstrating the presence of estrogen receptors in rodent mammary glands [100,105] suggest that estrogens have a direct action on the gland. That an EGF-receptor pathway might be activated by estrogens in the ductal growth program is suggested by experiments that showed that local implants containing either EGF or TGF-α stimulate end-bud structures in ovariectomized mice [106,107]. This effect could not be achieved with other polypeptide factors such as insulin, platelet-derived growth factor, transforming growth factor-β, and transferrin. Autoradiographic analysis of ^{125}I-EGF binding on sections of the mammary ducts during morphogenesis revealed labeling along the cap cell layer, which contains the apparent stem cells of the mitotically active terminal end buds [106]. Since end-bud regeneration could be achieved with either EGF or TGF-α in castrates, it is assumed that the EGF receptor at this site is linked to a mitogenic pathway and that synthesis of the receptor and postreceptor intermediates can occur independently of ovarian steroids. In this regard, growth of isolated mouse mammary terminal end buds in collagen gel was also stimulated in defined medium supplemented only with EGF [108]. Immunolocalization of EGF in the subadult

mouse mammary gland revealed immunoreactivity in some animals in luminal cells of the ducts and in cells of the interior layers of the end buds; positive staining was absent in the cap cell layer [107]. By contrast, TGF-α immunoreactivity was evident in the cap cell layer and in stromal fibroblasts at the base of the end buds, which suggests that this polypeptide serves as a positive growth regulator at this site. Since apparent estrogen receptors were detected in adjacent stromal cells but not directly in cap cells [105], it is speculative as to how a putative TGF-α autocrine loop in these cells is 'activated' by estrogens. The data from one study provide strong evidence for an estrogen-responsive element (ERE) in the 5′ flanking region of the TGF-α gene [109]. Such *cis*-acting regulatory elements could act as a convergent point for ovarian steroids and local growth factor pathways (figure

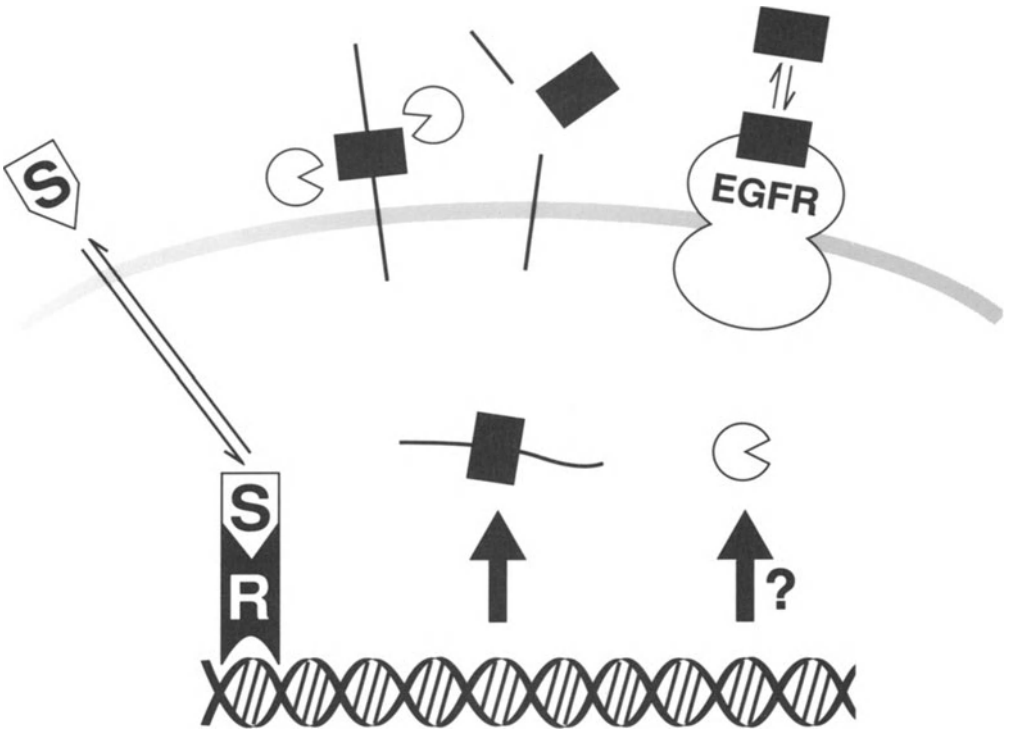

Figure 5. Proposed major steps involved in activation of an EGF receptor pathway by ovarian steroids. The steroid (S), such as estradiol, diffuses across the cell membrane of the target cell to interact with a complementary receptor (R), such as the estrogen receptor. The SR complex binds to a specific responsive element in the 5′-flanking region of the growth factor gene. This interaction leads to enhanced synthesis of the growth factor precursor, which is translocated to the cell membrane where it is processed by a specific protease(s). Expression of processing proteolytic enzymes (Pac-man symbol) might also be stimulated by ovarian steroids. The mature or soluble form of the growth factor interacts with the EGF receptor (EGFR) on the same or proximal cell.

5). As was mentioned above, the targeted overproduction of TGF-α in the mammary gland of transgenic mice can lead to hyperplasias and carcinomas [70], which further substantiates that ligands capable of activating the EGF receptor can function in positive growth regulation in the mammary organ. Numerous studies have been made of the putative role of TGF-α and other growth factors as autocrine mediators of estrogen-stimulated proliferation of breast cancer cells in vitro and in vivo, and the reader is referred to reviews on this specific topic [110–112]. Again, a major difficulty in ascertaining the role of EGF-like polypeptides as autocrine factors in normal or neoplastic cells is the paucity of specific inhibitors. The development and use of specific antibodies, chemicals, or molecular strategies that compromise the action of ligands or receptors and their signal transduction pathways are essential to understanding the functions of members of the EGF family and other growth factors [113,114]. In this context, a recent study demonstrated that the estrogen-stimulated proliferation of human breast carcinoma MCF-7 cells can be partially comprised in cells expressing a TGF-α antisense mRNA [115].

Following puberty and the formation of the mammary ductal tree, cyclical secretion of ovarian hormones continues to stimulate proliferation of mammary gland epithelial cells. Pharmacological studies of ovarian steroids in ovariectomized adult mice showed that maximal DNA synthesis occurred in the mammary gland when both 17β-estradiol and progesterone were administered [116,117]. Prolactin and adrenocorticosteroids are also required in many species to stimulate the lobuloalveolar growth manifested during pregnancy [101,118]. Progesterone and prolactin were both required to provide maximal proliferation of adult virgin mouse mammary epithelial cells in collagen gel; EGF can equal the combined potency of these hormones in this in vitro system, but exhibits no synergism when combined with progesterone and prolactin [13]. There is little understanding as to how endocrine hormones collaborate to stimulate mammary gland growth in adult animals and whether an EGF-like or other growth factor pathway is activated. Progesterone can stimulate the number of EGF receptors and also prepro-EGF transcripts in some human mammary carcinoma cells, but this does not result in increased proliferation of these cells [119,120]. A recent study examined binding of ^{125}I-EGF to mammary microsomal preparations from 5- and 10-week-old female mice [121]. Microsomes from intact or ovariectomized animals exhibited similar binding properties; however, treatment of the 10-week-old mice with estradiol and progesterone resulted in a twofold increase in EGF-receptor concentration when compared with that from ovariectomized controls. Treatment with estradiol or progesterone alone had no effect. It was also demonstrated that the increase in ^{125}I-EGF binding observed in mammary microsomes from estradiol-progesterone-treated mice was the result of a specific increase in the numbers of receptors in the epithelial compartment.

The mammary glands from immature female mice primed with estradiol/

progesterone undergo lobuloalveolar development in vitro in the presence of a defined medium containing insulin, prolactin, aldosterone, and hydrocortisone (see [101] for review). Withdrawal of the hormones, except for insulin, results in alveolar regression; a second round of alveolar development can be initiated provided that EGF is included in the culture medium with the original hormone cocktail [122]. Further studies with this explant system revealed that extracts of glands from the primed animals contained EGF-receptor reactivity but not EGF immunoreactivity [123]. The permissive action of EGF in inducing lobuloalveolar growth in mammary gland explants can also be achieved with TGF-α or a factor(s) in the extract of glands from estrogen/progesterone-primed mice; an extract of glands from unprimed mice has no effect [123]. Whether these results accurately reflect participation of an EGF receptor pathway in lobuloalveolar growth in vivo remains to be determined.

EGF transcripts can be detected in the mammary gland at different stages of development, making it somewhat difficult to assign a causal role to a particular stage of growth or differentiation [107,124]. There are, however, several studies demonstrating that mammary gland EGF expression in the rodent [20,21,125], and probably the human [126], is highest in the lactating, or fully differentiated, gland. Northern blots of RNA from mammary glands of adult virgin or pregnant female mice do not reveal the 4.7-kb EGF transcript [21,127]; a positive band, however, was observed in glands obtained at day 1 through day 18 of lactation [21]. Maximal induction of prepro-EGF mRNA in mammary gland explants from estrogen/progesterone-primed mice required the combination of insulin, hydrocortisone, and prolactin in the medium [128]. In addition, pituitary isografts placed in the mammary fat pads of female mice led to the localized induction of EGF immunoreactivity in the epithelium [125]. Thus, the expression of mammary EGF in the adult virgin mouse appears to be stimulated by elevated levels of prolactin and perhaps other lactogenic hormones. The appearance of EGF in the ducts of some mice during morphogenesis of the gland [107] might reflect elevation of blood growth hormone, a lactogen, which occurs postnatally in mice [129]. Immunolocalization studies of the mouse lactating gland with antibodies to mature EGF or the cytoplasmic domain of the EGF precursor reveal that this protein is synthesized by differentiated alveolar cells [21,125]. This increased relative abundance of EGF-specific transcripts during lactation accompanied by synthesis and processing of the precursor would account for the relatively high concentrations of mature EGF in milk [130,131]. The physiological function of the alveolar cell EGF precursor or milk EGF is not known. Exogenous EGF can inhibit casein and α-lactalbumin production by mouse mammary cells cultured in the presence of insulin, hydrocortisone, and prolactin [7,132,133]. Since the fully differentiated gland exhibits very little proliferation [134], EGF might function locally to regulate a specific transport system at the membrane of the alveolar cell or cells lining the gastrointestinal tract of the newborn.

Transcripts for TGF-α have been detected in the normal mammary gland by in situ hybridization with a labeled TGF-α antisense RNA probe [78]. Expression was found in ductal and alveolar cells of the virgin, pregnant, and lactating rat mammary glands and in nulliparous and parous human mammary glands. In pregnant rats, TGF-α mRNA expression was also present in a subpopulation of fibroblasts in the interlobular connective tissue and in the stroma adjacent to ductal epithelial cells. Northern blot analysis of poly(A)$^+$ mRNA isolated from the rat lactating gland revealed bands at ~4.8 kb and 1.6 kb. The level of transcript (in situ) increased between two- and threefold during pregnancy and lactation [78]. In accord with this, the lactating gland had the highest level of extractable biologically active TGF-α among the stages of mammary development examined [135]. TGF-α immunoreactivity has also been detected in human milk, but at much lower levels than that of EGF [136,137].

Uterus

The importance of estrogens in both the normal cyclical proliferation of epithelial cells of the endometrium and in endometrial cancer risk in humans is well known [6]. The proliferative response of rodent uterine cells to EGF in vitro [8] and the stimulation of uterine EGF receptor levels in vivo by estrogens [138,139] has prompted further study of members of this growth factor family in this organ. Prepro-EGF mRNA (4.7 kb) was detected in the immature CD-1 mouse uterus, although at levels that were orders of magnitude less than that in the submaxillary gland; transcript levels could be significantly increased by treatment with 17β-estradiol [12]. Positive immunostaining with EGF antiserum was present in uterine luminal and glandular epithelial cells, and Western blots of uterine membranes exhibited an EGF-immunoreactive band at Mr ~140,000. Mature EGF was found in the luminal fluid of estrogenized mice, suggesting that proteolytic processing of the uterine epithelial EGF precursor had occurred. Another laboratory demonstrated EGF immunolocalization in the uteri of ovariectomized adult CD-1 mice following treatment with estrogen [140]. Positive staining was observed in the luminal epithelium between 12 and 24 hours and then in the glandular epithelium by 48 hours after continuous exposure to 17β-estradiol. This effect could not be produced with progesterone. In situ hybridization experiments with cRNA and oligonucleotide probes confirmed that EGF-specific transcripts were restricted to epithelial cells of the mouse uterus. In accord with a specific response to estrogen, uterine epithelial EGF immunoreactivity was detected in intact female mice during late proestrus, estrus, and in days 1 and 4 of pregnancy [140]. In this regard, it is interesting that in the uterus of the cycling female rat, EGF receptor content is highest at proestrus compared to other days of the cycle [141]. The estrogenized mouse uterus can also form labeled mature EGF when this organ is incubated

with ^{35}S-Met/Cys [140], which further supports that de novo synthesis and processing of the EGF precursor occurs in this organ. DEAE cellulose column chromatography of extracts of the estrogen-stimulated mouse uterus revealed that a substantial portion of the EGF radioreceptor activity did not coelute with the EGF immunoreactivity. On this basis, it was proposed that another polypeptide with EGF-like bioactivity, such as TGF-α, could be present in the uterine extract [140]. Recent studies confirmed that the uterus synthesizes TGF-α. In one study, estrogens (diethylstilbestrol or 17β-estradiol) stimulated immature mouse uterine TGF-α mRNA levels in a dose- and time-dependent manner; expression was observed by six hours after administration of estrogen, which precedes the peak for DNA synthesis following this steroid [142]. Transcripts for this growth factor were not detected in uteri of mice treated with dexamethasone, progesterone, or 5α-dihydrotestosterone. The mature form of TGF-α was found in uterine luminal fluid of estrogenized mice at a level more than 20-fold greater that that previously found for EGF [12]. Treatment of the mice with TGF-α antiserum produced partial inhibition of estrogen-stimulated uterine cell DNA synthesis. Unlike the restricted localization of EGF in the epithelium of the murine uterus [12,140], TGF-α transcripts in this organ were detected in both the stromal and epithelial compartments [142]. However, the relative contribution of blood-borne cells to the distribution of uterine TGF-α mRNA needs to be evaluated. Another study examined TGF-α expression in the mouse uterus during the peri-implantation period [143]. TGF-α specific transcripts gradually increased in the uterus from day 1 to day 4 of pregnancy; in the first two days, transcript was localized primarily to epithelial cells, and on days 3 and 4, transcript also occurred in subepithelial stromal cells. In the postimplantation period (days 5–8), localization of TGF-α mRNA was found in the decidua at the implantation sites, which suggests that TGF-α has a role in both implantation and decidualization. The spatial and temporal differences between TGF-α and EGF in the rodent uterus suggest that these factors have different functions. Evidence for mRNAs encoding EGF and TGF-α in the human endometrium has been obtained by the reverse transcriptase-polymerase chain reaction (RT-PCR) on biopsy specimens [144]. Immunolocalization of EGF was found in glands and stroma of the human endometrium, but the intensity of staining was not related to the phase of the menstrual cycle [145,146].

Vagina

There are very few specific studies of the vagina related to EGF-like polypeptides. However, the existing findings related to this family of growth factors in this organ and other tissues with stratified squamous epithelia may provide significant clues as to the function of some of these polypeptides. During the menstrual cycle, the epithelium of the vagina undergoes cyclical

changes associated with varying levels of ovarian steroids. Ovariectomized rodents have a thin vaginal epithelium composed of only a few cell layers. The epithelium increases markedly in thickness and becomes keratinized following treatment with estrogens. As with other stratified squamous epithelia, such as the epidermis, this increased thickness results from proliferation and differentiation of cells in the basal layer. Immunohistochemical studies of the mouse vagina with EGF antisera revealed localization confined to the granular layer (stratum granulosum) that lies beneath the cornified layer [51]. This pattern of localization was only observed in the multilayered epithelium of estrogen-treated ovariectomized mice or in intact cycling mice; immunostaining was not detected in the thin epithelium of untreated castrales. Further studies showed that an antiserum to the cytoplasmic domain of the EGF precursor reacts with the borders of cells of the vaginal granular layer and that in situ hybridization with a prepro-EGF cDNA probe revealed transcripts associated with suprabasal cell layers.[2] These findings provide strong evidence for de novo synthesis of the EGF precursor at this site and contradict a role for EGF as a mediator of estrogen-stimulated proliferation of cells in the vaginal basal layers. The synthesis of this growth factor apparently occurs as a consequence of differentiation of this tissue, where it might have a regulatory role in the formation or posttranslational modification of cornified envelope proteins. The synthesis of the EGF/EGF precursor in the granular layer of the vaginal epithelium appears to be a common property of stratified squamous epithelia, since positive EGF immunolocalization was also found in the granular layer of mouse skin, buccal mucosa, esophagus, and tongue.[2] These findings are in accord with the EGF-immunoreactivity found in the suprabasal cells of the human esophageal mucosa [147] and sheep epidermis [148]. Localization was not observed in the pseudostratified epithelium of the trachea or in the transitional epithelium of the bladder of the mouse.[2] The granular layers of mouse skin have been reported to sequester Ca^{2+} extracellularly [149]. Ca^{2+} is important in the terminal differentiation of skin [150], and the influx of Ca^{2+} into cells of the granular layer may trigger aggregation and enzyme-catalyzed cross-linking of envelope proteins [151]. It is tempting to speculate that the EGF precursor in stratified squamous epithelia operates as both a reservoir of Ca^{2+} (through its putative Ca^{2+}-binding domains) and an activator of EGF receptor-mediated influx of Ca^{2+} (by elaboration of mature EGF). Processing of the EGF precursor in the granular layer probably occurs, since mature EGF is detected in the lavage fluid of the mouse vagina.[2] It is likely, then, that EGF is elaborated in the granular layer and influences either the cell where it originated or other cells in this layer. It is well known that the interaction of EGF with the EGF receptor can rapidly stimulate Ca^{2+} influx into cells in vitro [40,152–154]. One recent study showed that this property of EGF might occur as a result of activation of phospholipase-A_2 and the subsequent 5-lipoxygenase-catalyzed formation of leukotriene C4, which was proposed as acting directly on Ca^{2+} channels

[155]. It is not known whether this pathway exists in the granular layer of stratified squamous epithelia. Binding sites for ^{125}I-EGF have been demonstrated mostly in the basal layers of human skin [156,157] and mouse vaginal epithelium [158]. By contrast, studies with various EGF receptor antibodies revealed recognition in all the viable layers of skin [156,157]. This descrepancy might reflect occupancy or down-regulation of EGF receptor by an EGF-like polypeptide in the suprabasal cells. Understanding the role of the epidermal EGF/EGF precursor and cognate receptor systems might provide insight into a common function for this growth factor in other organs as well. Figure 4 depicts the putative functions of the precursor in both sequestering Ca^{2+} and activating Ca^{2+} influx through elaboration of EGF.

The expression of TGF-α by human keratinocytes in vitro has favored the notion that this polypeptide is a positive growth regulator for these cells and their vivo counterpart [74]. In contrast to the limited residence of EGF in the granular layer, TGF-α immunoreactivity in the vaginal epithelium of estrogen-treated ovariectomized mouse gave a diffuse pattern throughout the viable cell layers [51], which was reminiscent of the patterns observed in the epidermis with either TGF-α antisera or a TGF-α cDNA probe [74,159, 160]. Targeting TGF-α overexpression to the basal cell compartment of the mouse epidermis was accomplished with a keratin K14 promoter and resulted in transgenic mice with increased basal cell proliferation and thickening of the epidermis in many areas; the vaginal epithelium of mature females also exhibited increased thickening [161]. Such studies suggest that activation of basal cell EGF receptors is utilized in the normal positive regulation of cell proliferation in stratified squamous epithelia. However, more direct evidence is required to establish whether or not the TGF-α/TGF-α precursor confers the natural signal for this process.

Conclusions

Through the synthesis and secretion of such hormones as the estrogens and progestins, the ovaries markedly influence the pattern of gene expression in target organs. This influence can be manifested by changes in the rate of cell proliferation, morphology, permeability, and composition. These changes occur as a consequence of a transcription factor formed in part by the binding of the steroid and its cognate receptor in the nucleus. Since growth factors such as EGF and TGF-α can supplant some of the actions of ovarian steroids and are synthesized by target organs, it is reasonable to propose that some of the effects evoked by these steroids are mediated by activation of the EGF receptor. Activation of the EGF — or a related — receptor by increasing the bioavailability of the EGF-like ligand might represent an important rate-limiting step in vivo and a point of convergence of steroid action in an autocrine or paracrine circuit. The cumulative data, so far, suggest that each member of the EGF family synthesized by the mammary

gland has a different hormonal — or local signal — requirement for gene expression. In addition to influencing the level of growth factor transcript, endocrine hormones might also affect the postranslational steps required for sorting or the elaboration of the mature species of the growth factor. We currently have little knowledge of the nature or regulation of proteolytic enzymes that participate in vivo in the processing of the transmembrane precursors of EGF-like growth factors. It is now clear that in some cell populations the EGF receptor is linked to a mitogenic pathway, whereas in other cell populations this receptor provides a nonmitogenic function, perhaps regulating ion flux at the cell surface. Is there a division of labor among the EGF family members? Does EGF have equivalent functions in the mammary gland and in the kidney? Does TGF-α provide a proliferative signal in both the mammary gland and skin? Some conceptual hints as to growth factor function might be readily gleaned from observing sites and level of synthesis while the target organ, such as the mammary gland, goes through various stages of development or observing how the autocrine components respond to exposure or withdrawal of one or more of the ovarian or pituitary hormones.

The discovery of numerous growth factors and complementary receptors affirms that multiple homeostatic mechanisms are mediated by ligand recognition at the cell surface. In essence, growth factors and cytokines have become the language of cells. The current array of molecular and biological tools available should enable investigators to understand the precise functions of the EGF-like growth factors and the signal transduction pathways they excite or inhibit to modulate cell behavior. These experimental pursuits should ultimately provide a rationale for decreasing the risk or progression of neoplasms in the mammary gland.

Given the thousands of publications related to the EGF family, the author was compelled to limit the citation of many excellent studies for relevance and brevity. Forgiveness is asked of those who have made seminal contributions in this field and were not cited.

Notes

1. The terms such EGF-like, EGF-related, or EGF-type have taken hold in the literature to broadly describe a polypeptide with either structural or biological properties that resemble those of the parent EGF.
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9. Molecular and clinical aspects of the Neu/ErbB-2 receptor tyrosine kinase

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Tumorigenesis in model experimental systems involves multiple steps that include activation of cell growth and inactivation of inhibitory processes [1,2]. Tumor development in humans is thought to reflect the multiplicity of events in the accumulation of independent mutations that affect clonal growth of the cancerous cell [3]. The identity of the group of genes that confer malignancy in vitro appears to be similar to the set of genes that are mutated in animal and human tumors [4]. All the genes whose products undergo mutational activation (oncogenes) encode key regulatory elements in signal transduction pathways that lead to cell growth [5]. Thus, oncogenic proteins may be growth factors, their membrane receptors, cytoplasmic effector proteins, or nuclear factors that control gene expression. In animal model systems, altered forms of receptors for growth factors can induce tumorigenesis. Examples are the products of the *fms* and *erb*B retroviral genes that encode portions of the receptors for colony stimulating factor 1 (CSF-1) [6] and epidermal growth factor (EGF) [7], respectively. However, the possibility that modifications of growth factor receptors may be one of the molecular steps that are crucial for tumor development in humans remains open.

Cellular proliferation and progressive acquisition of a specialized phenotype involve complex intracellular signaling networks and intercellular communication mechanisms, which include surface- and matrix-anchored cell adhesion molecules as well as soluble polypeptide growth factors. These are usually secreted proteins of low molecular weights that function in paracrine or autocrine fashions [8]. Growth factors cause cells in the resting or G_0 phase to enter and proceed through the cell cycle. This progression usually requires the coordinated action of two or more growth factors, and oncogenes appear to replace the action of these growth factors [9]. Certain polypeptide factors can also promote differentiation and morphogenic processes [10]. One of the major incentives for studying the processes of proliferation and differentiation stems from the recognition of their potential contribution to tumorigenesis [8,9,11,12]. Indeed, many transformed cells have been found to produce growth factors, suggesting that autocrine stimulation of cell growth may lead to transformation. In addi-

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tion, overexpression of growth factors and their receptors can lead to transformation of certain established cell lines [13], and lastly, blocking of autocrine stimulation of tumor cells may result in decreased growth in vitro [14].

Cells of most tissue types are targets of growth factors that mediate their effects by binding to surface receptors. The oncogenic receptors belong to a family of cell surface molecules that is characterized by intrinsic tyrosine kinase activity [15]. Binding of the ligand to the extracellular domain of the receptor activates the cytoplasm-facing catalytic activity by means of dimerization and autophosphorylation [16]. This results in recruitment of signal-transducing enzymes from the cytosol to the inner surface of the plasma membrane [17]. Each tyrosine kinase receptor appears to be characterized by a unique combination of signal-transducing molecules that collectively identify the specific message to the nucleus [18]. However, structural and functional landmarks define subtypes of receptors. One of these groups includes EGF-receptor, Neu/*Erb*B-2 and *Erb*B-3/HER-3 proteins [19,20]. The Neu protein, which is also called HER-2, was implicated more than any other growth factor receptor in human cancer. The present review will try to combine molecular analyses of Neu with clinical and experimental data from animal model systems in order to provide a comprehensive basis for understanding its potential role in cancer.

Oncogenic forms of Neu

The Neu protein, like other receptor tyrosine kinases, is an allosteric enzyme whose catalytic function is regulated, either positively or negatively, by noncatalytic sequences at the extracellular-, transmembrane-, or carboxy-terminal portion. Accordingly, mutations that affect these regions influence the enzyme and thereby may activate its dormant transforming potential. Originally, the oncogenic potential of the receptor was unveiled by a carcinogen-induced point mutation within the *neu* gene [21,22]. Later studies that employed transfection of the artificially modified gene showed that truncations and overexpression can also release the transforming ability (figure 1).

Point mutation

BDIX rats that were injected with nitrosoethylurea 15 days after conception gave birth to offspring that developed tumors and disorders of the nervous system [23]. The DNA of 4 out of 6 cell lines that were derived from these tumors induced the formation of foci in a monolayer of mouse fibroblasts [24]. The transfected cells were tumorigenic in appropriate host mice and elicited a strong immune response against a 185-kilodalton (kDa) phosphoprotein [25]. The transforming gene was isolated in cDNA and genomic

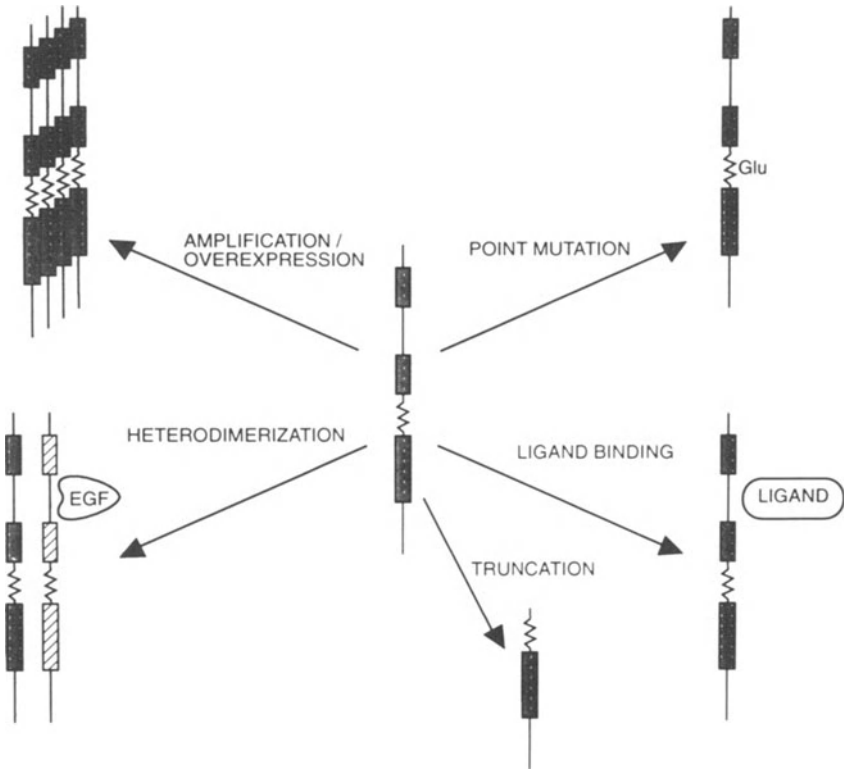

Figure 1. Oncogenic activation of Neu. The Neu protein is schematically represented. The zig-zag line indicates the transmembrane domain that connects the extracellular portion (the two cysteine-rich domains are shown by boxes) with the cytoplasmic part that contains a tyrosine kinase core sequence (boxed). The Neu-related receptor for EGF is shown with hatched boxes.

forms, and was found to encode a p185 transmembrane tyrosine kinase related to the EGF receptor. The cellular homologue of *neu* was unable to transform murine fibroblasts at comparable levels of expression, and construction of in vitro recombinants between the oncogene and the proto-oncogene identified a single point mutation that was transforming [22]. This changed an A to a T and resulted in substitution of a valine residue at the transmembrane domain with glutamic acid. Interestingly, out of a few other amino acids that replaced this valine, only glutamine, in addition to glutamic acid, conferred transforming potential [26,27]. The human homolog of rat *neu* was independently isolated by virtue of its relatedness to the *erb*B gene and was termed *erb*B-2 or HER-2 [28–30]. Although no analogous point mutation was found in the human gene, site-directed mutagenesis confirmed that a similar change can activate *erb*B-2 as an oncogene [31].

Truncations

Both rat and human *neu*/*erb*B-2 can undergo oncogenic activation by artificial deletions of sequences. Thus, deleting most of the extracellular domain activated the normal gene, but double truncation at both termini appeared more efficient for the transforming potential [27,32]. Partial deletion of the carboxy tail of the transforming rat *neu*, however, reduced its oncogenic potential in vitro and in animals [33].

Overexpression

When overexpressed in mouse fibroblasts, the human gene conferred a transformed phenotype in vitro and tumorigenesis in vivo [32,34]. A threshold level of overexpression was found to be crucial for the oncogenic potential. Likewise, extreme overexpression of the rat *neu* also induced transformation [35].

Receptor–receptor interactions

Neu undergoes transregulation by other protein kinases, like protein kinase C [36,37] and the EGF-receptor tyrosine kinase [36,38,39]. The latter mechanism may be associated with increasing the transforming potential of Neu, since simultaneous overexpression of EGF-receptor and Neu in mouse fibroblasts induced cellular transformation [40]. Interestingly, at the examined level of expression neither receptor alone was transforming, implying that it is the interaction between the receptors that synergistically affected the cellular phenotype.

neu in transgenic animals

Before discussing the clinical implication of Neu in humans, it is worthwhile to review its effect on transgenic mice. When driven by the mouse mammary tumor virus (MMTV) promoter, the transforming version of the rat *neu* induced mammary adenocarcinomas in the entire epithelium of the glands of 1 out of 4 transgenic lines [41]. In contrast, the parotid gland and the epididymis developed benign hypertrophy and hyperplasia [41]. It was, therefore, concluded that the activated *neu* gene is sufficient to induce tumorigenesis in a single step. Contrary to this conclusion, similar experiments with transgenic mice indicated stochastic appearance of mammary tumors, implying that *neu* was necessary but insufficient to induce malignant transformation [42]. Apparently in accordance with this conclusion, replication-defective recombinant retroviruses that carried the *neu* oncogene frequently induced mammary carcinomas when applied in situ on the mammary gland. However, individual tumors occurred with varying latencies,

and not all the infected cells progressed to form carcinomas [43]. Human *neu*/*erb*B-2 that contained an oncogenic mutation and was driven by an immunoglobulin enhancer and SV40 early gene promoter induced lymphomas neonatally in all transgenic animals [44]. However, the normal variant of the gene only sporadically induced lymphomas that appeared after a relatively long delay [44].

Neu in normal tissues

Analyses of the expression of *neu* by Northern blots detected the transcript in midgestation rat embryos in a variety of tissues, including the nervous system, connective tissues, and secretory epithelium, but not in lymphoid tissues [45]. In contrast, in adult brain *neu* was virtually not transcribed, but the protein was detectable in secretory epithelial tissues and the basal cells of the skin. Similarly, the Neu protein was identified on cell membranes in the gastrointestinal, respiratory, reproductive, and urinary tract, as well as in the skin, breast, and placenta [46]. High expression of $p185^{neu}$ was also described in the mucosal epithelium of the stomach, small intestine and colon, the liver parenchyma, the endocrine and exocrine portions of the pancreas, and the salivary gland [47]. Interestingly, the pattern of expression of *neu* in the developing animal is reflected by the susceptibility to the induction of tumors by a chemical carcinogen; whereas neuronal tumors are developed in midgestation embryos [23], adult rats developed tumors of the mammary gland, kidney, lung, thyroid, and liver [48].

Neu in human tumors

The potent oncogenic capability of the mutated rat *neu* sparked extensive investigation of its relevance to human cancer. Such relationships were suggested by preliminary evidence showing that the gene was amplified 5- to 10-fold in a human mammary carcinoma cell line [28]. Later analysis of 189 primary human breast cancers revealed that the gene was amplified from 2-fold to greater than 20-fold in 30% of breast tumors, and that amplification was a predictor of overall survival and time to relapse [49]. This landmark study was later extended by the same group to include 526 patients, of whom 345 were node positive [50]. For the node-positive subset, the authors confirmed the association between *neu* expression and poor prognosis in terms of recurrence and survival. Although later studies showed that the percentage of tumors expressing *neu* is closer to 20% than to 30%, the prognostic value of *neu* is now more generally accepted than initially, and efforts are being made to extend this observation to other adenocarcinomas (reviewed in [51–53]). What follows is a summary of the major conclusions drawn from the more recent studies.

Breast cancer

For patients with operable breast cancer, prognostic factors are extremely important, since they may assist in decision making regarding the choice of treatment. Indices other than auxiliary nodal status, which is considered the most important, include estrogen-receptor status and determination of ploidy and S-phase fraction. The level of expression of *neu*, as well as other oncogenes like *myc* [54], may thus provide additional useful markers of breast tumors. Before describing the actual observations, it is worth-while to discuss methodological aspects, since much of the early confusion in this field can be attributed to the methods of analysis. In most studies the protein was detected histochemically by using specific antibodies, or by using Western blotting. This procedure imposes a potential problem of antigen accessibility in tissue sections and extracts. Another problem is the clinical characteristics of patients, since some studies restricted analysis to specific stages or nodal status, whereas other included patients with different stages of the disease. Perhaps the most serious problem is due to study size: at least some of the conflicting results, especially with regard to prognostic effects, can be attributed to group size. Therefore, larger studies that include hundreds of patients are more significant, and usually these studies are those that support an association between *neu* and poor prognosis.

Unlike benign [55] or male breast carcinomas that show low if any expression of *neu* [56], female breast cancers display a fraction that is *neu* positive. While 10%–30% of infiltrating carcinomas are positive, up to 60% of in situ ductal carcinomas (DCIS) express *neu* [57–59]. Positivity is higher in comedo-type DCIS, which is characterized by large cells and higher growth rate than the other histological variants of DCIS. This suggests that *neu*-expressing tumors have a growth advantage. Indeed, proliferative activity, as measured by thymidine incorporation and S-phase fraction, was found to be moderately correlated with *neu* overexpression [60–63].

The all-important and previously controversial question of whether *neu* expression can be used as a clinical predictor appears to be approaching a resolution now after extensive studies during the last seven years. This question will be discussed in relation to the association between *neu* expression and conventional prognostic factors, and on the other hand, the relationships with relapse-free interval and overall survival.

Prognostic factors

Nodal status. Positive association between *neu* expression and the number of involved auxiliary nodes was originally reported by Slamon and his colleagues [49], and it was confirmed by other studies [64–70]. In accordance with this observation, significantly higher expression of *neu* was observed in stage III/IV than in stage I/II tumors [71,72]. However, no association with nodal involvement was observed in other studies [62,73–81].

Size of tumor. This staging parameter was not examined by all studies. However, it usually shows positive but moderate association with *neu* expression [57,72,81]. Other studies, however, did not find such an association between large tumor size and *neu* positivity [49,67,74,78,79].

Differentiation markers. Histological grade, nuclear grade, and hormone-receptor status were also examined in relation to *neu* expression. Although the association is weak, it appears that the less differentiated tumors more frequently overexpress *neu*. In contrast with the highly significant positive association between histological grade and *neu* expression that was reported by some studies [80,82–84], other investigators reported only weak association [57,64,65,74,77,79]. Inverse association with steroid hormone receptors was reported by many groups. This is moderately strong for estrogen [65,74,77,82,84–86] and weaker for progesterone [62,67,77,82,86,87]. As with other differentiation markers, several studies detected no correlation with hormone receptors. However, this may be due to an association between hormone receptors and disease stage [80]. Statistically less significant is the weak, if any, association between *neu* expression and aneuploidy [68,78,88,89].

Lack of recurrence and survival. In contrast with a few studies that failed to find any prognostic effect of *neu* expression [55,62,71,90], the majority of groups found a strong correlation with disease-free survival [50,73,78,79,83, 91,92] as well as with overall survival [49,50,74,81,91]. Importantly, failure to detect significant prognostic effects by some studies was likely due to the small groups of patients that were analyzed [52].

In summary, *neu* expression appears to enable some prediction of disease behavior in certain types of breast cancer. This conclusion not only is relevant to prognosis and diagnosis of early recurrence but also may help direct attempts to localize micromestastases and treat the disease, as will be discussed below.

Ovarian cancer

Like breast cancers, ovarian tumor cells contain steroid hormone receptors, and the vast majority of tumors arise from epithelial rather than stromal cells. In addition, these diseases appear to share etiologic factors, since women with one kind of tumor have increased risk of developing the other tumor. These similarities appear to extend to include overexpression of *neu*, since 26% [50] or 32% [93] of ovarian cancers showed significant overexpression of the protein when 120 or 73 ovarian cancers, respectively, were examined. Others reported that up to 60%–70% of ovarian tumors overexpress *neu* [94,95]. Immunoreactive cells were mostly epithelial, and overexpression was in general in agreement with gene amplification. Unlike breast cancer, some ovarian tumor cell lines display rearranged genes or

Table 1. Summary of reports that analyzed the amplification and/or overexpression of *neu* in various types of human neoplasia

Type of tumor	No. samples	No. positive	Comments	Ref.
Baldder	14	5 (36%)	One tumor with rearranged gene	[188]
	116	22 (19%)	Mostly in grade III and invasive tumors	[189]
	35	26 (74%)	8% with gene amplification	[190]
	54	20 (37%)	More frequent expression in high-grade and high-stage tumors	[191]
Urinary tract	Transitional cell carcinoma		Protein detected in plasma membrane and cytoplasm	[192]
	86	12 (13%)	Association between overexpression and tumor recurrence	[193]
Prostate	66		Benign and malignant	[194]
	34 benign	6 (18%)	No significant correlation with tumor	[195]
	29 malign.	6 (21%)	stage or grade	
Head and neck	34 (cell lines)		Correlation between *erb*B amplification and *neu* amplification	[196]
	46	0	No prognostic significance	[197]
Salivary gland	131	5 (3.8%)	Infrequent expression	[198]
	27	1 (3.7%)	No amplification detected in most adenomas	[199]
Hepatocellular	26	2 (8%)	Overexpression is unlikely to contribute to the malignant phenotype	[200]
Colorectal	89		Expression predicted hepatic metastases	[201]
	64	17 (27%)	Indicator for liver metastasis	[202]
	44	5 (11%)	Rare overexpression	[203]
Renal cell	30		Inverse relationship between *erb*B (high) and *neu* (low)	[204]
Pancreatic	6 (cell lines)	3 (50%)	One tumor with rearranged gene	[205]
	25	7 (28%)	Association with invasion	[206]
Endometrial	95	9 (9%)	Overexpression associated with absence of estrogen receptor and high mortality	[207]
Thyroid	17	12 (70%)	Expression in papillary thyroid carcinomas	[208]
			Enhanced protein expression	[209]
Cervix	30	9 (30%)	No correlation with clinical outcome	[210]
	49	7 (14%)	Correlation with tumor grade	[211]
	34	1 (3%)	Overexpression is a rare event	[212]

Note: See text for description and references related to studies of Neu in breast, ovary, lung, and gastric tumors. Unless otherwise indicated, primary tumors were examined, mostly by using immunohistochemistry. Where available, the fraction of tumors that showed overexpression of Neu is indicated.

variant transcripts of *neu* [94,96]. While a statistically significant association between *neu* overexpression and short survival was reported in two studies [49,93], others observed no clinical correlation [97,98]. In addition, xenografts of ovarian cancers grew in nude mice independently of the level of

overexpression [96]. Therefore, it appears that *neu* expression in ovarian tumors is potentially a prognostic marker, but more studies will be required to firmly establish this possibility.

Lung cancer

Overexpression of *neu* was reported in one third of lung adenocarcinomas and squamous cell carcinomas; however, only for the adenocarcinomas was *neu* expression a significant determinant of survival ($p = 0.04$) [99]. Overexpression of *neu* in a portion of all subtypes of non-small cell carcinomas was also confirmed in cultured cell lines and biopsies [100].

Gastric cancer

A relatively small proportion (9%–12%) of adenocarcinomas of the stomach overexpress *neu* [101–106]. Immunohistochemical analysis almost invariably localized the protein to the plasma membrane and correlated overexpression with well- or moderately differentiated cells. Controversy, however, exists as to the clinical relevance of overexpression. While two groups concluded that overexpression is an indicator for poor short-term prognosis and correlated it with lymph node metastasis [102,103], others reported that patients with Neu-positive intestinal-type tumors may have significantly better prognosis than those with Neu-negative tumors [104,105]. However, a recent study concluded that *neu* overexpression may not serve as a prognostic indicator, since patients with positive and negative Neu staining had similar five year survival rates [106]. Certainly additional work will be needed in order to clarify this issue.

Other cancers

Table 1 summarizes studies that examined Neu in other types of human cancer. As can be seen, these analyses are in general limited as to the number of patients that were analyzed, and their prognostic significance is less apparent than in breast cancer.

Biochemical aspects of Neu

Tyrosine kinase activity

The key to understanding the mechanism of oncogenesis by Neu, at least in experimental model systems, is its intrinsic tyrosine kinase activity. Artificially constructed kinase-defective mutants of *neu* fail to induce cellular transformation [31,107,108]. Similarly, the transforming activity of an overexpressed human Neu is strictly dependent on the tyrosine kinase activity of

the receptor [109]. On the other hand, enhancement of the catalytic function of Neu, both in vitro and in living cells, is shared by all the oncogenic forms of the receptor, including the point-mutant [26,110], the overexpressed normal protein [111,112], and the ligand-activated receptor [113,114]. Furthermore, in primary human breast tumors that overexpress the *neu* gene, the protein was found to be constitutively phosphorylated on tyrosine residues [115]. This implies that the tyrosine kinase function of Neu is critically involved not only in animal model systems but also in human cancer.

Autophosphorylation sites

Tyrosine phosphorylation sites of receptor tyrosine kinases appear to function as contact points with cytoplasmic effector enzymes [116]. Mapping analyses localized five sites of in vitro autophosphorylation to the carboxy-terminal tail of the Neu protein [31,109,117]. These are Tyr-1023, -1139, -1221/1222 and 1248. Replacement of the terminal tyrosine residue with phenylalanine reduced the transforming potential of the mutationally activated Neu protein [31,33]. However, the transforming ability of an overexpressed normal Neu protein was not significantly altered by individual mutations at tyrosine sites 1139, 1196, and 1248 [109]. However, in a chimeric EGF-receptor/Neu protein, substitutions of all five phosphorylation sites with phenylalanine residues markedly reduced phosphotyrosine content and the mitogenic activity of the receptor [118]. Thus, multiplicity of autophosphorylation sites may be more critical for normal Neu than for the mutated variant. The carboxy tail of Neu, like the corresponding part of the EGF receptor, may exert also an inhibitory effect, since a deletion of the distal 230 amino acids partially activated the transforming potential of the wild-type Neu [31]. However, a shorter deletion of 122 amino acids of the transforming rat Neu [33], and stepwise deletions in the human protein [119], did not activate the transforming potential but rather reduced it.

Signal transduction mechanisms

Unlike other receptor tyrosine kinases, whose ligands were characterized prior to receptor isolation, Neu was discovered long before its endogeneous ligand. Therefore, in order to study signal transduction in the absence of a known ligand, alternative approaches were undertaken. These include the use of agonistic monoclonal antibodies [110,120–123] and chimeric receptors in which the extracellular domain was derived from the EGF receptor and the cytoplasmic domain of Neu [111,113,114,124,125]. The possibility that the transforming Neu is biochemically equivalent to a ligand-activated receptor, in analogy to other oncogenic receptors like *Fms* and *Erb*B, provided further insights into the mechanism of signal transduction by comparison of the wild-type and the mutant proteins [26,110,111,127]. As

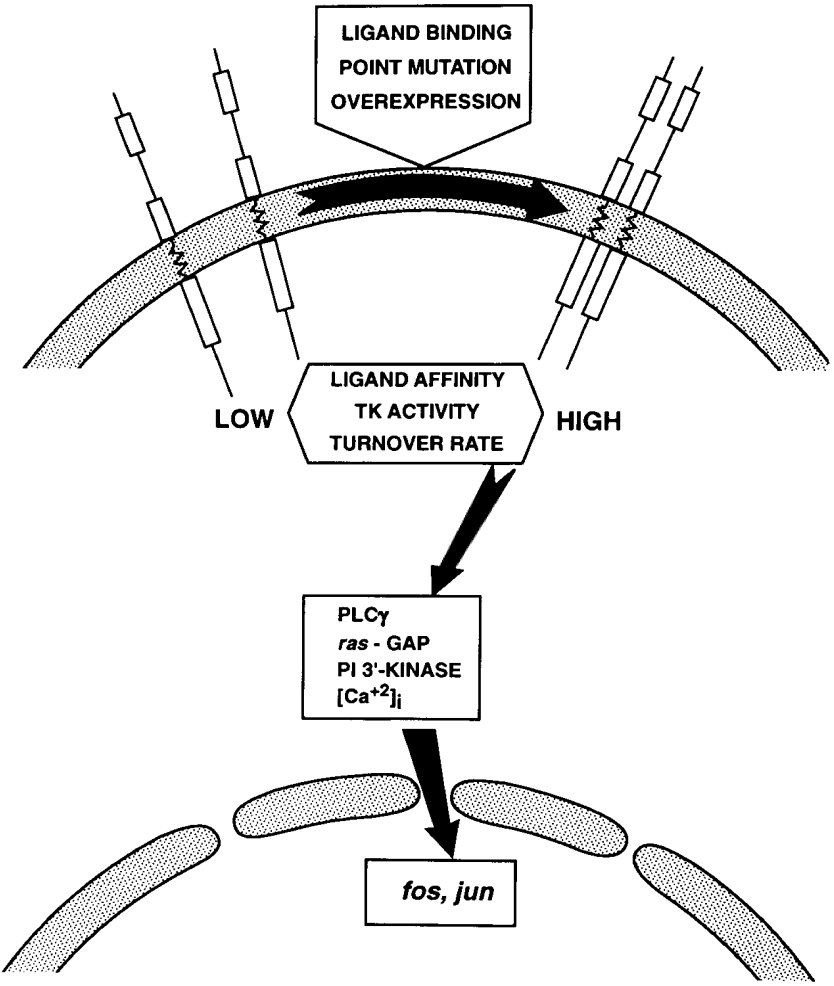

Figure 2. Mechanism of signal transduction by Neu. According to this model, activation of the tyrosine kinase of Neu is achieved through receptor dimerization. Once activated, the receptor affects a series of biochemical functions in the cytoplasm that culminate in transcriptional events in the nucleus. The stippled areas represent the plasma and the nuclear membranes. Abbreviations: TK — tyrosine kinase; PLC — phospholipase C; GAP — GTPase activating protein; PI — phosphatidylinositol.

will be discussed below, these studies revealed functional similarities between the various oncogenic forms of Neu that probably utilize the same pathway of signal transduction (figure 2).

Receptor dimerization. Conformational energy analysis of the transmembrane domain of Neu predicted that the wild-type protein contains a sharp bend in this region, whereas the sequence of the transforming mutant is

present in a helical form [128]. Another model argued that the glutamic acid present in the transmembrane domain of the transforming Neu forms hydrogen bonds that stabilize receptor dimers [129]. However, the three-dimensional structures of synthetic peptides corresponding to the membrane-spanning regions failed to support this model [130]. Covalent cross-linking of Neu was indeed able to detect dimers of the transforming protein, but the normal Neu displayed no dimer form [127]. This led to the possibility that the oncogenic mutation mimics the function of a ligand by inducing a constitutive dimer. This possibility was recently supported by using EGF-receptor/Neu chimeras with and without the transmembrane mutation [131]. Although human tumors do not contain an analogous mutation [132], overexpression at the cell surface may induce spontaneous receptor oligomers. This possibility is supported by theoretical considerations [128] and also by cross-linking experiments [133]. In analogy with other growth-factor receptors, dimerization of Neu activates autophosphorylation on tyrosine residues, as was shown by using chimeric receptors [113,114] and kinase stimulatory monoclonal antibodies that are inactive in their monovalent form [110]. Most likely, autophosphorylation of Neu is coupled to rapid endocytosis and lysosomal degradation of the receptor–ligand complexes. This was indicated by following the degradation of a chimeric receptor and radiolabeled EGF [131] and supported by the constitutively rapid turnover of Neu proteins carrying an oncogenic mutation [110,134]. However, antibody-induced internalization of Neu was shown to be independent of receptor phosphorylation [135].

Effector systems. Chimeric receptors in which the cytoplasmic domain was derived from Neu proved particularly useful in the study of intracellular signals induced by the activated tyrosine kinase. These receptors, when stimulated with the heterologous ligand, convey a strong mitogenic signal to murine fibroblasts [113,114]. In an attempt to identify the underlying biochemical events, it was found that marked increases in both intracellular calcium concentrations and plasma membrane potential followed kinase activation [136]. In addition, the turnover of inositol lipids was accelerated, as was glucose transport across the plasma membrane. The latter was due to transcriptional activation of the transporter gene [137]. Other transcriptional events that followed Neu activation were rapid induction of *fos* and *jun* expression, as well as of a group of other early-response genes [138]. These biochemical events are probably initiated by a set of proteins that undergo rapid tyrosine phosphorylation by either ligand-, antibody-, or mutation-activated Neu kinase [111,114,122,124]. Some of the proteins also form noncovalent complexes with the receptor. The identity of these substrates is only partially known, and they include phospholipase C_γ (PLC_γ), the GTPase activating protein of *ras* (*ras*-GAP) and the p85 subunit of phosphatidylinositol 3′-kinase (PI3K) [111,124,126]. They all share sequence motifs, called *src* homology 2 (SH2) domains, that are likely to enable

binding to phosphorylated tyrosine residues of activated receptors [116,139]. On the basis of these observations, it is predictable that Neu catalytically activates protein kinase C, affects the activity of *ras* proteins, and elevates the intracellular levels of 3′-phosphorylated inositol lipids. Analysis of mAb activation revealed that the activity of PI 4′-kinase is also elevated by Neu [122]. Interestingly, inhibition of dephosphorylation induced the appearance of a similar set of tyrosine phosphorylation substrates in a human mammary cell line [140], and overexpression of normal Neu led to constitutive phosphorylation of PLC_γ [111]. These findings, therefore, suggest the involvement of Neu substrates in cancers that overexpress the receptor.

Transregulation of Neu. Neu and EGF-receptor are often co-overexpressed in human breast carcinomas [51]. A third receptor that belongs to this family, *Erb*B-3, was found to be overexpressed in a subset of human mammary tumors [19]. The relevance of these findings to the malignant state is unknown, but as discussed before, in a model experimental system co-overexpression of Neu and EGF-receptor led to transformation and tumorigenicity of murine fibroblasts [40]. The underlying biochemical mechanism may involve transregulation of Neu by the EGF-receptor. Binding of EGF to its receptor was shown to induce rapid phosphorylation of Neu on tyrosine residues [36,38,39,141,142]. Covalent cross-linking revealed a high-molecular-weight complex that was formed after EGF binding. This was identified as a heterodimer of EGF receptor and Neu [143–145]. The complex is characterized by high affinity to EGF and by high tyrosine kinase activity, and its formation apparently results in accelerated degradation of Neu in lysosomes [146]. Through the use of a kinase-defective Neu, it was concluded that the Neu receptor is a substrate for the EGF-receptor kinase [147]. Similarly, a kinase-defective EGF-receptor caused dimerization and tyrosine-phosphorylation of Neu [148], implying that both inter- and intramolecular phosphorylation takes place within the heterodimer. In theory, heterodimers may elicit biological signals that are distinct from the signaling by homodimers of the EGF receptor and Neu. Otherwise, they could provide an amplification machinery. Another transregulatory mechanism that apparently reduces, rather than activates, the enzymatic function of Neu is mediated by protein kinase C and involves serine and threonine phosphorylation [37,149].

Ligands that interact with Neu

Although Neu and the EGF receptor share extensive structural homology, Neu binds none of the known ligands of the EGF receptor, including transforming growth factor-α (TGF-α). Based on the possibility that a ligand of Neu may share structural and functional characteristics with ligands of the EGF-receptor, we screened biological sources of TGF-α. An activity that

fulfilled some of the expected properties of a ligand for Neu was identified in the medium of *ras*-transformed fibroblasts [150]. Partial purification of the activity that induced tyrosine phosphorylation of Neu in mammary cells revealed that it was a heat-stable and disulfide-containing glycoprotein [151]. The homogeneously purified 44-kDa factor was active at picomolar concentrations and apparently underwent covalent cross-linking to Neu [152]. When tested on certain mammary tumor cell lines (MDA-MB453 and AU-565) the factor arrested cell growth and also induced phenotypic cellular differentiation to mature milk-secreting cells. Molecular cloning of the Neu differentiation factor (NDF) revealed that the precursor is a transmembrane molecule with a long (157aa) cytoplasmic domain that is connected through a single hydrophobic stretch to an extracellular portion that contains an EGF-like motif with six cysteine residues and an immunoglobulin-like domain [153]. The amino acid sequence of rat NDF turned out to be almost identical to the sequence of one of the variants of heregulin, a growth factor that was isolated from MDA-MB231 human mammary cancer cells [154]. This activated *ras*-containing fibroblast-like cell line was previously found to secrete a 30-kDa glycoprotein (gp30) that stimulated tyrosine phosphorylation of Neu, and at high concentrations inhibited the growth of Neu-overexpressing mammary cells [155]. Currently, it is not known whether gp30 is identical to NDF/heregulin. However, unlike the latter, gp30 was reported to bind to both EGF-receptor and Neu. Nevertheless, gp30, like NDF, was found to induce differentiation of mammary tumor cells in vitro [156]. A recombinant portion of heregulin that contained only the EGF-like domain bound at high affinity to mammary tumor cells and displayed a biphasic cellular growth effect, namely, stimulatory at the picomolar range of concentration and inhibitory at nanomolar concentrations [154]. Although native and recombinant NDF/heregulin appear to undergo covalent cross-linking with $p185^{neu}$ [152,154], we recently observed that the factor did not bind to $p185^{neu}$ on fibroblasts and ovarian cells [157]. The restricted cell-specificity of NDF may be due to the participation of a still unknown cellular molecule in NDF-$p185^{neu}$ interaction, and raises the possibility that Neu ligands with broader specificity may exist. Several molecules that have not yet been completely characterized may be qualified. Affinity purification on a recombinant ectodomain of Neu resulted in the isolation of a 75-kDa protein from the medium of SKBR-3 mammary carcinoma cells. The factor induced tyrosine phosphorylation of Neu and proliferation of Neu-overexpressing cells and showed no binding to the EGF-receptor [158]. Another putative ligand was isolated from bovine kidney [159]. The acid-labile 25-kDa factor was able to stimulate tyrosine phosphorylation of Neu in murine fibroblasts and was mitogenic to epithelial cells and fibroblasts that express Neu. In another study, human T-cell lines were used to obtain secreted molecules (8 to 24 kDa) that stimulated tyrosine phosphorylation of Neu and also induced dimerization and down-regulation of the receptor [160,161]. Yet another cellular source of a putative Neu ligand are mouse

peritoneal macrophages. The activated cells secreted a factor that could down-regulate Neu, and its activity was inhibited by the soluble ectodomain of the receptor [162]. Although Neu, like EGF-receptor, may have multiple distinct ligand molecules, complete purification, and even molecular cloning, will be required in order to determine which of the partially purified factors indeed functions as a ligand for Neu.

Potential therapeutic molecules directed at Neu

Monoclonal antibodies (mAbs)

Unconjugated antibodies. With the description of hybridoma techniques, it became apparent that mAbs can constitute novel reagents with potential applications in the diagnosis and treatment of malignant diseases. The knowledge gained by studying cell proliferation and the implication of growth factors and growth-factor receptors in human malignancies suggested that pathological cell proliferation could be impeded by mAbs directed to growth-factor receptors. Neu is a candidate cell-surface antigen for antibody targeting, although it should be stressed that the receptor is present on certain cancer cells as well as on many normal epithelial, neuronal, and fibroblast cells. The first mAbs against Neu were generated by Greene and his colleagues [163]. Out of these, mAb 7.16.4 was described in more detail, and it was shown that exposure of *neu*-transformed cells to this mAb caused down-modulation of p185 from the cell surface and resulted in loss of capacity for anchorage-independent growth. Monovalent fragments prepared from the 7.16.4 mAb were found to have no direct effect in inhibiting the growth of the tumorigenic cells in soft agar. Other lines of evidence also suggested that cross-linking of $p185^{neu}$ molecules by bivalent antibodies is required for biological activity [110]. In vitro, 7.16.4 mAb mediated multiple antitumor effects, including complement-dependent lysis of antibody-coated tumor cells and targeting of murine lymphoid cells to mediate modest levels of antibody-dependent cellular toxicity [164]. Treatment with this mAb was able to significantly inhibit the tumorigenic growth of *neu*-transformed NIH-3T3 cells in nude mice and syngeneic BDIX rats [165]. In an effort to elucidate the mechanism by which the anti-p185 antibodies inhibit tumor growth, a number of other mAbs were raised and studied, and it was concluded that the ability to directly inhibit the neoplastic growth of *neu*-transformed cells is primarily responsible for the antitumor activity [166,167]. In an attempt to generate mAbs that will mimic the action of the putative ligand of Neu, another panel of mAbs was generated against the rat Neu protein [110]. Some antibodies indeed stimulated tyrosine phosphorylation of Neu in living cells and significantly accelerated the rate of degradation of the receptor.

In order to explore the potential clinical use of mAbs to the human Neu,

a large number of mAbs were raised by immunizing mice with a variety of cell lines that overexpress the receptor [119,121,168–171]. One of these mAbs, 4D5, was generated against a mouse fibroblast cell line that was transfected with the human *neu* gene. It inhibited the growth of tumor cell lines in vitro, and sensitized them to the cytotoxic effect of tumor necrosis factor (TNF-α) [168]. The mAb also inhibited the growth of cells that overexpressed Neu, but not cells that expressed low levels of the receptor. Despite its antiproliferative effect, the antibody had agonist-like properties and competed with a putative ligand-gp30 [155]. It was also shown that the 4D5 mAb stimulated Neu phosphorylation on tyrosine, serine, and threonine residues [120]. Short-term exposure of breast carcinoma cells to 4D5 increased the intracellular levels of the second messengers inositol polyphosphates and diacylglycerol [122]. On the other hand, continuous growth of cells in the presence of 4D5 reduced the steady-state levels of p185 phosphorylation in a dose-dependent manner, inhibition that could not be completely accounted for by down-regulation of the receptor [120]. In vivo assays with a nude mouse model have shown that the mAb can localize at the tumor site and can inhibit the growth of human tumor xenografts [172].

Another panel of mAbs against the extracellular domain of the HER-2/Neu receptor was raised by immunization of mice with the breast carcinoma line SKBR-3 and was used to study the biological function of the receptor [121]. These antibodies induced either positive or negative growth effects on tumor growth in athymic mice, and had a limited effect on the growth of tumor cells in culture. However, a correlation between the growth-stimulatory effect of mAb N28 in vivo and activation of the tyrosine kinase function of the receptor was found. The mAbs that inhibited tumor growth had little effect on Neu phosphorylation. In accordance with these results, a more recent report described the isolation and characterization of four mAbs to the human *erb*B-2 protein [123]. Binding of the antibodies did not stimulate DNA synthesis, but treatment of two cell lines with three mAbs led to a rapid increase in phosphotyrosine content of the receptor. The fourth mAb inhibited the growth in culture of SKBR-3 cells [123]. Likewise, one of two mAbs that were raised by immunization with a lung adenocarcinoma line, Calu-3, was shown to reduce cell number in vitro, and displayed internalization capability [171]. Another study focused on the synergistic effect of a combination of two anti-Neu [173]. The combination of two mAbs directed to the extracellular domain of the Neu protein was tested in a nude mouse animal model of gastric cancer. The combination, but not each mAb alone, inhibited tumor growth. While complete regression of pre-existing tumors was observed, the treatment was not curative, in that tumors regrew after six weeks.

The molecular mechanism underlying inhibition of growth of tumor cells by Neu-specific mAbs was addressed by several studies. Two possibilities, which are not mutually exclusive, were raised. These are accelerated down-

regulation of Neu and the induction of cellular differentiation. Incubation of cells expressing transforming rat Neu protein with anti-Neu mAbs was followed by rapid loss of cell-surface receptors that could underlie the reversion of the transformed phenotype [162]. Treatment of NIH-3T3 cells expressing a transforming human Neu protein with mAbs also led to 50%–65% down-modulation of the receptor, whereas the wild-type protein was only marginally down-regulated [174]. The rate of turnover of Neu was also increased in the presence of mAb 4D5 [168], and this effect was linked to the antiproliferative potential. Results of another study indicated that inhibition of cell growth was associated with reduced immunostaining for the HER-2/Neu protein at the cell surface membrane, and with a transient increase in immunostaining in the cytoplasm [175]. It is also relevant that the mAb MGR-3 to human Neu was found to induce internalization of the receptor and to inhibit cell growth [171]. However, some other mAbs could induce receptor down-regulation with no correlation with their tumor-inhibitory potential [121,123]. Comparison of the cellular effects of various tumor-inhibitory antibodies (N12, N24, and N29) with a single stimulatory antibody, N28 [121], established a correlation between tumor inhibition in vivo and the induction of cellular differentiation in vitro [176]. While N28 had no effect, the tumor-inhibitory action of the other antibodies correlated with the extent of differentiation they induced. The antibody-treated breast cancer cells presented a growth-arrested mature phenotype characterized by flat morphology, large nuclei, and secretion of milk components. The inhibitory antibodies also induced redistribution of the receptor from the cell membrane to the cytoplasm and perinucleus. Predictably, the ability of certain antibodies to Neu to affect tumor growth through the induction of differentiation may have clinical implications.

Radiolabeled antibodies. The feasibility of targeting the Neu protein with radioiodinated murine mAbs was analyzed in a recent study [177]. Monoclonal antibodies 4D5 and 7C2 were used, and the rates of internalization and catabolism were analyzed. Treatment of mice that were injected with Neu-transformed fibroblasts with 400–700 μCi ^{131}I-4D5 caused marked inhibition of tumor growth, although no mouse was cured. In these experiments, the antitumor responses were most likely a result of radiation damage induced by ^{131}I, since unlabeled antibody did not inhibit neoplastic growth at the doses used.

Immunotoxins. Conjugates of mAbs and toxins have been used in preclinical trials as antitumor agents [178]. A recent study reported the construction of several immunotoxins using various anti-Neu antibodies that have been coupled to Lys PE40, a recombinant form of Pseudomonas exotoxin that lacks its cell-binding domain [179]. One antibody was selected for construction of a single-chain immunotoxin, which was more active than the chemical conjugate. To further increase the activity of this recombinant protein, the

toxin part of the molecule was altered, and it was shown to inhibit the growth of the human gastric cell line N87 in immunodeficient mice. Since the Neu protein is expressed in many tissues, a similar therapeutic approach may require administration of doses that will be sufficiently high to kill cells that overexpress the receptor, but will have only limited toxicity to normal cells. Use of multiple immunotoxins directed against different antigenic determinants was also described. In vitro assays indicated that a combination of two immunotoxins in which the recombinant ricin A chain was conjugated with mAbs that bound the transferin receptor and the Neu receptor exhibited synergistic cytotoxic activity in growth inhibition of SKBR-3 human breast tumor cells [180].

Humanized and bispecific antibodies. A major limitation to clinical use of murine mAbs is an immune response during therapy so that high doses of antibody are required in order to compensate for the rapid clearance of the mAbs from the patient's body. In addition, the murine mAb may have impaired effector functions. Chimeric and humanized antibodies are a solution to this problem. Humanized antibodies are obtained by transplanting the complementarity-determining regions (CDRs) from rodent antibodies into a human framework [181]. A humanized antibody, containing only the antigen-binding loops of the 4D5 anti-Neu mAb and human variable regions, together with IgG_1 constant domain, was constructed [182]. One of the humanized variants, designed by molecular modeling, bound the antigen threefold more tightly than the murine antibody and preserved its ability to inhibit tumor cell proliferation in vitro. It was also more efficient in antibody-dependent cellular toxicity assays. Recent studies have shown that human T-cells can be targeted with bispecific antibodies to react against human tumor cells in vitro. A bispecific humanized $F(ab')_2$ fragment [183] or a whole antibody [184] were constructed with arms of anti-CD3 antibody and an anti-Neu mAb. The presence of the bispecific $F(ab')_2$ fragment caused up to fourfold enhancement in the cytotoxic activities of human T-cells against tumor cells overexpressing the Neu receptor [183], whereas a combination of the whole bispecific antibody with CD4+ helper/killer cells showed a strong in vivo antitumor effect against human colon carcinoma cells implanted in nude mice [184]. These results suggest that the Neu oncoprotein may be a target for bispecific mAb-directed adoptive immunotherapy.

Cytokines

Several clinical trials indicated that treatment of selected ovarian cancer patients with interferons (INFs) can result in an antitumor response. This effect of INF may involve alterations in the expression of several proto-oncogenes [185]. One of these proteins is Neu, since it was shown to be modulated by interferons in ovarian carcinoma cells [186]. INFs inhibited the expression of HER-2/Neu in 6 out of 7 ovarian carcinoma cell lines, but

not in breast cancer cells. Tumor necrosis factor alone did not affect the expression of Neu, but slightly amplified the effect of INFs. Another study showed that treatment with INFγ enhanced the sensitivity of Neu-positive ovarian cells to cytotoxicity by lymphokine-activated killer (LAK) cells [187]. Increased sensitivity to lysis was associated with an increase in effector-target conjugate formation, the induction of target cell intercellular adhesion molecule 1 (ICAM-1) expression, and down-regulation of Neu. Another cytokine that may directly regulate Neu is NDF/heregulin [152–154]. Potentially the induction of cellular differentiation by NDF, and possibly also down-regulation of Neu, may be exploited for clinical purposes.

Conclusions

The major incentive for biochemists, molecular biologists, and clinicians who study various aspects of Neu is its potential prognostic value. The clinical information that was collected over the last seven years appears to support a correlation between amplification and overexpression of the gene and clinical outcome, at least in breast cancer, and probably also in ovarian and other human adenocarcinomas. Fortunately, animal model systems of oncogenic Neu proteins exist, and they significantly add to our understanding how Neu may contribute to malignancy of epithelial tumors. On the basis of multiple lines of biochemical and molecular lines of evidence, it is possible to propose the following model of Neu activation in human tumors. As a result of overexpression at the plasma membrane, spontaneous dimerization of the receptor occurs frequently, and this results in constitutive phosphorylation of the Neu protein on tyrosine residues. These sites of autophosphorylation reside at the carboxy-terminal portion of the receptor, and they function as anchoring sites for a group of cytoplasmic proteins that can produce second messengers. The latter stimulate a variety of cellular functions that include Ca^{++}-dependent processes, phospholipid turnover, and activation of protein kinase C, and they culminate in accelerated cell growth. Consequently, cells that overexpress Neu acquire a growth advantage, and probably also an improved invasive capability.

This simplified working model is complicated by the interaction of Neu with other proteins, including its own ligand(s) and other membrane proteins (e.g., EGF receptor). The effects of these interactions on the transforming potential of Neu, especially in human cancers, are currently unknown. Nevertheless, the proposed model may identify the protein complex of Neu and specific signal transduction enzymes as targets for pharmacological intervention of the action of the receptor in human cancer. This includes the use of antibodies, cytokines, and probably also compounds that affect tyrosine phosphorylation and specific enzymatic pathways. The clinical potential of this model and its therapeutic implications will have to be addressed by future research.

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10. MDGF1: A multifunctional growth factor in human milk and human breast cancer

Mozeena Bano, William R. Kidwell, and Robert B. Dickson

Endocrinologists have long recognized the existence of systemic-acting or endocrine hormones, as well as locally acting paracrine and autocrine factors. In addition, another body of literature documents the ability of chemical substances to act between individuals to modulate their behavior, physiology, or development. The initial exposure of individuals to such substances is, of course, *in utero*, where the baby is influenced by the mothers systemic endocrine hormones as well as by placental factors. After birth, salivary and mammary secretions continue to provide sources of nutrition, hormones, and even behavior-modifying substances. Cohen was the leader in identification of such substances with his seminal work identifying epidermal growth factor (EGF) in maternal salivary secretions and its role in eyelid opening of the neonatal rodents [1]. It is possible that, later in life, pheromonal substances may continue to exert interpersonal behavioral influences, particularly in reproductive aspects. In this chapter, we will focus on hormonal factors of possible physiological relevance in the milk.

Recent attention has focused on the presence of growth factors in milk and their potential physiological roles. Milk-derived factors could theoretically be synthesized in the mammary gland (in particular, in the secretory epithelium), or transferred from the blood via the epithelial cells to the milk. We have undertaken a novel research approach: identification of milk-derived factors with the potential to regulate normal and malignant mammary function in addition to a possible role for the neonate. In our laboratory, initial characterization of human milk-derived growth factor activity has indicated the presence of a high-molecular-weight (62 kDa), tyrosine kinase receptor-associated polypeptide growth factor with an isoelectric point of 4.8. We have named the factor *mammary-derived growth factor 1* (MDGF1), and the factor has been purified to homogeneity from human milk as well as from human primary breast tumors; it appears to be identical from the two sources [2]. MDGF1 stimulated cell proliferation and collagen IV synthesis in normal and malignant human mammary epithelial cells. It was demonstrated that MDGF1 binds to normal and malignant human mammary epithelial cells with moderate to high-affinity binding sites [3]. Also, in our previous experiments, it was observed that type I collagen

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synthesis in normal fibroblasts is stimulated by the factor in the absence of growth stimulation [4].

In this review, we will discuss the possible role of MDGF1 as an autocrine and paracrine growth factor in normal and malignant mammary tissues. Detailed description of growth factors in milk, other autocrine and paracrine growth factors in breast cancer, and collagen synthesis in breast cancer stroma are also included in this review. We will present our recent experimental data, which suggest MDGF1 to be an N-glycosylated product whose receptor might be a member of the family of tyrosine kinase-associated growth factor receptors [5,6].

Growth factors in milk

Human milk proteins are of nutritional and physiological significance to the newborn infant. The superiority of human milk has many facets: from built-in host defense factors and digestive enzymes to a well-balanced supply of amino acids, carbohydrates, lipids, minerals, and vitamins, and also as a source of biologically active molecules such as immunoglobulins, transferrins, casein fragments with opiate activities, hormones, and growth factors [7–9].

Human milk is a biological fluid that has the ability to stimulate the growth of cells in culture [10,11]. The growth-promoting properties of milk may be due in part to the presence of growth factors, mitogens capable of stimulating DNA synthesis and cell division in cultured cells [12,13]. A series of studies on the isolation and detection of the growth factors in milk has been performed by many investigators [14–16]. Shing and Klagsbrun had purified and biologically characterized three different growth-factor-containing fractions from human milk [10,17]. EGF appears to be a predominant growth factor, accounting for about 75% of the total growth-promoting activity in human milk [10,18–20]. Sinha and Yunis had isolated a colony-stimulating factor (CSF) from human milk using *in vitro* bone marrow culture [21]. Zwiebel partially purified a transforming growth factor-α (TGF-α)-like molecule in human milk using anchorage-independent growth assay of normal rat kidney (NRK) cells and radio-receptor assay for EGF receptor [22]. The presence of other growth factors such as insulin-like growth factor-I (IGF-I) [23,24], platelet-derived growth factor (PDGF), TGF-β [25,26], an epithelial transforming growth factor activity (TGFe) [27], and nerve growth factor (NGF) in milk have also been demonstrated [28].

Recent cellular localization studies have begun to compliment these earlier investigations. Liscia observed that TGF-α and EGF receptor are detected by *in situ* hybridization in biopsies of proliferating human and rat mammary epithelium during lobuloalveolar development of pregnancy [29]. Snedeker has examined the mouse mammary epithelium for EGF and TGF-α in studies ranging from virgin through lactational stages [30]. While TGF-α and

EGF were both detected in virgin and midpregnant glands, EGF strongly predominated in lactation. Additional hormones detected include growth-hormone-releasing hormone (GHRH), thyroid-stimulating hormone (TSH), luteinizing-hormone-releasing hormone (LHRH), thyrotropin-releasing hormone (TRH), insulin, erythropoietin, prolactin, relaxin, delta-sleep-inducing peptide (DSIP), and casein fragments with opiate activities (casomorphins and morphiceptin), some with immunological activities [9]. The function of most of these factors remains to be fully determined.

Our work now involved detection, purification, and characterization of a mammary-derived growth factor (MDGF1), a human-milk-derived, acidic, 62-kDa, N-glycosylated growth factor [2,4]. The amino terminal sequence of MDGF1 is as follows [5]:

I-P-V-K-Q-A-V-H-G-Q-F-L-L-P-K-Q-E-K

In addition to its effect on stromal fibrosis, MDGF1 stimulated collagen synthesis and proliferation of normal and malignant human mammary epithelial cells [3,4]. These activities may be mediated through unique membrane receptors that exhibit rapid phosphorylation on tyrosine of a membrane-associated protein [3,6]. An 18-amino-acid N-terminal partial sequence of the factor did not show any homology to other known growth factors or proteins [5]. More detailed characterization of the factor is described later in this chapter.

In summary, the presence of growth factor activities in human milk would be of physiological interest, since it would suggest that defined macromolecular factors in human milk are capable of having an effect on the gastrointestinal tract of the human infant and on the human mammary gland. However, the significance of the milk-derived growth factors *in vivo* is speculative at present and will require further studies.

Autocrine and paracrine growth factors in breast cancer

Endocrine hormones such as estrogens, progestins, glucocorticoids, thyroid hormones, and prolactin have been demonstrated to play important roles in the development and growth of the mammary gland. Estrogens appear to play one of the most critical roles in the development of breast cancer and appear to induce TGF-α-like molecules and IGF-related proteins in some human breast cancer cell lines [31,32]. Both of these growth factors are mitogenic for some human breast cancer cell lines. [33,34], and the mitogenic activity is mediated through the receptors for EGF/TGF-α and insulin-like growth factors [35,36]. Recent studies have also shown that antibodies directed against the growth factor receptors can suppress the proliferation of the responsive normal and malignant breast epithelial cells [31,37,38] suggesting that these growth factors may be involved in breast cancer cell proliferation through autocrine and paracrine loops [39].

Many breast cancer cell lines produce a TGF-β-related activity [40], contain receptors for TGF-β, and are inhibited by both TGF-β1 and -β2 [41]. TGF-β may be acting as a growth-inhibitory factor of breast cancer cells through autocrine and paracrine loops. Actions of TGF-β *in vivo* are also likely to involve tumor–host interactions. Recent studies have suggested that predominant actions of TGF-β in the nude mouse implanted with human breast cancer cells include immunosuppression, fibrosis, and cachexia [42,43].

IGFs, TGF-β, and PDGF, which are synthesized by many breast cancer cell lines, are potent mitogens for fibroblasts, smooth muscle cells, and other stromal cells [44]. All three factors may play a paracrine role in breast cancer development, possibly at the level of desmoplasia.

Since MDGF1 stimulates not only the growth and synthesis of type IV collagen of human mammary epithelial cells, but also type I collagen synthesis of normal fibroblasts, the factor may be involved in the growth and development of breast cancer through both autocrine and paracrine mechanisms.

Collagen synthesis in breast cancer stroma

An abnormal deposition of extracellular matrix in the proximity of tumor nests and cords is often recognized in invasive ductal carcinoma of the breast [45]. Biochemical and immunohistochemical analysis of the stromal component in breast cancer indicates that collagen in the stroma is primarily composed of embryo–fetal type I-trimer collagen, in addition to some regular type I, III, and V collagen. Recently, type IV collagen has also been detected immunologically in interstitial elastosis in breast cancer tissues [46].

Several growth factors including EGF, TGF-α, TGF-β, and PDGF are known to increase proliferation, but also to selectively increase glycosaminoglycans or collagen synthesis by fibroblasts [47]. One striking effect of TGF-β in certain fibroblasts is to stimulate anchorage-independent growth [48]. TGF-β also potently stimulates the synthesis of several extracellular matrix proteins, including type I collagen and fibronectin [49–52]. TGF-β was shown to induce a rapid fibrotic response, chemotaxis, and to elevate the rates of biosynthesis of collagen in vitro.

Type IV collagen is one of the major components of the basement membrane. Its synthesis appears to be important for the growth and/or survival of the normal epithelium and of well-differentiated epithelial tumors [53]. It has also been suggested that the enzymatic degradation of type IV collagen, initiated by type IV collagenase, may be an important characteristic of invasive neoplasms. This process is closely linked to invasive and metastatic potential in several experimental models [54].

We previously reported that MDGF1 strongly stimulates type I collagen production of NRK fibroblasts by an amplification of its protein synthesis

rather than by a decrease of its protein turnover [2]. Interestingly, when the fibroblasts were plated on type IV collagen-coated dishes, there was no effect of MDGF1, whereas the cells that had been plated on type I collagen-coated dishes or directly on tissue-culture plastic dishes differentially increased their production of collagen I by three- and fourfold, respectively, in response to MDGF1 [2].

MDGF1 also stimulates type IV collagen synthesis of normal rat, mouse, and normal and malignant human mammary epithelial cells [2–4]. Since human mammary tumors contain MDGF1, and since immunoreactive MDGF1 has been observed in the cytoplasm of human mammary tumor cells, the factor may be produced by many human mammary tumors [55]. Immunoreactive MDGF1 has also been detected in the concentrated medium prepared from normal and malignant human mammary epithelial cells [5]. These experimental data suggest that MDGF1, secreted by normal or malignant epithelial cells, might regulate type IV collagen deposition on the basement membrane between the cells and stroma, and that abnormal secretion of MDGF1 by malignant cells might stimulate stromal fibroblasts in a paracrine manner to produce excessive type I collagen in the extracellular matrix of breast cancer tissues. Moreover, inappropriate deposition of type IV collagen secreted by malignant cells might modulate the invasive and metastatic ability of the cells.

MDGF1 in the normal mammary gland and primary human breast cancer

Using polyclonal antiserum raised against the N-terminal synthetic peptide, we previously demonstrated that conditioned media from some human breast cancer and normal mammary epithelial cell lines contain the factor. The antibody could be used to adsorb the biological activity of the factor present in the conditioned medium [5]. In recent studies, the presence and distribution of MDGF1 in benign and malignant human breast tissue is being determined by ELISA technique and also by immunohistochemical analysis using high titer polyclonal anti-MDGF1 antisera [55]. Frozen sections of normal and malignant breast tissue were used for immunostaining, while homogenized soluble fractions were used for ELISA testing. Antigen absorption confirmed the antibody staining to be specific. Preliminary results indicated that MDGF1 is localized in the duct and lobular epithelial cells of the mammary gland. Staining was both nuclear and cytoplasmic [55]. The relative localization of MDGF1 in tumor and normal mammary tissue sections may be significant in the control of breast development and/or tumor formation and progression. At present, the study of the tissue distribution of MDGF1 is incomplete. However, we know that it is also synthesized in the human placenta by immunohistochemical analysis, Western blot detection and *in vitro* translation of mRNA (unpublished data).

Response of normal and malignant mammary epithelial cells to MDGF1

Our earlier results showed that MDGF1 stimulated cell growth and collagen synthesis of normal rat and mouse mammary epithelial cells, freshly isolated from mammary ducts and alveoli [2,4]. MDGF1 had a synergistic effect with estrogen, since our results showed that the growth of epithelial cells in culture was very low if the cells were isolated from animals depleted of estrogens by ovariectomy. Thus MDGF1 may act synergistically with estrogen by some mechanism that is not yet defined. We have begun to study such growth regulation by analysis of cell lines in vitro.

We carried out a systematic screening of biological effects of MDGF1 on normal and tumorigenic human mammary epithelial cells (figure 1). As depicted in the figure, at a concentration of 25 ng/ml the factor stimulated by 50% the growth of hormone-dependent tumorigenic MCF-7 breast cancer cells and hormone-independent tumorigenic MDA-MB 468 cells. Normal 184 mammary epithelial cells and benz(a)pyrene-immortalized 184 A1N4 cells were also stimulated by 50%. However, transfection of SV40-T, v-Ha-*ras*, and/or v-*mos* oncogenes into 184A1N4 cells resulted in a diminution of growth responsiveness to MDGF1 [3]. Three possible reasons for this decreased sensitivity seemed possible: transformation could result in a loss of function or expression of MDGF1 receptors or MDGF1 could be produced in sufficient quantities so that exogenously added MDGF1 would be

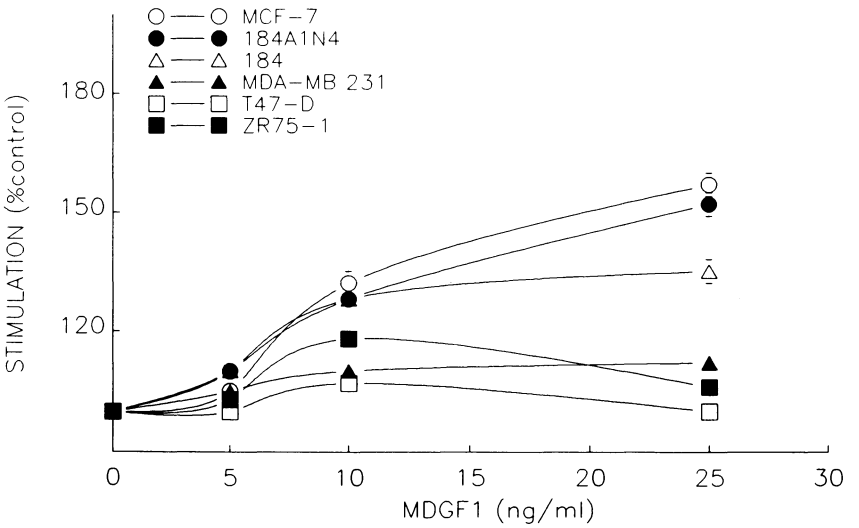

Figure 1. Effect of MDGF1 on human mammary cell growth. Cells were grown on tissue culture plastic dishes. Cells were incubated with the factor at the indicated concentrations. After 96–144 hours, cells were trypsinized and cell count was taken using a Coulter counter. Data are mean cell numbers expressed as percent of untreated control cell numbers. (Reproduced from [3].)

ineffective. Alternatively, different growth-regulatory mechanisms may be operant after transformation. Significantly, we observed that the responsive human breast cancer cell lines (MCF-7 and MDA-MB 468) possessed detectable MDGF1 receptors, whereas the nonresponsive cell lines (ZR75-1, T47-D, and MDA-MB 231) lacked significant numbers of the receptors.

MDGF1 in normal and malignant breast cell lines

To determine whether MDGF1 might be an autocrine or paracrine growth factor produced by and acting on normal and malignant human breast epithelial cells, we examined whether human breast epithelial cells produce the growth factor. A 62-kDa putative MDGF1 was immunologically detected in the concentrated conditioned medium and in cell lysates prepared from the MDA-MB 231 human breast cancer cell line, HBL-100 human milk-derived but non-tumorigenic mammary epithelial cell line, and a reduction mammoplasty-derived normal mammary epithelial cell strain designated 184 [5]. In contrast, three other cell lines (MCF-7 human breast cancer, MDA-MB 468 human breast cancer, or 184A1N4 immortalized human mammary epithelial strain) did not show any immunologically detectable MDGF1. Polyclonal antiserum raised against the synthetic polypeptide (N-terminal 18 amino acid sequence of MDGF1) recognizes native milk-derived MDGF1,

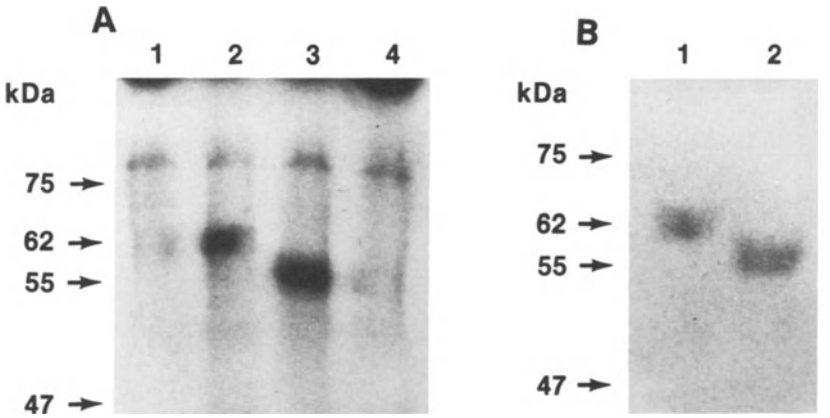

Figure 2. (A) Tunicamycin treatment. HBL-100 cells were grown to confluency and were treated with tunicamycin for 4 hours at 37°C. Metabolic labeling and immunoprecipitation using anti-MDGF1 antibody were performed. Immunoprecipitates were analyzed by SDS-PAGE and subsequent fluorography. Lanes 3 and 4 denote tunicamycin treatment and lanes 1 and 2 the absence of tunicamycin. Results for anti-MDGF1 sera are shown in lanes 2 and 3 and those for prebled sera in lanes 1 and 4. (B) N-linked glycosylation of 62-kDa MDGF1. Purified sample was incubated with N-glycanase (lane 2) or buffer only (lane 1) and subjected to Western blot analysis and silver-stained. (Reproduced from [5].)

adsorbs MDGF1 biological activity from mammary cell-derived conditioned medium, and has been used in these experiments [5].

Immunoprecipitated product derived from *in vitro* translation of mRNA from the HBL-100 cell line or the MDA-MB 231 cell line yielded a protein band with a molecular mass around 55 kDa. In order to establish that MDGF1 might be a glycoprotein, tunicamycin treatment, metabolic labeling, and immunoprecipitation were carried out, and the result is illustrated in figure 2A. Lane 2 depicts the 62-kDa band in the absence of tunicamycin, and lane 3 shows a lower-molecular-mass band (55 kDa) after tunicamycin treatment. Treatment of purified MDGF1 with N-glycanase also led to a reduction in molecular mass to 55 kDa (figure 2B). These results indicated that the mature 62-kDa MDGF1 contains N-linked carbohydrates.

Cellular receptors for MDGF1

To determine whether MDGF1 might have specific receptors to human mammary epithelial cells, purified MDGF1 was iodinated, and binding experiments were conducted on cell monolayers in the presence of varying concentrations of unlabeled factor. Specific high MDGF1 binding was detected in the case of MCF-7, MDA-MB 468, and 184A1N4 cells (figure 3). As shown in the figure, for MCF-7 cells an affinity constant of 6.2×10^{-9} M was calculated by Scatchard analysis, which yielded a linear plot indicating a single class of receptor sites (figure 3A). About 25,600 sites/cell were obtained.

MDGF1 binding appears to be specific, since an excess of EGF, basic FGF, and IGF-1 in the radio-receptor assay failed to inhibit the binding of labeled MDGF1 to cell membranes [3]. Figure 3B depicts the ability of labeled factor to bind to MDA-MB 468 cells with an affinity constant of 6.3×10^{-9} M. Binding of the factor with low affinity was seen with 184 and 184A1N4 cells. With other human breast cancer cell lines (MDA-MB 231, ZR75-1, and T47-D), the receptors were undetected.

The results of SDS-PAGE of [^{125}I]-MDGF1, covalently cross-linked to MCF-7 or MDA-MB 468 cells, is depicted in figure 4. The cross-linking experiments using DSS indicated that labeled MDGF1 was associated with a protein migrating with an apparent molecular mass of 130–140 kDa in size.

MDGF1 tyrosine phosphoprotein: Possible autophosphorylation of receptor subunit

Further characterization of the mechanism of MDGF1 action was begun by study of its potential to stimulate accumulation of phosphotyrosine on cellular proteins as determined by Western blot using a monoclonal anti-phosphotyrosine antibody. The results, as shown in figure 5, indicated that

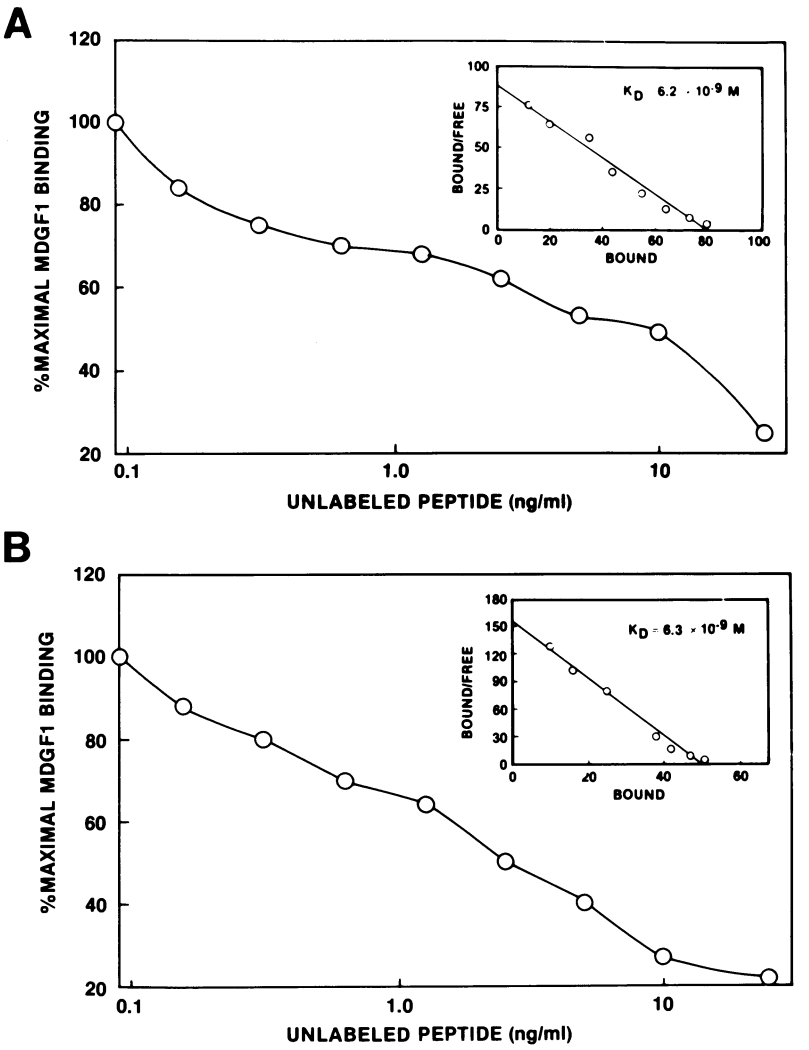

Figure 3. Binding of [^{125}I]-MDGF1 to cells. Aliquots of cells were incubated for 2 hours at 37°C with [^{125}I]-MDGF1 in the presence of various concentrations of unlabeled MDGF1. Nonspecific binding was determined with excess of unlabeled MDGF1. Data represent the percent maximal binding after nonspecific binding is subtracted. Inset shows the Scatchard analysis. (A) MCF-7 cells; (B) MDA-MB 468 cells. (Reproduced from [3].)

MDGF1 induced the appearance of phosphotyrosine in a 180–185-kDa protein in MDGF1 receptor-positive cell lines (MCF-7, MDA-MB 468).

Immunofluorescence staining and membrane fractionation showed that phosphorylation induced by MDGF1 may be plasma-membrane associated [6]. Phosphorylation by MDGF1 was not blocked by an antibody directed

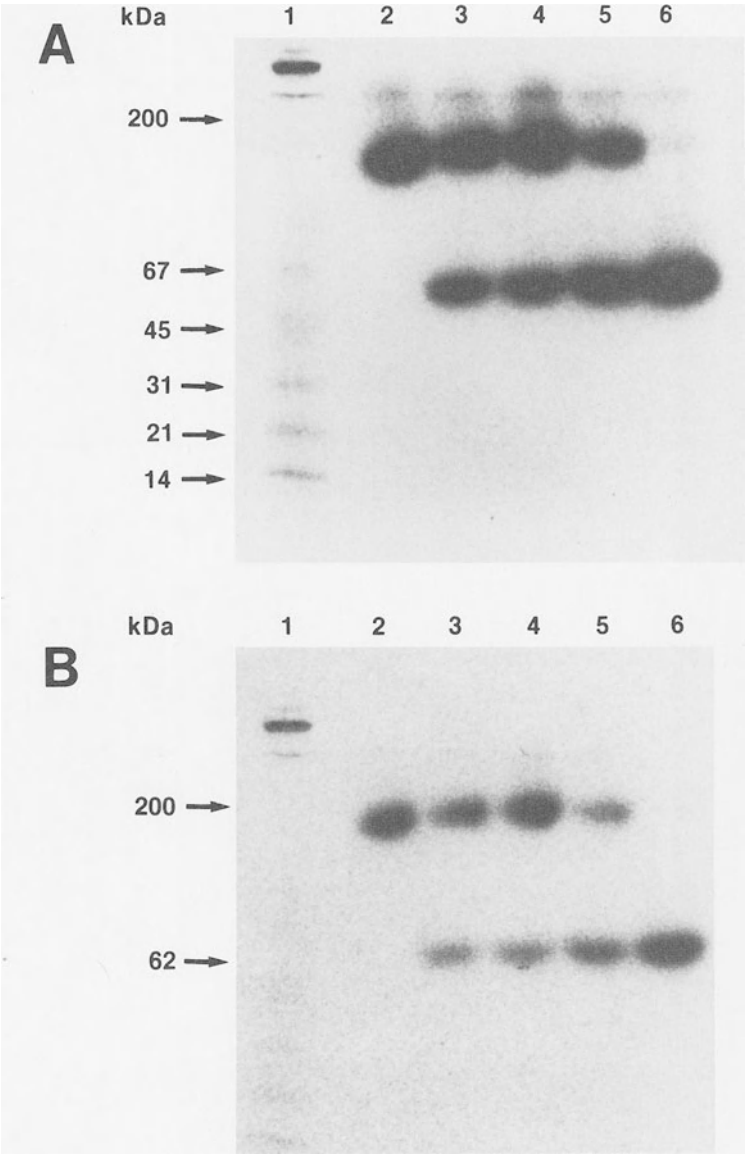

Figure 4. Cross-linking experiments. Cells were incubated with [^{125}I]-MDGF1 without any other additives (lane 2) or in the presence of excess unlabeled MDGF1 (2.5, 5.0, 10, and 25 ng, lanes 3–6). The cells were then treated with a cross-linking agent DSS. Dissolved cells were subjected to SDS-gel electrophoresis. Lane 1 denotes molecular mass markers. (A) MCF-7 cells; (B) MDA-MB 468 cells. (Reproduced from [3].)

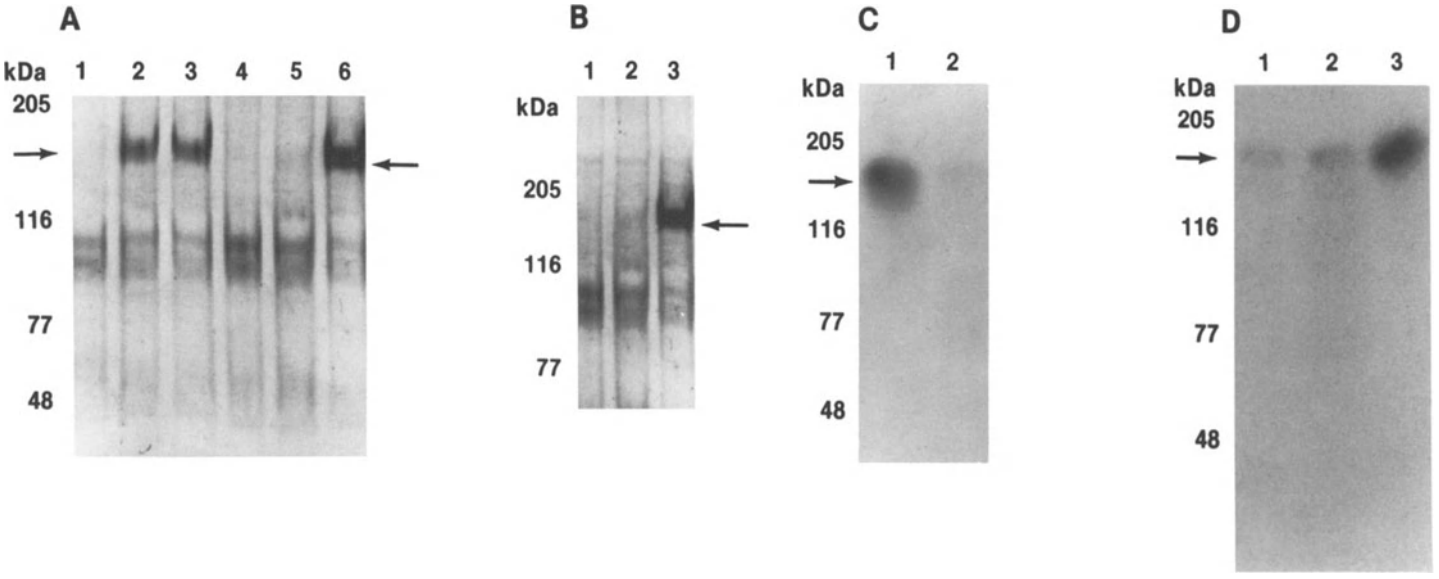

Figure 5. (A) Western blot of phosphotyrosine in MDA-MB 468 cells. Adequate amounts of cells (100,000) were incubated with no addition (*lane 1*), 10 nM purified MDGF1 (*lane 2*), MDGF1 in the presence of an excess of anti-EGF receptor monoclonal antibody 528 (*lane 3*), 5 nM EGF (*lane 6*), EGF in the presence of an excess of anti-EGF receptor monoclonal antibody 528 (*lane 5*), and 100 nM monoclonal antibody 528 alone (*lane 4*). Cells were solubilized in sample buffer and subjected to gel electrophoresis (7.5% gels) and Western blot analysis using a commercially available monoclonal antiphosphotyrosine antibody (2.5 μg/ml) and visualized with a chromogenic reagent. The *arrow* at *left* indicates a MDGF1-induced 180–185-kDa-size band (*lanes 2* and *3*) and, at *right*, an EGF-stimulated 170-kDa band (*lane 6*). (B) Stimulation of tyrosine phosphorylation of MCF-7 cells by MDGF1. Confluent MCF-7 cells were treated with 0 (*lane 1*), 10 nM EGF (*lane 2*), or 10 nM MDGF1 (*lane 3*) for 20 minutes at 37°C, and then the lysates were subjected to SDS-PAGE, Western blotting, and incubation with monoclonal antiphosphotyrosine as above. The *arrow* at *right* indicates a 180–185-kDa-size MDGF1-induced band (*lane 3*). (C) Autoradiogram of phosphorylated proteins in MDA-MB 468 cell lysates. Total cell lysates were analyzed by 7.5% SDS-PAGE gels, followed by immunoblotting with polyclonal anti-phosphotyrosine antibody as explained above. The blots were then processed with [^{125}I]-Protein A, dried, and autoradiographed. *Lane 2* denotes control lysate, and *lane 1* denotes cells treated with 10 nM MDGF1. The *arrow* indicates the 180–185-kDa band. (D) Detection of the EGF-R autophosphorylation activity in MDA-MB 468 cells. Cultures of untreated (*lane 1*), MDGF1-treated (10 nM, *lane 2*), or EGF-treated (10 nM, *lane 3*) cells were lysed and then immunoprecipitated with an anti-EGF receptor antibody. The washed immunoprecipitates were incubated with 20 mM HEPES. pH 7.4, 0.15 M NaCl, 0.1% Triton X-100, 10% glycerol, 10 nM $MnCl_2$, and 5 μM ATP containing 0.1 μCi of [γ^{32}P]-ATP (Amersham) for 5 minutes at 25°C, leading to autophosphorylation. Reactions were stopped in sample buffer, and the samples were electrophoresed on 7.5% PAGE followed by autoradiography. (Reproduced from [6].)

Table 1. MDGF1 production and properties by various cell lines

Cell line or strain	Receptor binding	Growth	Stimulation of phosphotyrosine	MDGF1 production
MCF-7	++	++	++	−
MDA-MB 468	++	+/−	++	−
184A1N4	+	++	+	−
184	+	+	+	++
MDA-MB 231	−	−	−	++
HBL-100	+	++	+	++

Radio-receptor assay and growth stimulation assays were performed as described earlier [2,3]. Binding studies showed high- (++) and low- (+) affinity binding sites for MDGF1 on different cell lines [3]. For growth assays, (++) denotes above 60% stimulation above control and (+) denotes 10–30% stimulation. A biphasic growth effect is depicted by (+/−), which means growth stimulation at low concentration and growth inhibition at high concentration. Western blot of phosphotyrosine in various cell lines were performed after MDGF1 stimulation using anti-phosphotyrosine monoclonal antibody [5]. Immunoreactive MDGF1 was detected in the conditioned medium and cell lysates of the three cell lines using Western blot with polyclonal anti-MDGF1 antibody [5]. (Reproduced from reference [6].)

against the binding sites of the EGF receptors. Two-dimensional gel electrophoresis and immunoblotting with antiphosphotyrosine antibody indicated that the tyrosine phosphoprotein induced by MDGF1 is distinct from that induced by EGF [6].

We hypothesize that the MDGF1 receptor exists as two components, one for ligand binding and the other a substrate for tyrosine phosphorylation, a situation similar to the tyrosine-kinase-associated receptors such as the insulin [56,57]. However, the nature of the phosphorylation site is under investigation to elucidate if alternatively it might be a cellular substrate of the MDGF1 receptor.

Table 1 summarizes the properties and production of MDGF1 by various cell types. Although MDGF1 is apparently not secreted by some responsive cell lines (like MCF-7 with moderate affinity binding sites) and some apparently nonresponsive cell lines secrete MDGF1 (like MDA-MB 231, which does not show detectable levels of MDGF1 receptors), we cannot yet rule out an intracellular mechanism of receptor/MDGF1 sequestration and autostimulation. MDGF1 might also serve a paracrine action in normal and/or malignant breast tissue, since we have previously shown that it is a potent stimulator of collagen synthesis in fibroblasts [4].

Summary

Preliminary results indicated that MDGF1 differentially stimulated the incorporation of proline into collagen-sensitive protein by 2.5–10-fold in NRK,

rat, mouse, and human normal and malignant mammary epithelial cells. Pulse-chase studies indicated that the factor stimulated collagen synthesis rather than blocked collagen turnover. It was also shown that production of type IV collagen is controlled at the level of increased biosynthetic rates with no alteration in degradative rates [2,58].

The biochemical studies indicated that MDGF1 is likely to be a novel N-glycosylated growth factor. Also of importance is the fact that a possible receptor for MDGF1 has been detected. Its binding site appears to be associated with a 120–140-kDa polypeptide chain [3]. It is not yet clear whether the 180–185-kDa phosphotyrosine-containing protein detected after stimulation of responsive cells with MDGF1 is a receptor subunit with tyrosine kinase activity, or itself a primary or secondary substrate of protein kinase activity initiated by MDGF1 binding to the 120–140-kDa binding site [6]. Current studies are addressing the molecular cloning of cDNA of MDGF1 to assist in further characterization of the factor.

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11. The role of polyamines in the hormonal control of breast cancer cell proliferation

Andrea Manni

The diamine putrescine (usually referred to as a polyamine) and the polyamines spermidine and spermine are small aliphatic amines that are essential for cell growth and differentiation [1,2]. Because of their positive charge, they interact with negatively charged phosphate groups on molecules such as DNA and RNA. They have, indeed, been found to effect important properties of DNA such as stability and condensation [3,4]. However, despite these recent findings, the specific mechanisms by which polyamines influence a variety of cellular functions remain largely unknown. Although polyamines are ubiquitously present in mammalian cells, their level in tumors is significantly greater than that of surrounding normal tissue [5,6]. Consequently, polyamines could be regarded as an attractive target for anticancer therapy.

The polyamine biosynthetic pathway is illustrated in figure 1. The enzyme ornithine decarboxylase (ODC) is of particular importance, since it catalyzes the formation of putrescine from ornithine, the first and rate-limiting step in polyamine biosynthesis. The availability of specific inhibitors of this enzyme, particularly α-difluoromethylornithine (DFMO), has been instrumental in establishing the significance of polyamines in cell growth and differentiation. Administration of DFMO has, indeed, been found to have a significant antiproliferative effect in a variety of experimental tumor models both in-vitro and in-vivo [7,8]. The usefulness, however, of DFMO as an effective antitumor agent in-vivo is limited by lack of major suppression of the distal polyamines, particularly spermine [9]. This is related to the well-known occurrence of multiple compensatory events aimed at preserving cellular polyamine pools [10] including transport of exogenous polyamines from the tissues of the host into the tumor cells [11–13]. To circumvent these problems, considerable effort has recently been placed in developing polyamine analogues [14]. These compounds inhibit both ODC and SAM-DC activity [15] and accelerate the degradation and secretion of polyamines by inducing the enzyme spermidine/spermine-N′-acetyltransferase [16–19]. Furthermore, they are able to replace the naturally occurring polyamines at intracellular sites without being able to exert their functions [20]. Administration of polyamine analogues has, indeed, been found to be associated with more profound suppression of cellular polyamine contents and, at times, increased antitumor activity compared to DFMO [21,22].

R. Dickson and M. Lippman (eds.), Mammary Tumorigenesis and Malignant Progression.

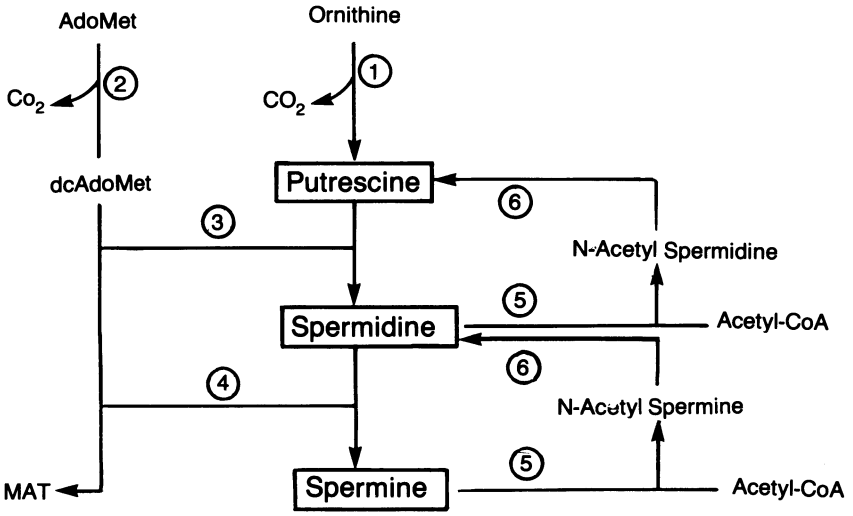

Figure 1. Metabolic pathways for polyamine biosynthesis and catabolism as it occurs in mammalian cell systems. The key enzymes include 1) ornithine decarboxylase, 2) S-adenosylmethionine decarboxylase, 3) spermidine synthase, 4) spermine synthase, 5) spermine/spermidine N^1-acetyltransferase, and 6) polyamine oxidase. (From [14], with permission.)

Over the last several years, our laboratory has been interested in investigating the role of polyamines in the control of breast cancer cell proliferation. It is the purpose of this chapter to summarize our findings as well as those of other investigators in this field. Particular emphasis will be placed on the interaction between the polyamine pathway and endocrine mechanisms controlling tumor growth. The potential role of polyamines in breast cancer progression from a hormone-dependent to a hormone-independent, more aggressive phenotype will be discussed. Finally, we will present some preliminary evidence indicating that manipulation of the polyamine pathway may increase hormonally induced synchronization of breast cancer cell proliferation. This maneuver could potentially enhance the antitumor action of cytotoxic drugs.

Polyamine involvement in breast cancer growth and tumorigenesis

In our initial experiments conducted in the hormone-responsive N-nitrosomethylurea- (NMU) induced rat mammary tumor cultured in soft agar, we demonstrated that DFMO administration exerted a dose-dependent inhibitory effect on tumor colony formation [23]. The specificity of the DFMO effect through the polyamine pathway was supported by the ability of exogenous putrescine addition to reverse the DFMO effect in a dose-dependent fashion [23]. Subsequent experiments demonstrated that admin-

istration of escalating doses of DFMO to tumor-bearing rats (0.5%, 1%, 2%, 3% in drinking water) exerted a significant growth-inhibitory action in vivo, which was also rescued with exogenous putrescine [9]. DFMO treatment caused a major suppression in putrescine but only a modest decrease in spermidine levels, while spermine content was not affected [9]. These results are well in line with the well-known compensatory events discussed above, which occur in-vivo following DFMO administration. Since DFMO has been reported to inhibit ovarian ODC activity [24], we tested the influence of this compound on the hormonal milieu of the host. We observed that the treatment did not effect the estrous cycle of the rats and did not alter serum levels of estradiol and prolactin or ovarian and uterine weights [25]. Thus, our results do not suggest an endocrine mechanism accounting for the antitumor action of the drug.

Recently, Thompson and co-workers have demonstrated a protective effect of DFMO administration in the promotion stage of chemically induced mammary carcinogenesis in the rat [26–28]. These investigators reported that doses of only 0.125% in drinking water decreased the incidence and number of cancers per rat following NMU injection [27]. This protective effect was associated with suppression of ODC activity and polyamine levels [26,28] and was blocked by feeding putrescine in conjunction with DFMO treatment [27].

Taken collectively, these results indicate that polyamines play an important role in the growth of established breast cancers as well as in mammary tumorigenesis.

Polyamines as mediators of hormonally stimulated events in breast cancer

The growth of the NMU mammary tumor is under multihormonal control. When tumor is cultured in soft agar in the absence of serum, administration of estradiol, prolactin, growth hormone, and progesterone similarly stimulates tumor colony formation in a dose-dependent manner [29–31]. We observed that under these experimental conditions, administration of 1 mM DFMO completely abolished the growth-promoting action of these mitogens. In every case, the growth-inhibitory effect of DFMO was completely reversed by exogenous polyamine administration [29–31]. Subsequently, several laboratories, including our own, have investigated the role of polyamines as mediators of estradiol-stimulated growth of several human breast cancer cell lines in liquid culture [32–35]. In some [32,33] but not all of them [34,35], ODC activity was stimulated to variable degrees by estradiol administration. In every case, however, DFMO treatment was found to consistently suppress ODC activity, cellular polyamine levels, and estradiol-stimulated growth [32–35]. These observations provide support for the contention that polyamines play a critical role in mediating hormonal effects on human and experimental breast cancer cell proliferation in culture.

Next, we tested whether the antitumor effect of the antiestrogen tamoxifen could be reversed by exogenous polyamine administration. We hypothesized that if polyamine biosynthesis is under estrogen control and polyamines are critical mediators of estrogen action, antiestrogen administration might, at least in part, exert its antitumor effect through induction of polamine depletion. In initial experiments conducted in NMU tumors cultured in soft agar, we observed that the inhibitory effect on colony formation exerted by tamoxifen was reversed in a dose-dependent fashion by exogenous administration of putrescine (0.1–2.5 mM) as well as spermidine and spermine (1–100 μM) [36]. Subsequent experiments conducted in human breast cancer cell lines in liquid culture by us [37] and other investigators [32,33,38] have given conflicting results in this regard. While some investigators have observed reversal of the antiproliferative effect of tamoxifen with exogenous administration of polyamines [32,38], others, including ourselves [33,37], have failed to confirm this finding despite demonstration of moderate suppression of ODC activity and cellular polyamine levels induced by antiestrogen treatment [37]. In experiments conducted in vivo in NMU tumor-bearing rats, we observed that the antitumor action of tamoxifen but not that of ovariectomy could be inhibited by concomitant administration of

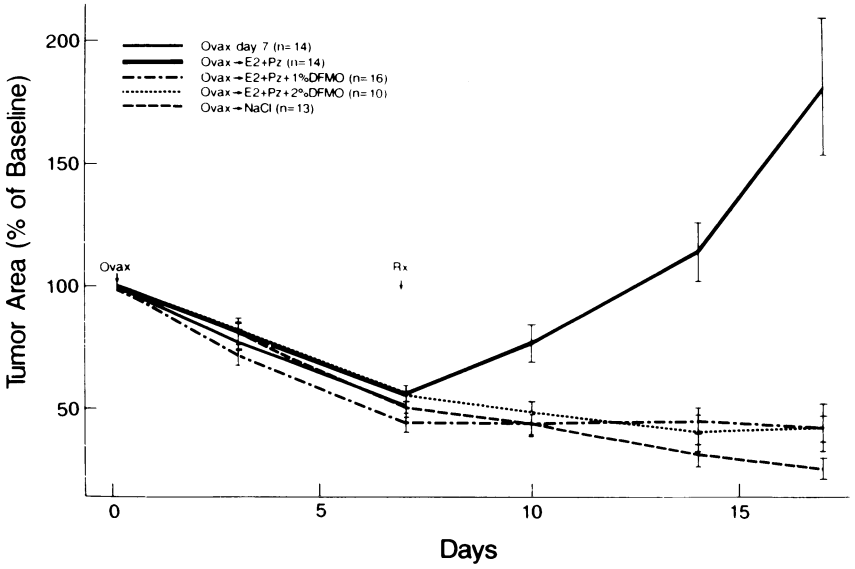

Figure 2. Influence of DFMO administration (60 mg/rat/day by Alzet minipump plus either 1% or 2% in drinking H_2O, as indicated) on hormone-stimulated NMU rat mammary tumor growth. Groups of rats (n = 8–10/group) harboring mammary tumors matched for size were randomly assigned to the indicated experimental treatments. Data represent means ± SEM. Ovax, ovariectomy; E2, estradiol benzoate (5 μg s.c. daily); P_z, perphenazine (1 mg i.m. daily, to stimulate endogenous prolactin release). (From [40], with permission.)

Table 1. Influence of DFMO administration on hormonal effects on NMU-mammary tumor levels of progesterone receptors (PgR)[a]

Experimental groups	Number	PgR (f moles/mg protein)
Control	15	303 ± 65
Ovax, d7	16	28 ± 8[b]
Ovax → E_2 + P_Z	11	378 ± 123
Ovax → E_2 + P_z + 1% DFMO	15	240 ± 47
Ovax → E_2 + P_z + 2% DFMO	8	286 ± 55
Ovax → saline	9	10 ± 5[b]

[a] Experimental design and abbreviations are as described in the legend to figure 2. Data represent means ± SEM.
[b] $p < 0.05$ vs. the remaining groups. (Adapted from [40], with permission.)

putrescine [39]. The mechanism of reversal, however, remained obscure, since chronic antiestrogen treatment did not effect tumor polyamine pools [39]. A similar lack of influence of tamoxifen treatment on NMU mammary tumor polyamine levels has also been reported by Thompson et al. [28]. Thus, at present, it is uncertain whether any of the antitumor action of tamoxifen is mediated through the polyamine pathway. It is the author's opinion that polyamines probably do not play a major role in mediating antiestrogen induced inhibition of breast cancer growth.

There is considerable evidence in the literature to suggest selectivity of polyamine involvement in hormone action. It is quite clear that hormonal stimulation of ODC activity and polyamine biosynthesis in endocrine target tissues cannot be equated to universal mediation by polyamines of every aspect of hormone action. In experiments conducted in NMU-mammary-tumor-bearing rats, we observed that DFMO administration, while able to inhibit hormone-stimulated tumor growth (figure 2), was unable to significantly effect estradiol-mediated progesterone receptor synthesis in the same tumors (table 1) [40]. A similar finding has been reported by Kendra and Katzenellenbogen in MCF-7 breast cancer cells in culture [41]. In addition, we also showed that DFMO treatment had either a minimal effect or no effect on estrogen-stimulated uterine growth in the same animals (figure 3) [40]. Other investigators have also reported a similar dissociation between hormone stimulation of polyamine biosynthesis and proliferation in rat and mouse uterus [42,43] and rat prostate [44].

Taken together, these results underscore the selectivity of polyamine involvement in hormonal action, despite evidence of endocrine control of polyamine biosynthesis in virtually every endocrine target tissue tested so far [45–48]. Such selectivity, if confined to hormonal mediation of neoplastic cell growth as suggested by our studies in the NMU tumor, could represent a major therapeutic advantage in the use of antipolyamine therapy in the treatment of hormone-responsive breast cancer.

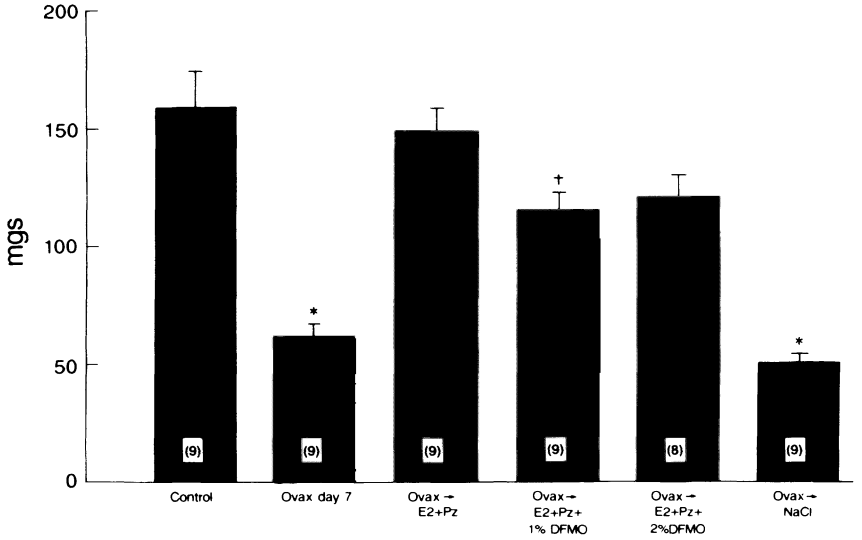

Figure 3. Effect of DFMO on hormone-stimulated uterine growth. Experimental conditions and abbreviations are as described in the legend to figure 2. Data represent means ± SEM. Numbers in parentheses indicate the number of determinations. *, $p < 0.05$ vs. the remaining four groups; +, $p < 0.05$ vs. 'control' and 'Ovax → E_2 + P_z.' (From [40], with permission.)

Interactions between polyamines and autocrine/paracrine effectors of breast cancer growth

Although polyamines are essential mediators of hormonal action on breast cancer growth, they are not able by themselves to reproduce such endocrine effects when added alone to tumor cells in culture [49]. Therefore, we postulated that polyamines may interact with additional mechanisms involved in hormonal stimulation of tumor growth. In considering these additional modes of hormone action, we focused our attention on autocrine/paracrine factors, which have been implicated in breast cancer growth [50]. In evaluating the potential sites of interaction between autocrine/paracrine effectors of tumor growth and the polyamine pathway, we chose to investigate polyamine involvement in the synthesis and action of hormonally regulated growth factors. Experiments conducted in the NMU mammary tumor in soft agar indicated that polyamines may play an important role in growth factor synthesis [51,52]. In these studies, we initially conditioned our media with E_2/oPRL ± DFMO ± polyamines and then evaluated their growth-promoting action. We hypothesized that if polyamines are essential for the synthesis of E_2/oPRL-induced growth factors, the conditioned media obtained from tumors treated with the hormones and DFMO should no longer have the growth-promoting activity usually observed with E_2/oPRL-CM. On the other hand, such growth-promoting activity should be restored

with conditioned media obtained from tumors exposed to E_2/oPRL, DFMO, and exogenous polyamines. The data, indeed, supported our hypothesis and suggested that polyamines may play an essential role in the synthesis of estradiol and prolactin inducible growth factors [51,52]. With the availability of monoclonal and polyclonal antibodies directed against known growth factors and/or their receptors, we investigated the nature of the secreted peptides in this experimental system. Using this approach, we observed that transforming growth factor α is responsible primarily for estradiol effects and to a lesser extent progesterone action [53,54]. TGF-α, on the other hand, does not appear to be secreted upon exposure to prolactin and is not involved in the mediation of the proliferative effects of this peptide hormone [53,54]. The secretion of insulin-like growth factors appears to be stimulated to a similar extent by estradiol, prolactin, and progesterone [55]. Furthermore, our results provide strong support for a role of endogenously produced IGFs as mediators of hormonal effects on NMU mammary tumor growth [55].

In human breast cancer cell lines in liquid culture, on the other hand, we found less evidence for a major role of polyamines in growth factor synthesis. Despite inducing a profound suppression of ODC activity, cellular polyamine levels, and estradiol-stimulated growth, administration of DFMO did not influence either basal or E_2-induced excretion of TGF-α [56] or immunoactive IGF-I [35]. Polyamines, on the other hand, appear to be involved in TGF-β production [57]. Addition of DFMO to MCF-7 breast cancer cells in culture increased the level of TGF-β detected in the cultured media to a similar extent as the antiestrogen 4-hydroxytamoxifen [57]. The effect was additive when the two compounds were added together. Although 4-hydroxy tamoxifen reduced cellular polyamine levels, polyamine repletion with putrescine did not prevent the antiestrogen-induced increase in TGF-β secretion [57]. These results indicate that polyamines are involved in basal TGF-β production by MCF-7 cells but do not mediate antiestrogen-induced TGF-β secretion.

Our results indicate that polyamines are more consistently involved in growth factor action under conditions of both anchorage-independent and anchorage-dependent growth. In experiments conducted in the NMU mammary tumor cultured in soft agar, we observed that DFMO administration inhibited the colony-stimulating effects of the hormone conditioned media [49,58]. Furthermore, we observed that exogenous polyamine administration markedly potentiated in a dose-dependent fashion the growth-promoting actions of estrogen- and prolactin-conditioned media [49,58]. In contrast, no inhibitory effect of DFMO or potentiating effects of polyamines were observed when control-conditioned media were used [49,58]. These experiments suggested that polyamines are essential for the action of hormonally regulated growth factors and markedly potentiate their effects. Similarly, in MCF-7 breast cancer cells in liquid culture, we observed that DFMO administration significantly inhibited the proliferative effects of exogenously added IGF-I [35] and TGF-α [56]. With regard at least to the latter growth factor,

Table 2. Effect of four-day treatment with DFMO (4 mM) on IGFBP secretion by MDA-MB-231 and BT-20 cells[a]

A. MDA-MB-231 cells

	36.5 kDa		25 kDa (IGFBP-1)		19.5 kDa	
	A (%)	B (%)	A (%)	B (%)	A (%)	B (%)
Control	100	100	100	100	100	100
DFMO	234 ± 49	259 ± 56	141 ± 11	155 ± 14	129 ± 5	143 ± 7

B. BT-20 cells

	31 kDa (IGFBP-2)		27 kDa	
	A (%)	B (%)	A (%)	B (%)
Control	100	100	100	100
DFMO	120 ± 7	162 ± 15	123 ± 19	162 ± 27

[a] Levels of secreted IGFBPs were estimated by densitometric analysis of ligand blotting performed in serum-free conditioned media. Values were normalized both by cell number (A) and protein loads on the gels (B). Data are expressed as percent of control and represent the means ± SEM of four replicate experiments for each cell line. (Adapted from [62], with permission.)

the degree of inhibition by DFMO appeared to be influenced by serum factors and clonal variability of MCF-7 cells [56]. The specific sites of interaction between polyamines and growth factor action remain to be elucidated. Evidence in the literature indicate that polyamines may be critically involved in several steps of the signal-transduction cascade, such as phospholipase C [59], protein kinase C [60], and numerous protein phosphorylations [61]. Recently, we have investigated the hormonal and polyamine regulation of insulin-like growth-factor binding proteins (IGFBPs) synthesis and secretion by human breast cancer cells in culture [62]. As can be seen in table 2, we observed that DFMO administration induced an increase in IGFBPs secretion by MDA-MB-231 and BT-20 cells. DFMO administration, on the other hand, did not affect secretion of IGFBPs by MCF-7 breast cancer cells. These results indicate that, at least under certain circumstances, polyamines may influence IGFs action by modulating the secretion of IGFBPs. Suppression of polyamine biosynthesis with DFMO could, therefore, inhibit IGF-I- and -II-stimulated breast cancer cell proliferation by raising the secretion of growth inhibitory IGFBPs.

Polyamine involvement in tumor progression

Loss of hormonal dependence in breast cancer is an inevitable phenomenon that is associated with the acquisition of an aggressive phenotype resulting in

the patient's death. At present, our understanding of this process remains fragmentary, thus preventing us from devising more effective therapies in the treatment of this malignancy. The hypotheses put forward to explain breast cancer transition from a hormone-dependent to a hormone-independent phenotype include oncogene amplification [63], mutated and/or truncated steroid receptor-like proteins acting as constitutive activators [64], and overproduction of several growth factors [65]. As we discussed above, polyamines interface with hormones in several key steps regulating breast cancer cell proliferation. It is then possible that constitutive activation of the polyamine pathway may enhance cell growth, thus bypassing the need for hormonal presence and leading to the development of the hormone-independent breast cancer phenotype. Several lines of circumstantial evidence suggest an association between increased polyamine biosynthetic activity and aggressive phenotypic characteristics of breast cancer. Glickman et al. [66] reported higher ODC activity levels in human breast cancer tissues with higher cellularity, lower histologic differentiation, and higher nuclear aplasia. Kingsnorth et al. [67] observed that increased intracellular polyamine levels in human breast cancer specimens were correlated with histologic grade 3, estrogen-receptor negative status, and direct extension to the skin or chest wall. In addition, these authors reported a positive correlation between high tumor polyamine levels and probability of recurrence within two years of mastectomy. Recently, T. Thomas et al. [68] studied amplification and expression of the ODC gene in the hormone-responsive MCF-7 and T47 D cell lines and in hormone-resistant MDA-MB-231 and BT-20 cells. The authors reported a higher ODC gene dosage in the hormone-independent cell lines, which was associated with a 2- to 3-fold increase in ODC mRNA levels. In addition, BT-20 cells manifested a higher cellular level of ODC activity and polyamines and, as also reported by other investigators [34], were insensitive to the antiproliferative effects of DFMO [68]. Nevertheless, increased polyamine biosynthetic activity and DFMO resistance cannot be equated to hormone unresponsiveness, since MCF-7 and MD-MB-231 cells have similar levels of ODC activity and polyamines and are equally sensitive to DFMO [68,69]. Therefore, the role, if any, of polyamines in tumor progression to hormone independence remains to be established. We are currently testing this hypothesis by evaluating the effects of induction of overexpression of ODC and SAM-DC in breast cancer cells through a transfection approach.

Polyamine manipulation as a means to enhance hormonally induced synchronization of breast cancer growth

Induction of tumor cell growth synchronization has been proposed as a potential means to enhance the action of phase-specific cytotoxic chemotherapy [70–72]. The estrogen dependency of human breast cancer provides the

unique opportunity to selectively manipulate tumor cell kinetics by hormonal means without affecting the normal tissues of the host. The ability to hormonally synchronize breast cancer cell proliferation may be limited, however, by cellular heterogeneity in sensitivity to the multistep activation of growth following initial hormone binding to its receptor. In experiments conducted in the NMU mammary tumor, we observed that following ovariectomy there was a reduction in cellular labeling indices as assessed by tritiated thymidine autoradiography (figure 4) [73]. Upon exogenous hormone administration to castrated rats, we observed a gradual increase in the labeling indices of the tumor cells, peaking on day 7 of treatment at

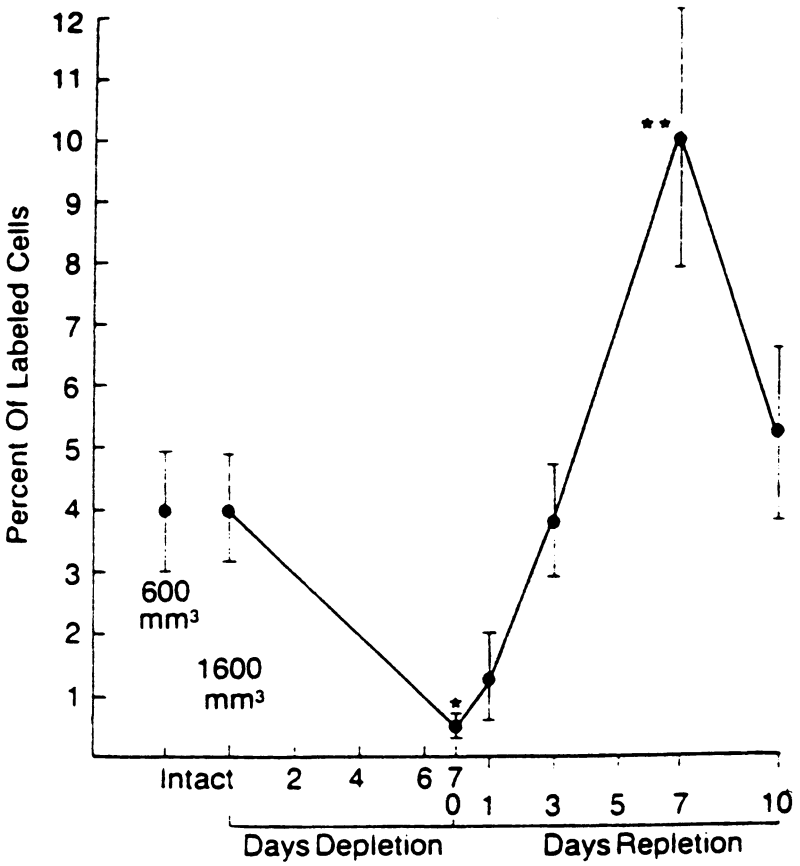

Figure 4. Effect of hormone depletion (ovariectomy) and repletion (estradiol benzoate, 5 µg s.c. daily and perphenazine 1 mg i.m. daily) on the labeling indices of NMU rat mammary tumor cells. The number of tumors was five in each case. Data represent means ± SEM. *, $p <$ 0.01 versus 'intact'; **, $p < 0.01$ versus 'intact' and '7-day castration.' The intact 600 mm^3 group was included on the basis of the estimated effect of 7-day castration on tumor growth to exclude the possibility that a smaller volume rather than the effect of treatment accounted for the observed alterations in cell kinetics. (From [73], with permission.)

levels twice as high as those observed in the tumors of control intact rats (figure 4) [73]. Although this 'overshoot' indicates that a certain degree of cell cycle synchronization had been obtained, the gradual increase in labeling indices between days 1 and 7 suggests that a significant degree of asynchronous growth was still occurring, with different populations of tumor cells being recruited into the cycle at different time intervals after hormonal exposure. Furthermore, by day 7 the tumors had also regrown essentially to their original sizes. Considering the complexity of hormone action on proliferation and the heterogeneity of breast cancer cells, it is not surprising that only suboptimal synchronous growth occurs following hormonal administration. Between the initial binding of the hormone to its receptor and the beginning of hormone-induced cell proliferation, numerous intermediate events need to take place. It is then not surprising that the activation of this cascade of events upon hormonal exposure requires different time intervals depending on the differential sensitivity to hormones of heterogeneous populations of breast cancer cells. We hypothesized that if we could intervene more distally in the hormone action pathway, we might be able to reduce the heterogeneity in cellular proliferative response to hormone administration. Since our data (discussed above) indicated that polyamines may be 'second messengers' of hormone effects, we speculated that manipulation of the polyamine pathway might improve our ability to hormonally synchronize breast cancer growth. In detailed time-course studies, we have, indeed, observed that a combination of ovariectomy and DFMO induced a faster and greater suppression of the labeling indices of NMU mammary tumor cells than the individual treatments, even though tumor regression was not superior to that produced by ovariectomy alone (figure 5) [74]. We have recently tested a combined hormone/polyamine manipulation strategy in the attempt to optimize synchronization of breast cancer growth [75]. In these experiments conducted in NMU-tumor-bearing rats, we induced an initial phase of hormone and polyamine depletion (ovariectomy plus administration of DFMO) to maximally block cell growth. This was followed by an intermediate phase of hormone repletion while polyamine biosynthesis was still blocked by DFMO. During this time interval, we expected to 'prime' all hormone-responsive cells up to the point (presumably distal in the proliferative cascade) where polyamines are involved. At this point, the polyamine blockade was removed and the cancer cells were allowed to initiate proliferation in the presence of continuing hormonal administration and endogenous polyamine repletion. The effects of this combined approach on the labeling indices of tumor cells were compared to those of individual hormone/polyamine depletion/repletion regiment. We observed that the combined approach yielded the highest degree of synchronization, with labeling indices of the epithelial cells being approximately twofold over intact control after only two or three days of the combined repletion. In contrast, hormone treatment alone restored the labeling indices of these cells only at best to control levels. In addition, although the rate of tumor

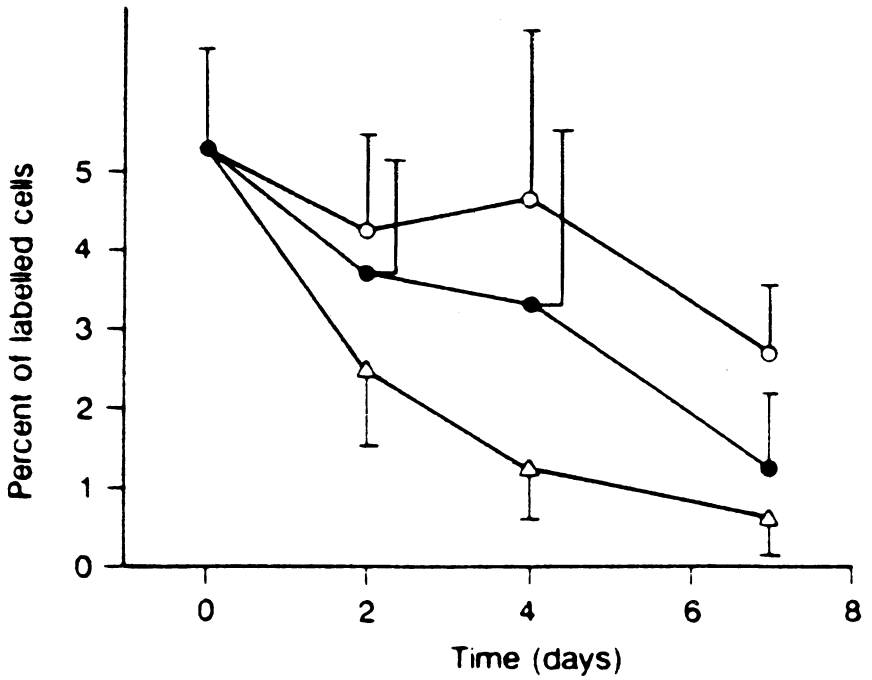

Figure 5. Effect of ovariectomy (●—), DFMO (2% in drinking H_2O) (○—), and ovariectomy plus DFMO (△—) on the labeling indices of NMU mammary tumor cells. Data represent means ± SEM of 4 or 5 determinations per experimental condition. Statistical analysis of change over time in the labeling indices: DFMO, $p = 0.18$; ovariectomy, $p = 0.0079$; DFMO + ovariectomy, $p = 0.0001$. (Adapted from [74], with permission.)

regrowth was highest with the combination treatment, the absolute tumor volumes did not differ significantly at the end of the repletion phase between the three regimens. Overall, these data suggest that combined hormone/polyamine manipulation may improve the implementation of kinetically designed chemotherapeutic regimens. Expansion of tumor volume represents a major deterrent from the application of this form of therapy, since it may cause potentially life-threatening toxicity to the patient. Our results indicate that combined hormone polyamine repletion was superior in inducing synchronization of tumor growth in the absence of a greater increase in absolute tumor volume. Thus, this combined approach appears to offer a superior 'therapeutic window' (labeling index/tumor volume) than sequential hormone depletion/repletion alone.

Conclusions

Overall, the data summarized in this chapter indicate that polyamines play an important role in breast cancer growth. They appear to be critical

mediators of the proliferative effects on mammary tumor cells induced by hormones and growth factors. The specific steps in the signal-transduction system effected by polyamines still remain to be largely established. There appears to be selectivity of polyamine involvement in hormone action, since these compounds do not appear to be required for estrogen stimulation of progesterone-receptor synthesis and growth of some normal tissues such as uterus and prostate. Such selectivity could be exploited therapeutically with the use of antipolyamine therapy. Inhibition of polyamine biosynthesis with DFMO has been shown to be quite effective in inhibiting the growth of established mammary tumors in rats as well as inhibiting promotion of tumorigenesis. Antipolyamine therapy could be employed in combination with standard endocrine treatment. This approach is supported by the greater suppression of tumor cell labeling indices with the combined use of DFMO and ovariectomy discussed above. The lack of greater reduction in tumor volume may simply reflect the short duration of our experimental treatment (1 week). Finally, our recent data indicate that polyamine manipulation could also be used to optimize hormonally induced synchronization of breast cancer growth with the goal of improving the efficacy of cytotoxic drugs.

Effective and safe methods of administration of DFMO to humans are currently being tested in clinical trials, with encouraging results [76]. Furthermore, potent polyamine analogues are being developed that circumvent many of the drawbacks associated with DFMO administration discussed above. It is expected that the clinical testing of these analogues will begin in the near future.

Acknowledgments

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IV

Cancer Cell Adhesion, Migration, and Proteolysis

12. Modulations of the epithelial phenotype during embryogenesis and cancer progression

Pierre Savagner, Brigitte Boyer, Ana Maria Valles, Jacqueline Jouanneau, and Jean Paul Thiery

In this chapter, we develop the idea that in order to be able to detach from the primary tumor, invade, and metastasize to distant organs, carcinoma cells must modify their adhesive status and change their cytoskeletal organization. Interestingly, such modifications of cell adhesion and communication systems have been shown to occur during embryogenesis and particulary during migratory process of epithelial–mesenchymal transition (EMT). These embryonic events therefore could represent the prototype of epithelial cell dispersion. Eventually, cells may switch back to a stable epithelial phenotype state that involves local growth and maintenance of this differentiated state, in coordination with the local environment. The delicate modulation of this equilibrium on a specific cell population represents a basic mechanism of embryogenesis. A similar mechanism of epithelial cell plasticity may apply to cancer cells. In this chapter, we first discuss this balance during a well-documented case of induced EMT in a bladder carcinoma. Then we expand the review to examples of EMT occurring during embryogenesis. Finally, we review cancer metastasis, with a special emphasis on breast cancer.

An in vitro model of induced epithelio-mesenchymal transition: Acidic FGF-activated NBT-II cells

Elsewhere we describe that acidic FGF (aFGF) treatment activates a rat bladder carcinoma cell line (NBT-II) to undergo EMT [1]. In a noninducing medium, NBT-II cells grow as epithelial monolayers. They are polarized and display keratin filaments and membrane-associated specialized junctions such as desmosomes. After activation by aFGF, they lose these epithelial features and acquire mesenchymal characteristics such as vimentin filaments and an elongated phenotype (figure 1). They start migrating randomly, and express gelatinase activity [2–4].

R. Dickson and M. Lippman (eds.), Mammary Tumorigenesis and Malignant Progression.

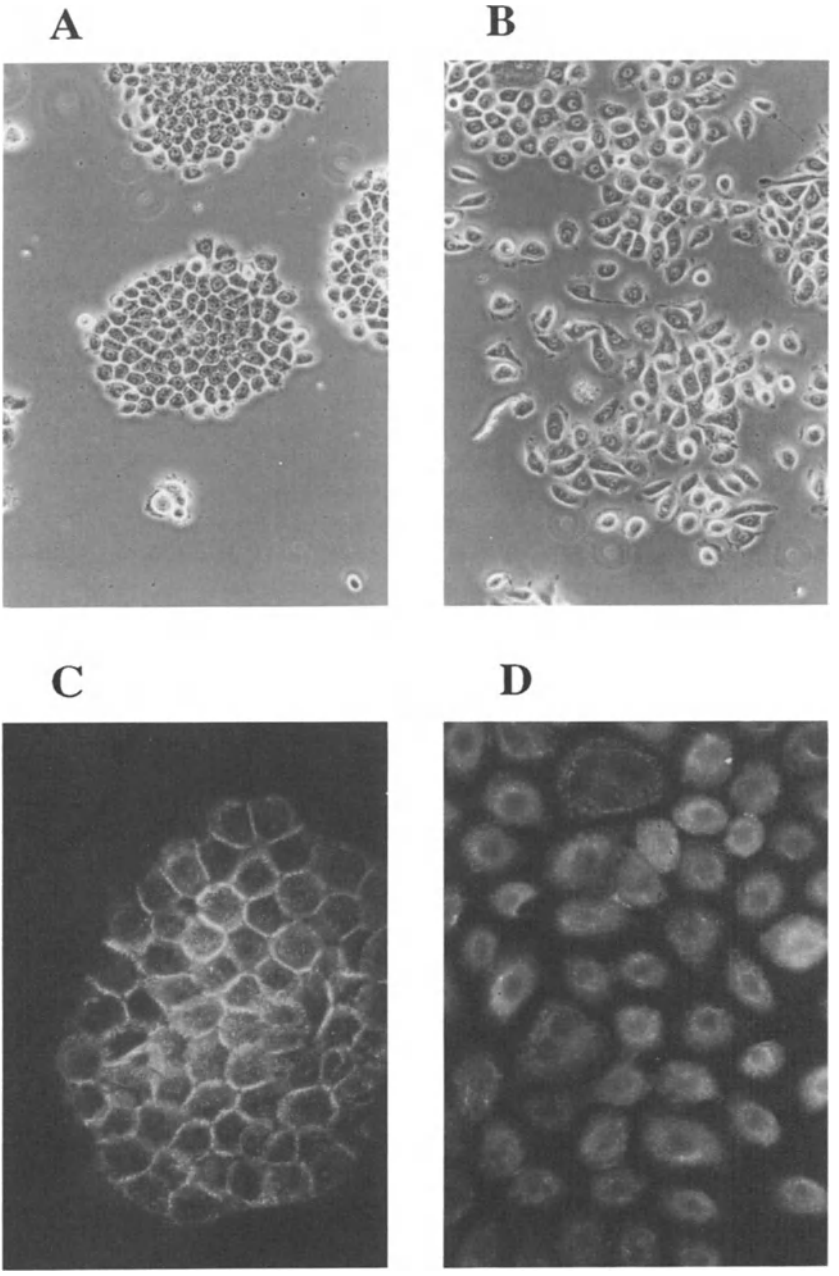

Figure 1. Epithelial-mesenchymal transition in NBT-II cells. (A) Rat bladder carcinoma cells express epithelial characteristics when cultured in standard medium. (B) They become scattered and express mesenchymal characteristics when cultured at low density in presence of aFGF (20 ng/ml) and heparin (10 ng/ml). (C) As indicated by using antibodies to desmoplakin protein, desmosomes are localized at cell–cell borders in most cells expressing an epithelial phenotype. (D) However, they are absent in cells treated for 24 hours with aFGF.

EMT activators and kinetics of activation

NBT-II cells can be activated by aFGF, but also by other related factors as FGF 5 and KFGF [5]. TGF-α and EGF are also active [6]. Other growth factors such as basic FGF (bFGF) and TGF-β were found to be inactive on EMT [1,7]. The phenotypic transformation occurs progressively, starting with cell spreading, desmosomes internalization and acquisition of cell motility, all of which are effective by 5 to 10 hours of induction. This transformation is reversible and dependent on the continuous presence of the activator. In the absence of further activation, cells revert to an epithelial phenotype by a mesenchymal–epithelial transition (MET).

Collagen induction

A different way to induce EMT activation is to grow the cells on collagen substrate [8]. NBT-II cells are able to adhere and spread on various substrates such as collagens, fibronectin, and laminin. However, only collagens of type I, III, IV, and V are active on dissociation, whereas fibronectin, laminin, or denatured collagen I are not. This activation is inhibited by antibodies against β1 integrin subunit (G.C. Tucker, personal communication).

Modulation of cell adhesion systems

NBT-II cells express distinct systems for cell-cell and cell-matrix interactions. The modulation of these systems during EMT is likely to be involved in the new properties of mesenchymal NBT-II cells. Among the cell adhesion molecules (CAM), E-cadherin is described as a necessary component for cohesiveness in different types of cells. Epithelial and mesenchymal NBT-II cells express similar amount of E-cadherin at their surface and the level of phosphorylation of E-cadherin and of catenins are not modified after EMT induction. However, E-cadherin is redistributed after EMT over the entire surface of the cells [9].

Cell culture density regulates NBT-II response

Induced EMT only occurs in cells grown at low density (figure 2 and [10]). Conversely, aFGF acts specifically as a growth-stimulating factor at high confluency (figure 3 and [10]). As shown in [10], this alternative responsiveness is not due to the different accessibility of aFGF to the cells. In vitro wound-healing assays suggest that the cell response depends on its localization within the cell population: cells located at the edge of the wound undergo EMT and do not incorporate ^{3}H-thymidine, whereas cells within the confluent monolayer remain cohesive and synthesize DNA. Consistently, cells located at the periphery of large colonies are the first to dissociate.

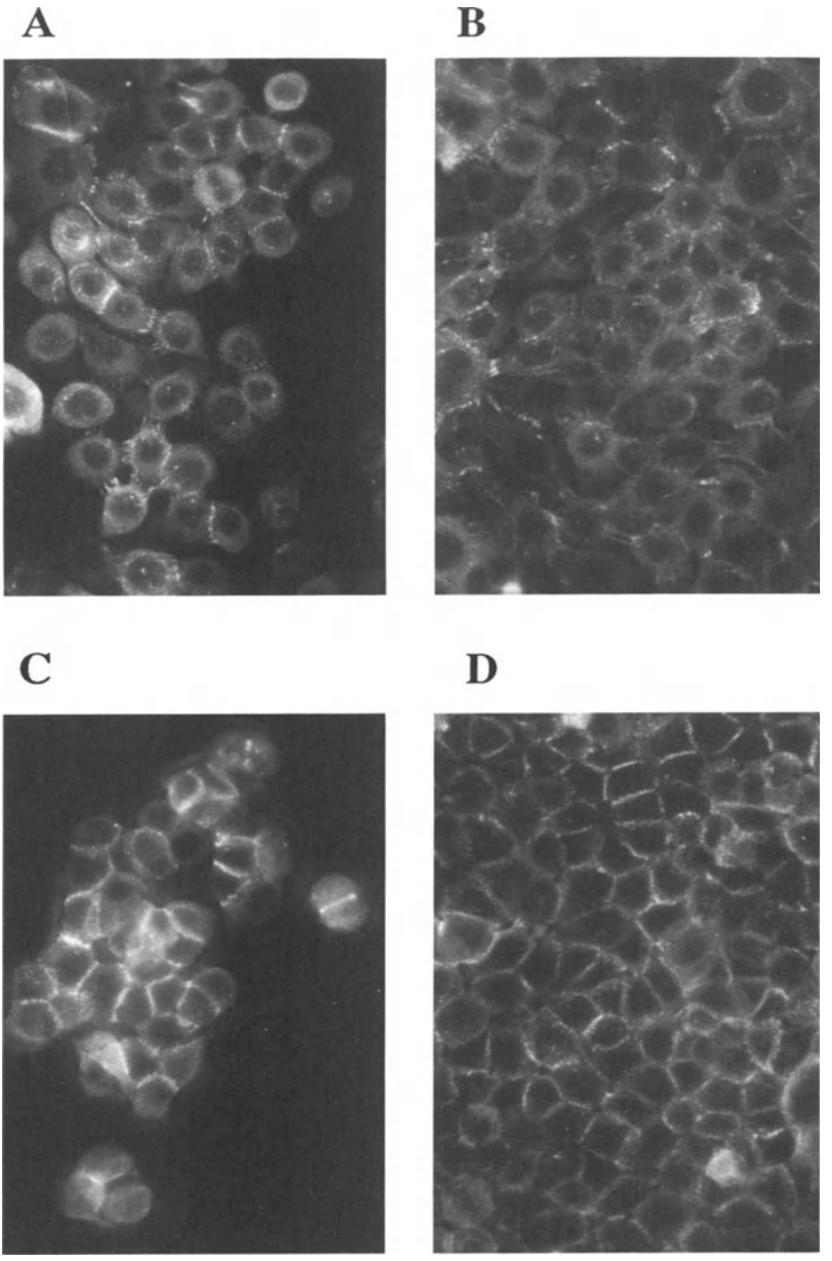

Figure 2. Culture density conditions control aFGF-induced EMT in NBT-II cells. When NBT-II cells are cultured in presence of aFGF (20 ng/ml) and heparin (10 ng/ml), biological response depends on culture density. Only cells grown at low density undergo an epithelial-mesenchymal transition [10]. Mesenchymal phenotype is still expressed by cells activated for 3 hours with aFGF up to 18 hours after replating at (A) low or (B) high density in standard medium. Under the same conditions, untreated cells express an epithelial phenotype, as found by desmosome localization, 18 hours after replating at (C) low or (D) high confluency.

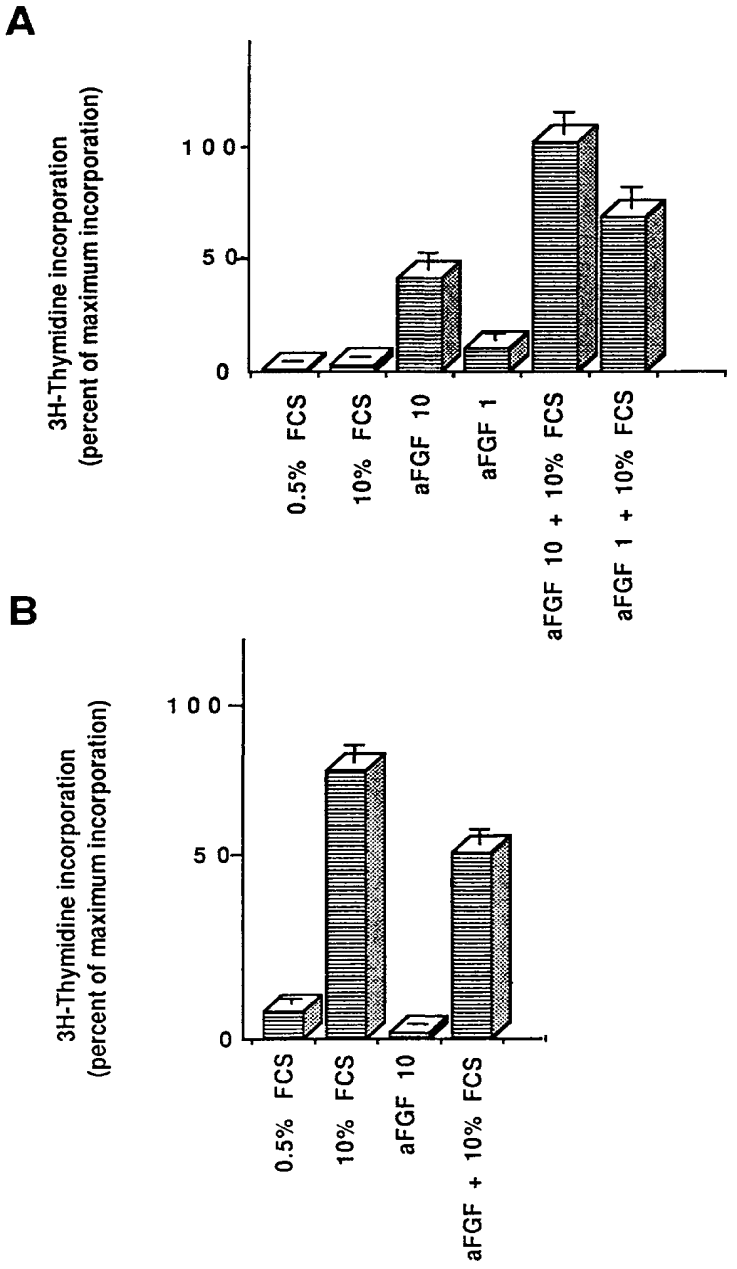

Figure 3. Culture density conditions also control aFGF-induced growth stimulation in NBT-II cells. (A) When NBT-II cells grown at high density are cultured in presence of aFGF (10 ng/ml), they show a strong growth stimulation as indicated by enhanced [^{3}H] thymidine incorporation. (B) Conversely, when NBT-II cells grown at low density are cultured in presence of aFGF, they show no growth stimulation. In both conditions, fetal calf serum is active on [^{3}H] thymidine incorporation.

Activation pathway

A distinct population of about 5000 high-affinity receptors recognizes specifically aFGF but not bFGF on NBT-II cells. EMT activation is therefore likely to be mediated through these high-affinity receptors. At least four related families of FGF receptors have been described in the recent past. They share a similar intracellular region, including a split tyrosine kinase domain. Autophosphorylation of the kinase domain induces the binding, phosphorylation, and activation of different signaling molecules. Growth factors triggering EMT in NBT-II cells act through a tyrosine kinase receptor, resulting in two alternate pathways leading to growth or EMT, depending on the confluency state of the cells [10]. Indeed, aFGF-induced EMT and growth are suppressed by tyrosine kinase inhibitors such as tyrphostin or genistein, whereas tyrosine phosphatase inhibitors such as orthovanadate actually mimic aFGF effects [7]. These findings suggest that tyrosine phosphorylations are involved in both pathways. A major difference between these two pathways is their regulation by intracellular cAMP levels. Increasing the level of intracellular cAMP by use of forskolin, 8-bromo cAMP, dibutyryl-cAMP, or cholera toxin potentiates the mitogenic effect of aFGF at confluency while inhibiting the EMT at low cell density. The same compounds also induce the reversion of mesenchymal-converted NBT-II cells to the epithelial phenotype [7]. This emphasizes the fact that at high and low cell densities, NBT-II cells have different transduction systems activated by the same growth factor. Therefore, the mutually exclusive signal-transduction pathways of aFGF can be distinguished by the cAMP pathway.

In addition, cycloheximide [2] and actinomycin D (P. Savagner, unpublished) studies suggested that transcription and early protein synthesis before 2 to 3 hours were required in both cases to allow EMT induction. It is conceivable that genes specifically associated with the program of EMT or cell proliferation should be transcribed after activation of the same FGF receptor in an otherwise different cell status.

EMT and invasiveness in in vitro and in vivo models

NBT-II cells are described to show tumorigenic and invasive potentials in different assays. When NBT-II cells aggregates confronted a bladder fragment, the invasiveness was accelerated when the cells were treated with aFGF, or when the cells were previously transfected with cDNAs encoding aFGF or TGFα [5]. This observation was confirmed in vivo: subcutaneous injection of aFGF- or TGF-α-transfected NBT-II cells in nude mice induced the appearance after 1 to 4 weeks, respectively, of massive adenocarcinoma-type tumors, while nonproducing cells induce tumors within 8–10 weeks (J. Jouanneau, unpublished). These results argue for the role of some growth factors in the progression toward malignancy.

Epithelial-mesenchymal transition during embryogenesis

During embryogenesis, EMT represents a basic mechanism involved in the making of the body plan and in tissue remodeling. We will discuss here several examples of this process.

Gastrulation

Gastrulation consists of a complex series of events during which predisposed blastula cell populations, triggered by specific inducers, reorganize into the three primary germ layers including the first mesenchymal component [11]. In a number of species, a typical EMT operates in the epiblast at a specific time, giving rise to the migratory mesenchymal cells, while in others there is either an incomplete EMT or an 'en bloc' migration of cells. During gastrulation, cell movements and delamination do not appear to be correlated with cell division. Blockade of cell division in Xenopus does not prevent the start of gastrulation [12].

In the sea urchin, the ability to move is linked to a modulation in cell adhesion systems including desmosomes [13], while in the chick, cadherins and N-CAM are modulated [14]. The extracellular matrix surrounding the site of EMT is clearly involved, either representing a mechanical obstacle that is locally disaggregated [13], or offering a migration substrate rich in fibronectin [15,16], or cytotactin [17] used by differentiating mesenchymal cells. Perturbation of these interactions was shown to generate a major scramble during cell layering [15].

Factors inducing gastrulation or similar processes have not been found. However, it is clear that the growth factors recognized as mesoderm inducers must be implicated in the commitment of mesodermal cells that will invaginate during the process of gastrulation. Interestingly, these factors include members of the TGF-β and FGF family [18–21]. The transduction of the message probably involves interacting pathways [22]. These factors can act in coordination with other multipotent factors, including members of the Wnt family, involved in transduction and cell signaling [23]. In the cascade of interactions following the inductive event, it has been recently found that goosecoid, a transcription factor containing a homeobox sequence, is central in the triggering of gastrulation [24]. Other morphogenetic agents such as retinoic acid have been shown to be present in vivo at the site of epithelial invagination in vertebrates [25].

Somitogenesis

In vertebrates, segmentation of the mesoderm is a later example of complex EMT, combined with the reverse process of mesenchymal–epithelial transition (MET) leading to the metameric somites and their derivatives. First, mesenchymal cells condense into metamerized epithelial structures. During

condensation of mesenchymal cells, N-cadherin (A-CAM) accumulates at the apex of epithelial cells in contact, while extracellular matrix molecules organize into a basal membrane surrounding the somites. Fibronectin has been shown to participate in epithelialization process [26]. Then sclerotomal cells dissociate from the somite through an EMT process and migrate as mesenchymal cells towards the notochord. This EMT step of sclerotomal cells is accompanied by a down-regulation of N-cadherin, whereas cells differentiating into dermamyotome, maintain the expression of N-cadherin.

Sclerotomal cells are also characterized by their abilities to migrate in vitro, on various collagen and fibronectin substrates [27]. Once again, EMT represents an in vivo step giving rise to a migratory population.

Prevalvular mesenchyme induction during heart morphogenesis

The cardiac valves are derived from specific areas of endothelium [28]. The myocardium from these regions induces an EMT on the endothelium to form the myocardial cushion tissue. Components, distinct from fibronectin, are secreted by the myocardium and accumulate in the cardiac jelly. Antibodies against insoluble basement membrane component inhibited the transition, whereas antibodies against fibronectin failed to do so [29]. In addition, antisense TGF-β3 RNA blocks heart EMT [30,31], suggesting TGF-β could be necessary during this EMT. Interestingly, pertussis toxin has also an inhibitory effect on this EMT [32], suggesting G-protein involvement.

Formation of the palate

In mammals, the secondary palate derives from the fusion of two epithelial sheets, leading to the disappearance of the medial edge cells forming the seam. Local apoptosis [33,34] or an EMT involving the medial edge cells [35] has been suggested to participate in this event. Recently, vital dye staining shows clearly migrating medial edge cells leaving the midline epithelial seam and migrating towards oral and nasal epithelia, into which they become incorporated [36,37]. This interesting case of temporary EMT concurred with extracellular remodeling, intracellular reorganization, and keratin intermediate filaments down-regulation [36].

Metanephros formation

During kidney organogenesis, metanephric tubules differentiate from a condensing mesenchyme located around the ureter. This MET is induced by the branching ureteral bud [38]. Several adhesion molecules have been described during condensation, including E-cadherin (uvomorulin), but antibodies against this molecule do not interfere with tubulogenesis [39–42]. In contrast, antibodies to laminin A chain, which is secreted during the condensation phase, inhibit the formation of polarized structure [40]. Epithelial-

mesenchymal interactions are also clearly involved, since antibodies against a mesenchymal membrane glycolipid, the disialoganglioside GD3, inhibit the transition [42].

Regression of Müllerian tract

In vertebrates, during the development of the reproductive duct in male, the Müllerian duct disappears progressively. This regression starts with the dissolution of the Müllerian duct basement membrane. Morphological studies suggest that this regression results partly from an EMT, during which epithelial Müllerian duct cells undergo a transition to mesenchymal cells being incorporated into the local mesenchyme [43]. Members of the TGF-β family have been implicated in this process [44].

Neural crest cells

The neural crest is a transient structure that appears at the apex of the neural epithelium. Crest cells undergo a typical EMT during their individualization from the neural primordium. Subsequently, they migrate towards different sites where they differentiate to give rise to various structures, including most of the peripheral nervous system, but also melanocytes and craniofacial tissues. Cell adhesion systems show an interesting sequential expression pattern. For example, N-cadherin is down-regulated in the newly formed neural crest cells (figure 4) [45]. It is re-expressed at the site where the cells reaggregate to differentiate into ganglia of the peripheral nervous system. The role of different extracellular matrix components in the adhesive and migratory behavior of crest cells have been studied in detail. For example, different adhesive domains of the fibronectin molecule and its cognate integrins are crucial for neural crest cell migration [46].

Epithelial-mesenchymal transition in cancer

Both destabilization of cell cytoskeleton and cell–cell adhesion systems are common features of EMT. During carcinoma progression, it is hypothesized that cells undergo transient EMT phases, accompanied by the loss of the differentiated epithelial phenotype, that may not be as pronounced phenotypically as in the case of NBT-II cells in vitro, and may be reversible. In the following section, we will examine the case of carcinosarcomas that help to understand the role of EMT.

Carcinosarcomas

In humans, these infrequent tumors can appear in different organs, including digestive tract [47–49], lung [50–55], bladder [56–60], uterus [61,62],

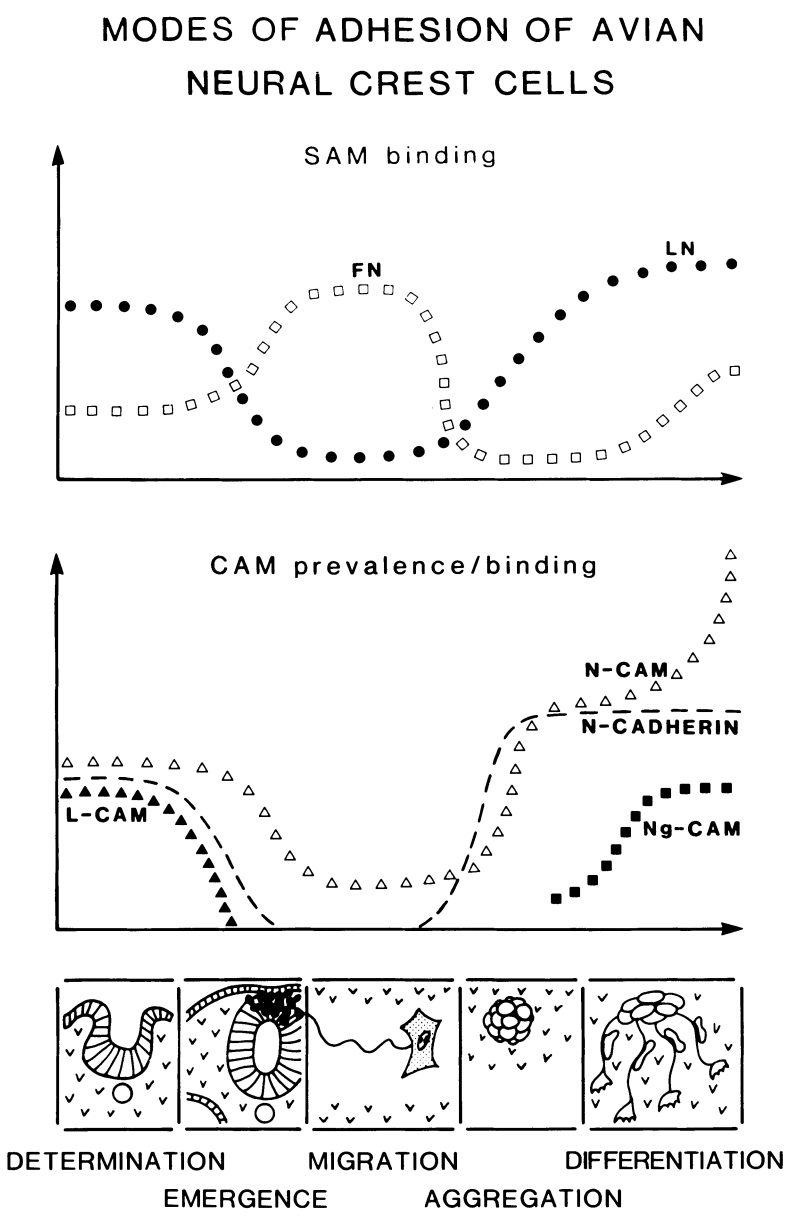

Figure 4. Modulation of adhesion systems during the ontogeny of neural crest. Crest cells progressively lose their calcium-dependent and calcium-independent intercellular adhesion (L-CAM, N-Cadherin, and N-CAM) prior to, during, and after dissociation from the neural epithelium. During migration, they use their cell substrate adhesion system mediated by integrins. In vitro, fibronectin is the most effective substrate for migration, possibly involving several integrins. At the site of arrest, crest cells aggregate to form the ganglia rudiment expressing de novo N-CAM, and N-cadherin, as well as other CAMs during their differentiation into neurons and glia.

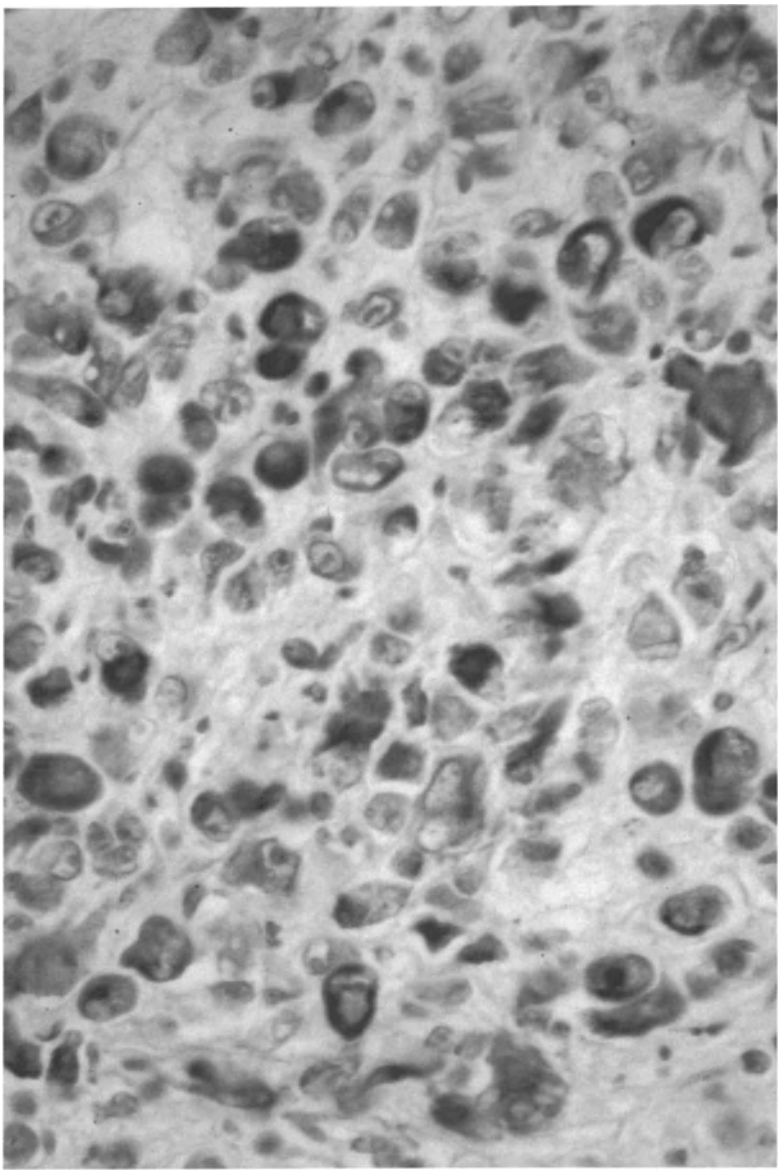

Figure 5. Recurrence of a breast carcinoma with a sarcoma phenotype. Immunoperoxydase staining for vimentin, kindly provided by Dr. Xavier Sastre from Institut Curie (Paris).

prostate [63–65], or breast [66–71]. They consist of a mixture of epithelial as well as mesenchymal cancer cells. It is not clear whether these two cell types derive from a common precursor or result from the independent transformation of epithelial and mesenchymal elements. The first hypothesis is supported by studies on clones of rat mammary cell lines: when injected into the animals, myoepithelial cell clones gave rise to sarcomatous and carcinomatous tumors [72]. Different cases have been documented, presenting evidences for both possibilities [72–74].

Chemically induced and spontaneous tumors in rat have shown more histological heterogeneity inside the tumor itself as compared to human breast tumors. However, the proportion of carcinosarcomas, as in humans, is very low — 0.6% in chemically induced tumors [73].

Breast carcinosarcomas were found to include mainly epithelial cells, myoepithelial cells, and polymorphous mesenchymal cells, the proportion of which is difficult to establish using standard epithelial or mesenchymal markers [67]. Some cells appear to be in an intermediate state between epithelial and mesenchymal. They may show immunoreactivity for keratin and vimentin filaments (figure 5). Interestingly, some authors estimate that most breast sarcomas actually include few epithelial cells that could have given rise to the mesenchymal component of the tumor by a process of EMT. This would represent a case of persistent EMT. Such progression was actually described in vivo during the sarcomatous transformation of mammary tumors in mice [75,76].

Spatio temporal succession between EMT and growth during tumor progression

In any case, it is clear that carcinoma epithelial cells have to undergo at least transiently some rearrangement of their cell–cell and cell-matrix adhesion, resembling EMT in order to become invasive and motile. Nevertheless, compared to carcinosarcoma, the epithelial phenotype of carcinomas seems to be stable. It appears, therefore, that cell phenotype is maintained by a powerful regulatory system that can only be overcome in particular situations.

Succession and combination of EMT and growth could represent an efficient strategy for metastases of tumor cells. In figure 6, we propose a schematic model of tumor growth and dissemination that includes succession of EMT/growth phases. In this model, EMT initiates local invasion and metastases. Migration may bring metastatic cells into a local environment where they will be subject to new stimulations, potentially triggering growth or further dissemination.

In this respect, the scatter factor (SF) that induces EMT and migration in several normal and tumor cells was found to be identical to the hepatocyte growth factor (HGF) that triggers proliferation in regenerating liver [77–79]. Interestingly, the factor is primarily synthesized by mesenchymal cells and is active on epithelial cells expressing the c-met receptor. A functional domain

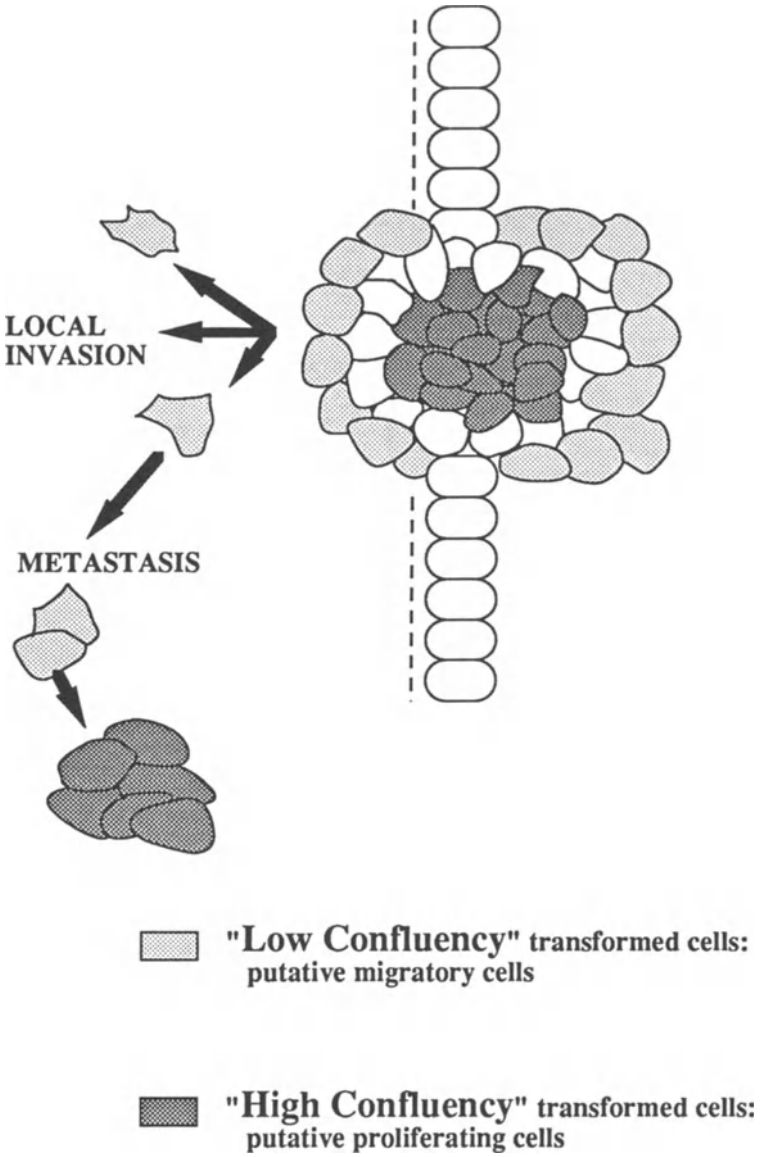

Figure 6. To move or to grow: a group decision for carcinoma cells. In this simplified model, we speculate on the role of cell density in the tumor. In the case of NBT-II, two cell conditions could be found in the tumor that could be reflected by their location. Cells in some tumor area, presumably inside the tumor, would be similar to in vitro high-confluency cells, and would be activated to grow by growth factors such as FGFs. Cells in other areas, presumably at the tumor periphery, would be similar to in vitro low confluency cells, and would be activated to undergo an epithelial-mesenchymal transition, initiating local invasion and metastasis.

in the factor could induce EMT by itself after binding to c-met, without triggering the mitogenic effect [80]. However, SF/HGF mRNA expression in human fibroblasts is down-regulated in the presence of co-cultured keratinocytes [81]. (See chapter 10 in this volume.)

Mechanisms by which cells discriminate between EMT and cell growth

Modulation of growth factors and receptors

So far, few pieces of evidence exist for the prevalence of growth factors or receptors as a mechanism for discriminating between growth and EMT. In explant cultures from newborn rat lenses, bFGF is able to induce proliferation, cell migration, or differentiation depending on its concentration [82].

Expression of receptors by a cell population may actually depend on the local microenvironment. In mammary epithelial cells, surface expression of erbB-2 is under the control of EGF, but also depends on the cell confluency state [78], expressing a 'density factor' similar to what we reported with NBT-II cells. This was observed with different cell types and growth factors [83–85].

Activation may be provided through a close cell population including mesenchymal elements. In vivo, several authors have shown how mixing mesenchymal with epithelial cells could accelerate the growth and shorten the latency period of growing epithelial tumors, potentially leading to development of metastases [86,87]. Different mechanisms have been suggested, mainly emphasizing a role for the growth factors secreted by mesenchymal cells, or for extracellular matrix components, that may actually have similar activity. A single cell type may, after undergoing EMT, allow similar self-activation to occur. This could explain how growth-factor-transfected NBT-II cells exhibit enhanced in vitro invasiveness and in vivo accelerated growth.

Determination of transduction pathway specificity

The problem is to determine at which point the message gets on the mitogenesis pathway rather than the EMT pathway, and which factors are committed to one specific pathway, rather than to the other. Most growth factor receptors include an intracellular kinase domain activated by ligand binding. For example, in FGF receptor, ligand binding leads to tyrosine phosphorylation. Phosphorylated tyrosines represent putative binding sites for specific SH2 domain-factors [88–90]. It was shown that phospholipase Cγ binds a phosphorylated tyrosine, residue 766 in the tyrosine kinase domain. A point mutation at this site inhibits phospholipase Cγ binding, and further induced phosphorylation. However, it has no effect overall on the FGF-induced mitogenesis, suggesting distinct but perhaps complementary activation pathways [91,92]. Through SH2 domain factors and probably

other receptor-binding factors, the activation eventually triggers other components of the transduction machinery. It was found in several cases, as with the NBT-II cells, that cAMP-mediated events were oriented towards proliferation, rather than towards EMT-like events or other cell differentiation steps. For example, bFGF was found to stimulate the proliferation of melanoblasts, without activating their differentiation pattern, when cultured in presence of dibutyryl cyclic AMP [93]. Conversely, EGF induced rat liver epithelial cell migration, but this activity was down-modulated by cyclic AMP and cholera toxin [94,95]. In the same situation, EGF also induced fibronectin expression. Similarly, when deprived of c-AMP, foreskin microvessel endothelial cells undergo in vitro an EMT, whereas they revert to an epithelial phenotype in the presence of c-AMP [96].

EMT inducers involve transcription apparatus

Several transcription factors have been described as specifically involved in EMT situations. Mammary epithelial cells were found to lose their epithelial differentiated phenotype when an inducible c-FosER fusion protein was activated [97]. Although the localization and activation pathways of c-fos appear as very general and ubiquitous, the function expressed in this system appear specifically linked to an epithelial-mesenchymal transition. Interestingly, c-fos is a general transcription factor involved in dimers including c-jun and other factors. It is activated rapidly and transiently by most growth factors. But it is phosphorylated by protein kinases that may be specifically triggered by some growth factors [98].

Other transcription factors show more restricted expression. Among the Ets family, represented in drosophila as well as in humans, Ets-1 is expressed in several types of cells undergoing morphogenetic changes. It is strongly expressed during embryogenesis in cranial neural crest cells and in early endothelium, at stages where hemopoiesis take place through EMT-like events [99]. In humans, Ewing's sarcoma is a tumor with undefined cell phenotype. Specific chromosome translocation in Ewing's sarcoma generates a gene combining Ets-1 DNA-binding domain with a part of EWS, a protein sharing some homology with RNA polymerase II proteins [100]. This fusion gene combines a specific DNA-binding domain probably involved in EMT-like events with an RNA polymerase-related activation domain. Such an arrangement could have a strong impact on the tumor phenotype and evolution.

Several nuclear factors, including LFB1, LFB3, Pax-2, and Wt-1, have been shown to be expressed in the kidney during tubule formation at MET sites. LFB1 and LFB3 are two homologous homeoproteins forming heterodimers [101,102]. LFB3 is expressed by condensed mesenchymal cells. It is strongly increased by retinoic acid treatment [101]. LFB1 transcripts appear afterwards, as soon as the epithelial structures differentiate [102]. Pax-2 codes for a transcription factor expressed specifically during kidney morphogenesis and in the developing neural tube [103,104]. More specifically,

Pax-2 is found in the nuclei of condensing mesenchymal cells and early transformed epithelial tubule cells, then abruptly disappears as tubules differentiate [103]. Interestingly, it is also highly expressed in the epithelial component of Wilms' tumors, suggesting an active role during EMT processes [103]. Wt-1 is the Wilm's tumor locus gene, and encodes a DNA-binding zinc finger protein. It is also expressed specifically by condensing mesenchymal cells and subsequent glomerular epithelial cells from the developing kidney [105]. Wt-1 binds specifically to EGR-1 binding site. EGR-1 is an Early Growth Response DNA-binding protein directly involved in cell proliferation [106]. By competing with EGR-1 for site-specific binding, Wt-1 could provide the cells with an alternative to growth activation, according to our observations on NBT-II cells. Such mechanisms may also result in the activation of genes directly controlling the EMT process, as opposed to a cell proliferation activation program. It would be of considerable interest to identify such genes if they actually provide the decisive signal triggering an EMT versus a proliferation program.

To undergo EMT or to grow appear to be two alternate pathways followed by a cell as a result of interactions with local environment, including growth factors availability, extracellular matrix structure and cell density. Further work is required to characterize this alternate activation model, potentially involved during tumor growth and dissemination.

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13. Cell–cell adhesion in invasion and metastasis of carcinomas

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Adhesive interactions of metastasizing cells

Metastasis of tumor cells involves a series of consecutive attachment and deattachment events that are based on a multitude of specific cell-to-cell and cell-to-substrate interactions. The process is initiated by disaggregation of invasive cells from the primary carcinoma — a step that requires a breakdown of intercellular adhesion (see below for a detailed discussion). Invasion then depends on novel adhesive interactions with extracellular matrix components of the basement membrane and the mesenchymal tissue. The hallmark of invasion (in particular in carcinomas) is the penetration of tumor cells through the surrounding basement membrane — a process that indicates the transition from a benign carcinoma in situ to a malignant invasive tumor. This step requires adhesion to and digestion of extracellular matrix molecules, like laminin and collagen type IV. It has been shown, for instance, that invasive tumor cells express elevated levels of type IV collagenase and of both laminin and collagen IV receptors [1,2]. A similar combination of changes in proteolytic and adhesive activities also aids the invasive cells in their movement through the interstitial stroma and through basement membranes of blood vessels. For instance, metastasis formation in vivo could be suppressed by injection of the cell attachment peptide YIGSR that is located in the laminin B1 chain [3]. Various experiments have shown that proteins containing the RGD amino acid sequence for recognition by integrin receptors serve as substrates for migrating malignant cells [4–6]. An important role of integrin receptors in metastasis formation was revealed by expression of the α_2 integrin cDNA in low metastatic rhabdomyosarcoma cells. The transfected cells expressed functional $\alpha_2\beta_1$ adhesion receptors, they exhibited enhanced adhesion to collagen and laminin in vitro, and they produced more metastatic tumor colonies in nude mice in comparison to the parental cells [7]. Interestingly, transformation of rodent cells by oncogenic viruses resulted in a decrease of integrin receptors [8], and overexpression of the $\alpha5\beta1$ integrin in transformed chinese hamster ovary cells reduced tumorigenicity and motility of the cells [9]. These results reveal a dual role of cell matrix adhesion in tumorigenesis: on the one hand, adhesion to

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extracellular matrix is required for cell locomotion, but on the other hand, it can impose constraints on motility and growth and thus must be minimized in certain cases [10].

The translocation of metastatic cells to distant organs takes place in blood or lymph vessels. Here, enhanced homotypic adhesion among the tumor cells and heterotypic interactions with certain blood cells are mediated by lectin-like cell surface proteins and may protect individual cells from physical damage [11]. The final escape of metastatic cells from the vascular system and the entry into the stroma of the target organ requires specific adhesion to endothelial cells. *Homing receptors* on the metastasizing cells and *addressins* on the endothelium (which are similar to the molecules identified in normal lymphocyte extravasation processes) might here play an important role. A candidate addressin molecule, Lu-ECAM-1, has been found in mouse lung endothelium where it probably mediates the specific colonization by B16 melanoma cells [12]. A recently identified splice variant of the homing receptor CD44 (the Hermes antigen) was shown to confer metastatic capability to nonmalignant rat colon tumor cells [13]. Overexpression of the CD44 variant in nonmetastasizing cells by cDNA transfection resulted in full metastatic potential [13]. Since there are several indications that the 'normal' CD44 protein is involved in cell adhesion processes [14–16], the CD44 variant protein might participate in a multitude of adhesive interactions required for metastasis formation. A consequence of these might be the arrest of tumor cells in lymph nodes, since CD44 splice variants have been observed in stimulated lymphocytes, and since antibodies to the variant proteins block antigenic stimulation [17].

Loss of cell–cell adhesion as a prerequisite for invasion of carcinoma cells

Normal epithelial cells are characterized by the tight association of individual cells through various junctional organelles. As a functional consequence, epithelial cells are often less mobile than cells of mesenchymal origin, and their invasive capacity is limited. Thus, the development of malignant carcinomas that arise from normal epithelia is likely to involve alterations of the intercellular adhesion system. Based on pathological observations, carcinomas can be subdivided by morphological and functional criteria: 1) well-differentiated carcinomas retain epithelial tissue structure, show well-developed intercellular junctions, and are generally weakly invasive, and 2) poorly differentiated carcinomas are characterized by a more amorphous tissue structure, have fewer cell-to-cell junctions, and are more highly invasive [18]. There are also heterogeneous carcinomas in which both differentiated and dedifferentiated areas are found within one tumor. Furthermore, it has been shown that the state of differentiation and the concommittant degree of invasiveness of carcinomas can determine cancer prognosis.

There are several reports showing that cell–cell adhesion molecules might play important roles in the development of specific types of tumors. For instance, the putative tumor-suppressor gene DCC (deleted in colon carcinomas) encodes a neural cell adhesion molecule (N-CAM)-like protein, and its loss in colon tumors not only might influence growth but also might allow cells to invade [19]. Reduced cell-to-cell adhesion might also occur in Wilms' tumors that reexpress the highly sialyated and less adhesive embryonic form of N-CAM [20]. Interestingly, expression of the carcinoembryonic antigen (CEA), which is another N-CAM-like cell adhesion molecule, is increased in colon carcinomas. It has been suggested that this might either result in disturbance of normal intercellular adhesion or provide advantages in further steps of metastasis [21]. In the past few years, it became clear that members of the cadherin family of cell adhesion molecules (in particular E-cadherin) play a prominent role in establishing adhesion in epithelial cells and may thereby counteract invasiveness. E-cadherin is a 120-kDa cell surface glycoprotein that is specifically expressed in epithelial cells [22]. E-cadherin function can be blocked by antibodies against the extracellular domain of the protein; this treatment leads to dissociation of epithelial monolayers in vitro and induces a shift from an epithelial to a fibroblastoid morphology of the cells [23,24]. Furthermore, forced expression of E-cadherin cDNA generates epithelium-like monolayers that exhibit polarized distribution of certain marker molecules [25,26]. These results indicate that E-cadherin is a powerful regulator of epithelial differentiation. Our group has found that nontransformed Madin–Darby canine kidney (MDCK) epithelial cells invaded collagen gels and embryonic heart tissue after disturbance of intercellular adhesion with anti-E-cadherin antibodies. Furthermore, MDCK cells transformed with Harvey and Moloney sarcoma viruses were found to be constitutively invasive, and they do not express E-cadherin. We concluded from these results that the loss of adhesive function of E-cadherin is a critical step in the promotion of epithelial cells to a dedifferentiated and invasive, i.e., more malignant, phenotype [27]. We also found that human carcinoma cell lines derived from differentiated tumors had an epitheloid morphology and expressed E-cadherin, whereas cells from dedifferentiated tumors exhibited a fibroblast-like morphology and were E-cadherin deficient [28]. In the collagen invasion assay, E-cadherin-expressing cells were generally noninvasive, whereas E-cadherin-deficient cells were invasive, which is in line with the results obtained with normal and transformed MDCK cells. Transfection of mouse cDNA into the dedifferentiated breast carcinoma cell line MDA-MB-435S reduced the invasive capacity of the cells, and invasiveness could be restored after treatment of the cells with dissociating anti-E-cadherin antibodies [28]. In similar experiments of other groups, invasiveness of transformed MDCK cells and mouse mammary gland cells into chick heart fragments and of L-cells (fibroblasts) into collagen gels was abolished following transfection of E-cadherin cDNA [29,30]. In the former case, the transfected cells produced differentiated tumors upon in-

jection into nude mice. Furthermore, down-regulation of the E-cadherin protein via antisense RNA techniques induced invasiveness in otherwise noninvasive cells [29]. In a study of various human breast cancer cell lines, absence of E-cadherin correlated with fibroblastoid morphology, vimentin expression, and high invasiveness of the cells [31]. Interestingly, down-regulation of E-cadherin expression was found in experimental tumors derived from homogeneously E-cadherin-positive transformed MDCK cells, indicating host influences on E-cadherin synthesis or stability [32]. A study on E-cadherin expression in mouse epidermal carcinoma cell lines of varying malignancy revealed a potential role for E-cadherin in the control of tumorigenicity. After transfection of E-cadherin cDNA in an E-cadherin-deficient highly malignant skin carcinoma cell line, the latency period and the growth of tumors produced by the transfected cells was reduced [33]. Interestingly, the *fat* tumor-suppressor gene in Drosophila has been identified as a member of the cadherin family [34].

To assess the role of E-cadherin in invasion of carcinomas in vivo, we examined E-cadherin expression in human squamous cell carcinomas of the head and neck. We found that E-cadherin expression was inversely correlated both with the loss of differentiation of the tumor and with lymph node metastasis (table 1; see [35]). Immunofluorescence staining for E-cadherin in well-differentiated tumors was nearly as strong as in the normal squamous epithelium, whereas the staining was reduced or heterogeneous in moderately well-differentiated tumors and completely absent in dedifferentiated cases. Most significantly, 7 of 8 tumor-infiltrated lymph nodes were E-cadherin negative, even in cases where the primary tumor still expressed the molecule (table 1). A clear correlation of E-cadherin expression to the differentiation state was also found in infiltrating lobular breast carcinomas, where all cases examined had lost expression of E-cadherin. In contrast, in ductal breast carcinomas several invasive cases retained E-cadherin expression, indicating different mechanisms of invasiveness in these two types of breast carcinomas [35A]. Studies by several other labs demonstrate that loss of E-cadherin is a common feature in carcinomas. For instance, a large proportion of human esophagus, stomach, and breast carcinomas and of fibroblastic meningiomas exhibited reduced or heterogeneous E-cadherin expression [36–38]. Thus, E-cadherin expression in many tumors is tightly linked to the invasiveness and the differentiation state of the cells. Clinical studies will now be important to assess the relevance of E-cadherin as a prognostical marker for risk of metastasis in human carcinomas.

Down-regulation of E-cadherin in dedifferentiated tumors might be due either to direct mutations of the structural gene and its regulatory regions or to indirect suppression of E-cadherin gene expression. Until now, no changes in the genomic organization of E-cadherin in tumors have been described. However, a new tumor-suppressor gene in hepatocellular carcinomas has been localized on human chromosome 16q22.1 to q23.2 [39],

Table 1. Properties of the squamous cell carcinomas of head and neck

Case	Age/Sex/Site[a]	TNM[b]	Differentiation Grade[c]	E-cadherin Expression	
				Primary tumor[d]	Infiltrated lymph node[e]
1	66/f/H	T2 NO MO	well	++	
2	69/m/L	T3 NO MO	well	++	
3	45/m/H	T3 NO MO	well	++	
4	43/f/H	T2 N1 MO	well	++	−
5	64/m/L	T3 N1 MO	well	++	
6	57/m/L	T3 NO MO	well	+	
7	67/m/L	T3 NO MO	well	+	
8	52/m/H	T4 NO MO	well	+	
9	57/m/H	T4 NO MO	well	+	
10	67/f/O	T4 NO MO	well	+	
11	64/m/O	T1 N3 MO	well	+/−	−
12	77/m/F	T2 N1 MO	well[f]	+/−	−
13	39/f/O	T4 NO MO	moderately	++	
14	51/f/O	T2 N1 MO	moderately	++/−	
15	50/m/L	Tis	moderately	+	
16	60/f/O	T2 NO MO	moderately	+	
17	57/m/L	T2 NO MO	moderately	+	
18	61/m/O	T3 NO MO	moderately	+/−	
19	55/f/O	T4 NO MO	moderately	+	
20	51/f/L	T2 N1 MO	moderately	+	
21	62/m/H	T2 N1 MO	moderately	+/−	
22	62/m/H	T2 N2b MO	moderately	+	
23	66/f/F	T3 N2c MO	moderately	+	+
24	72/m/O	T2 N2b MO	moderately	+/−	−
25	47/m/F	T4 N2b MO	moderately	+/−	
26	57/m/O	T2 N3 MO	moderately	+/−	−
27	54/f/L	T4 N2c MO	moderately[g]	−	−
28	62/f/O	T2 NO MO	poorly	−	
29	65/m/H	T3 N1 MO	poorly	−	
30	47/m/H	T2 N2b MO	poorly	−	
31	49/f/L	T4 N1 MO	poorly	−	
32	50/m/H	T3 N3 MO	poorly	−	−

[a] Site of tumor: F, floor of mouth; O, oropharynx; H, hypopharynx; L, larynx.

[b] TNM staging as performed by the Department of Otorhinolaryngology, Medical School, University of Essen. T, tumor size; N, lymph node infiltration; NO, no infiltration; N1, one infiltrated lymph node <3 cm; N2b, several infiltrated lymph nodes <6 cm on one side; N2c, same, but on both sides; N3, all infiltrated lymph nodes >6 cm; MO, no distant metastasis; Tis, carcinoma in situ.

[c] Tumor grading as determined by the Department of Pathology, Medical School, University of Essen.

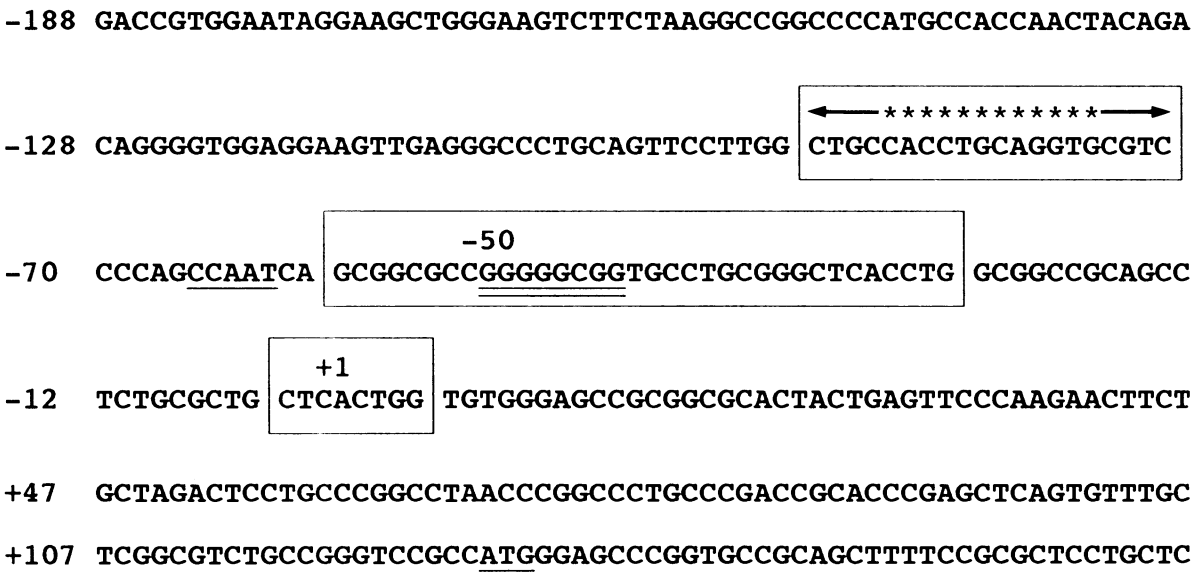
[d] E-cadherin expression as determined by immunofluorescence; ++, homogeneously high expression comparable with the staining of normal stratified squamous epithelium; +, homogeneously weak expression; −, no detectable expression, ++/−, +/−, heterogeneous staining.

[e] Only infiltrated lymph nodes were analyzed.

[f] According to our hematoxylin/eosin staining, we would classify this tumor as moderately differentiated.

[g] The area we have analyzed of this tumor was clearly poorly differentiated.

A


-188 GACCGTGGAATAGGAAGCTGGGAAGTCTTCTAAGGCCGGCCCCATGCCACCAACTACAGA
-128 CAGGGGTGGAGGAAGTTGAGGGCCCTGCAGTTCCTTGG CTGCCACCTGCAGGTGCGTC
-50
-70 CCCAGCCAATCA GCGGCGCCGGGGGGCGGTGCCTGCGGGCTCACCTG GCGGCCGCAGCC
+1
-12 TCTGCGCTG CTCACTGG TGTGGGAGCCGCGGCGCACTACTGAGTTCCCAAGAACTTCT
+47 GCTAGACTCCTGCCCGGCCTAACCCGGCCCTGCCCGACCGCACCCGAGCTCAGTGTTTGC
+107 TCGGCGTCTGCCGGGTCCGCCATGGGAGCCCGGTGCCGCAGCTTTTCCGCGCTCCTGCTC

B

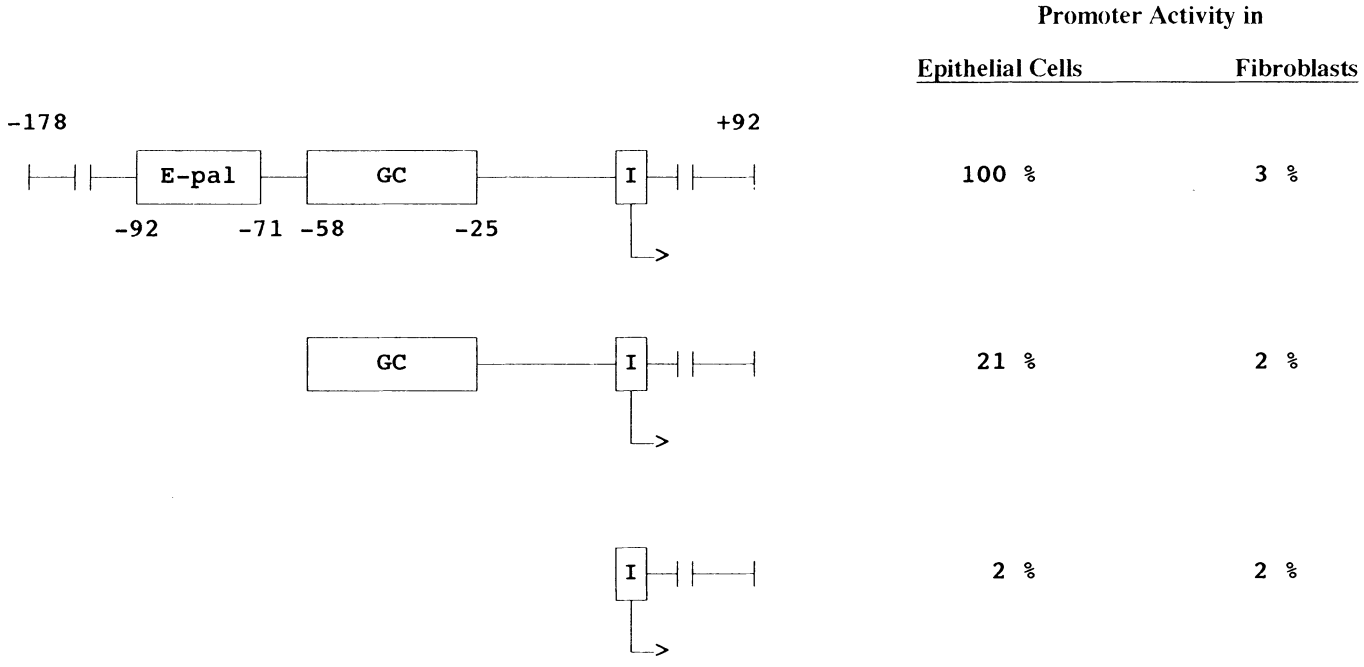


Figure 1. (A) DNA sequence of the E-cadherin promoter including the translational start site at +128, the transcription start site at +1 (the initiator sequence is boxed), the GC-rich area between −58 and −25 (boxed), and the palindromic E-pal sequence at −86 (boxed). Marked are also the CAAT-box and the Sp1-site (modified from [43]). (B) E-cadherin promoter activity in mouse CSG epithelial cells and in NIH 3T3 fibroblasts using the indicated deletion fragment/CAT constructs (from [43]).

the region where the human E-cadherin gene is located [40]. Furthermore, loss of heterozygosity on chromosome 16 was much more frequent in poorly differentiated (88%) than in well-differentiated (18%) carcinomas, suggesting E-cadherin as a candidate for this tumor-suppressor gene. Similar allelic losses have been reported for human prostate and breast cancer [41,42].

We have identified the E-cadherin promoter and found that a −178- to +92-bp upstream fragment of the E-cadherin gene induced specific expression of a CAT reporter gene in epithelial cells, whereas in nonepithelial cells the fragment was inactive (figures 1A and 1B; see [43]). A further deletion fragment (from −58 bp to +92 bp) exhibited fivefold reduced activity that was still epithelium specific (figure 1B). Importantly, both the −178-bp and the −58-bp fragments were highly active in differentiated breast carcinoma cells, but were inactive in dedifferentiated carcinoma cells (figure 2). By DNAse footprinting and gel retardation analysis, we could identify binding of nuclear factors to two regions within the −178-bp E-cadherin promoter

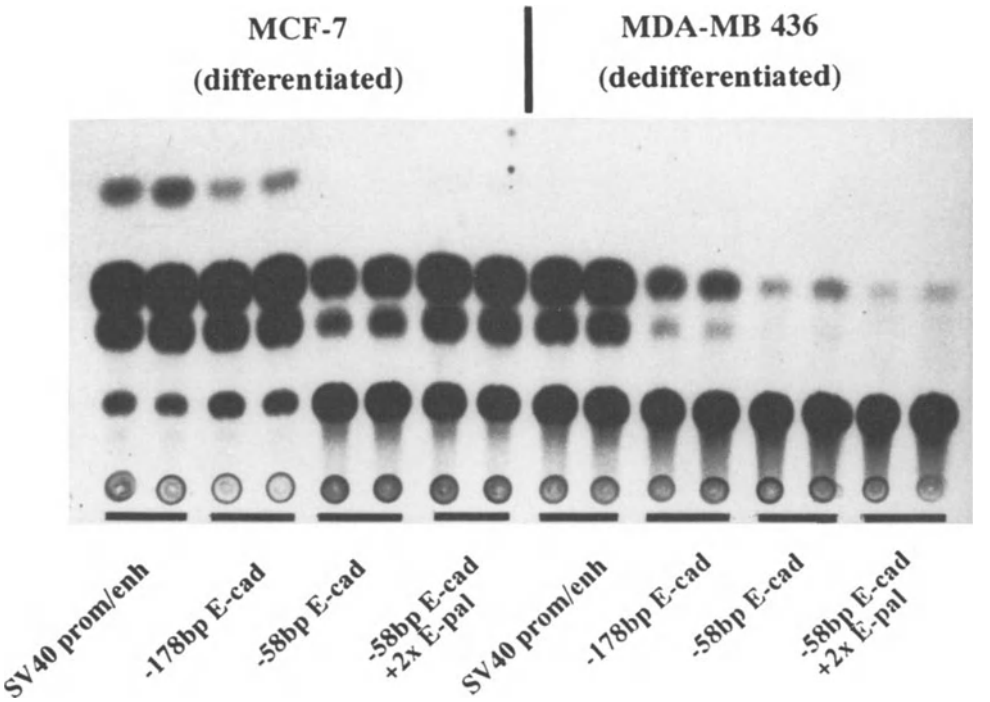


Figure 2. CAT activities in cell extracts of differentiated (MCF-7) and dedifferentiated breast carcinoma cell lines (MDA-MB 436) after transfection of CAT constructs containing the −178-bp E-cadherin promoter fragment, the −58-bp fragment, and the −58-bp fragment with two copies of the E-pal element. Note the stimuation of the −58-bp promoter activity by the E-pal element in differentiated, but not in dedifferentiated breast carcinoma cells.

fragment; first, a GC-rich region harboring an Sp1 site, and second, a palindromic sequence at −86 (named E-pal) that shows similarities to recently identified promoter elements of keratin genes and contains two copies of the CANNTG consensus sequence for binding of helix-loop-helix transcription factors [44,45]. When linked to the −58-bp proximal promoter, the E-pal element activated transcription in differentiated but not in dedifferentiated breast carcinoma cells (figure 2). In contrast, in nonepithelial cells, the E-pal element inhibited transcription from a heterologous promoter, and mutation of E-pal resulted in increased activity of the E-cadherin promoter, indicating that this element mediates both stimulatory and inhibitory signals, depending on the cell type. Accordingly, we did not observe any significant difference in nuclear factor binding to the here-identified E-cadherin promoter elements when extracts of epithelial and nonepithelial cells or of differentiated and dedifferentiated carcinoma cells were compared. We assume either that both stimulating and inhibitory factors might bind to the regulatory elements, or that additional factors might generate specific promoter activity by protein–protein interactions. Alternatively, transcription factors might be differentially modified in various cell types.

It should be noted that there are certain examples of poorly differentiated, nonadhesive carcinomas with unchanged amounts of E-cadherin, indicating that the invasive phenotype in these tumors was not due to reduced expression of the molecule but rather due to interference with its cell adhesive function [37,46,47]. Such a loss of function might be a consequence of mutations of the E-cadherin gene, e.g., in the extracellular adhesive part of the molecule. Alternatively, the interaction of the cytoplasmic part of E-cadherin with the cytoskeleton might be affected. This interaction is crucial for E-cadherin function and depends on the association of E-cadherin with cytoplasmic proteins, the catenins [48–50]. The cDNAs for α and β catenin have recently been cloned and show sequence similarities to the junction-associated proteins vinculin and plakoglobin, respectively [51–53]. Deletion of parts of the cytoplasmic portion of E-cadherin abolishes both the binding of catenins and the cell adhesive function of E-cadherin in transfected fibroblasts [49]. Furthermore, overexpression of a mutant form of N-cadherin that lacks the extracellular domain in Xenopus embryos disturbed cell–cell adhesion and inhibited catenin binding to E-cadherin [54]. We have recently found that the E-cadherin/catenin complex might be subject to negative regulation by the $pp60^{v\text{-}src}$ tyrosine kinase [55]. We showed that Madin–Darby canine kidney (MDCK) epithelial cells transformed with a temperature-sensitive mutant of v-src exhibit an epithelial phenotype at the nonpermissive temperature for $pp60^{v\text{-}src}$ activity but rapidly lose cell-to-cell contacts and acquire a fibroblast-like morphology after culture at the permissive temperature (figure 3). Furthermore, the invasiveness of the cells into collagen gels or chick heart fragments was increased at the permissive temperature (not shown). Concomitantly to these changes, we noticed increased tyrosine phosphorylation of the E-cadherin/catenin complex, while

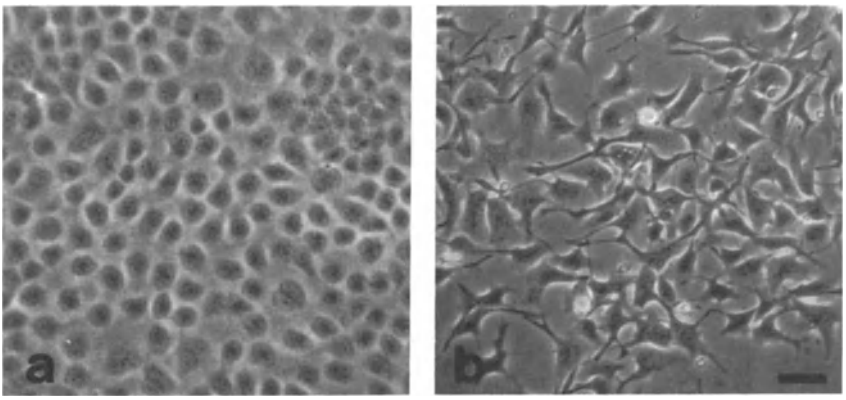


Figure 3. Dissociation of epithelial monolayer of ts-src MDCK cells after the switch from the nonpermissive to the permissive temperature. MDCK cells transformed with a temperature sensitive RSV mutant (ts-src MDCK) were cultured either (A) at 40.5°C or (B) at 35°C for 14 hours. Note the loss of cell contacts and the fibroblast-like morphology of cells cultured at 35°C, the temperature permissive for src activity. Bar, 40 mm.

the amount of these proteins was unchanged (figure 4A). Time course studies showed that increased tyrosine phosphorylation of β-catenin could be detected as early as ten minutes following the shift of cells from the nonpermissive to the permissive temperature (figure 4B). Tyrosine phosphorylation of β-catenin and alterations of cell–cell adhesion has also been observed in P-cadherin-expressing fibroblasts after introduction of the v-src gene [56]. Interestingly, treatment of normal MDCK cells with tyrosine phosphatase inhibitors leads to an increase of phosphotyrosine-containing proteins at the cell contact areas and to the subsequent disruption of the adherens junctions [57]. Based on these results, we might hypothesize that tyrosine phosphorylation of β-catenin by junction-associated tyrosine kinases — such as src or members of the src family — alters its interaction with E-cadherin and thereby interferes with E-cadherin function. Thus, in dedifferentiated tumors that show normal E-cadherin expression, E-cadherin function might be abolished by similar biochemical modifications. Because of the reversibility of such mechanisms, differentiated epithelial structures could reform in the metastasis after appropriate dephosphorylation of the adhesion system. As a consequence of our work, carcinomas should be screened for aberrant catenin expression. If misregulation of E-cadherin function through disturbances of catenins is indeed important for invasiveness of carcinoma cells, modifications, mutations, or loss of expression of catenins should be a frequent event in these tumors. Recently, a human cancer cell line, PC9, has been described that expresses E-cadherin and β-catenin but lacks α-catenin [58]. These cells exhibited reduced cell-to-cell adhesion that was restored after transfection of an α-catenin cDNA [59].

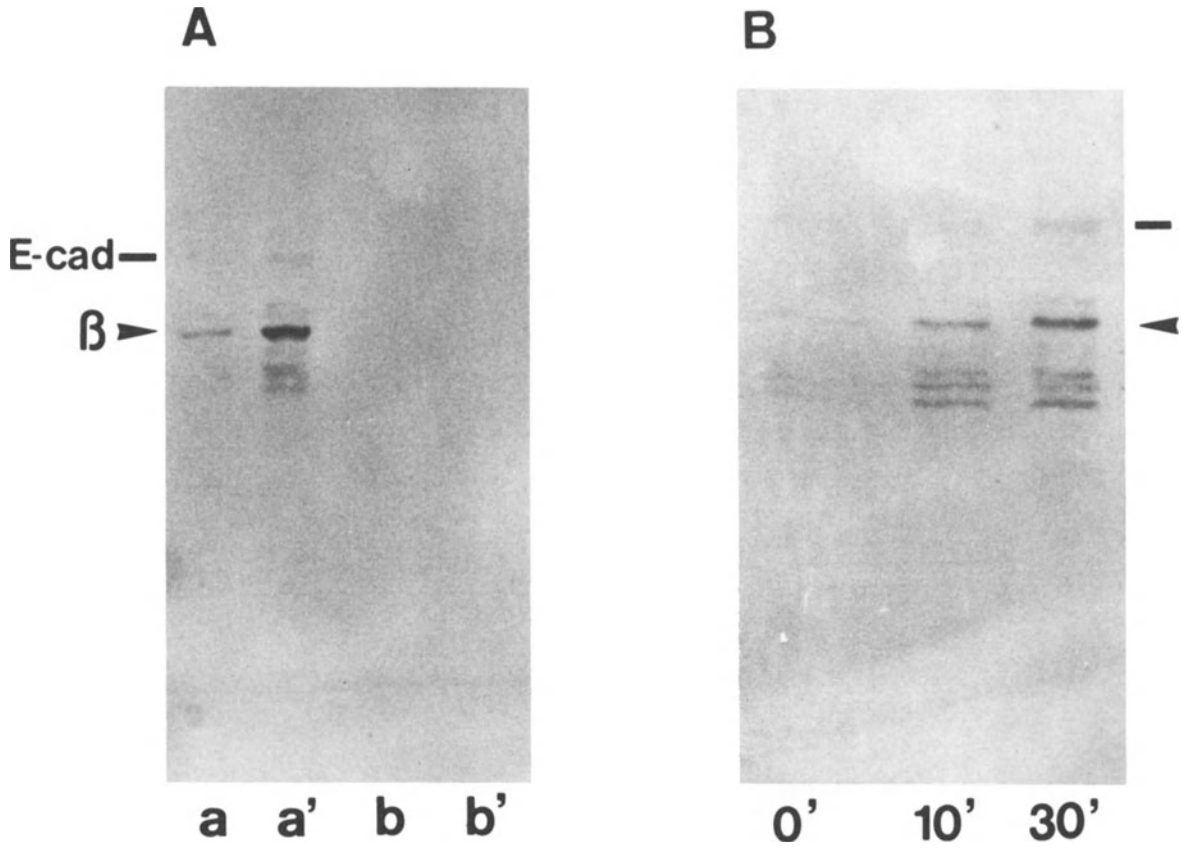


Figure 4. Tyrosine phosphorylation of the E-cadherin-catenin complex in ts-src MDCK revealed by antiphosphotyrosine antibody labeling. (A) Immunoprecipitates from extracts of ts-src MDCK cells cultured either at 40.5°C (lanes a, b) or at 35°C (lanes a′, b′) were subjected to SDS-PAGE, blotted onto nitrocellulose, and probed with antiphosphotyrosine antibodies. Immunoprecipitation reactions were performed using anti-E-cadherin antiserum (lanes a, a′), or preimmuneserum (lanes b, b′). Bands representing E-cadherin and β-catenin were identified by comparison with radioactively labeled immunoprecipitates run in adjacent lanes (not shown). (B) Anti-E-cadherin was immunoprecipitated from ts-src MDCK cells that had been either cultured at 40.5°C (lane 0′) or shifted from 40.5°C to 35°C for 10 and 30 minutes. (lanes 10′ and 30′, respectively). Reaction with antiphosphotyrosine antibodies was performed as in (A). Bands representing E-cadherin and β-catenin are indicated.

There is increasing evidence that the cell adhesion machinery is subject to regulation by hormone-like substances that lead to cell dispersion and enhanced cell motility and therefore might also promote invasion of tumor cells. Certain growth factors, e.g., EGF, PDGF, and TGF-β, exhibit both growth- and motility-stimulating activities. Acidic fibroblast growth factor (aFGF) has profound effects on cell cohesion and morphology of bladder carcinoma cells [60]. Cells that permanently express aFGF due to cDNA transfection show a constitutive fibroblastoid phenotype, exhibit enhanced motility, and express elevated levels of gelatinase [61]. Similar to aFGF, interleukin 6 (IL-6) can dissociate certain carcinoma cell lines. In particular, ductal breast carcinoma cells (which are growth inhibited by IL-6) are affected (see [62] for a review). The most potent and general cell-dissociating cytokine with respect to its promiscuous action on various normal and transformed epithelial cells is 'scatter factor' (SF). SF is produced by mesenchymal cells; it induces cell separation and enhances cell motility [63]. Our group has shown that SF promotes the invasion of carcinoma cells into collagen matrices, making it a likely candidate for an invasion-inducing factor of tumors in vivo [64]. SF is a 92-kDa heparin-binding glycoprotein that is converted into disulfide-linked 62-kDa and 34/32-kDa subunits by proteolytic cleavage [64]. SF is identical to human hepatocyte growth factor, and functional analysis has shown that both factors exhibit identical activities [65]. Similar to other growth factors, SF/HGF binds to and activates a tyrosine kinase receptor — the product of the c-*met* protooncogene [66,67]. Interestingly, SF/HGF does not seem to affect steady-state level, synthesis, degradation, or phosphorylation of E-cadherin [64], suggesting that the SF/HGF-triggered signal cascade bypasses the E-cadherin-mediated cell adhesion system. The action of SF/HGF and of other cell-dissociating cytokines might therefore represent a further molecular mechanism (besides loss of E-cadherin expression and phosphorylation of E-cadherin/β-catenin by junction-associated tyrosine kinases) that leads to morphological dedifferentiation and invasion of carcinomas. Thus, the elucidation of signal cascades and the identification of target genes activated by SF/HGF will be an important subject of future research.

Acknowledgments

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14. Enhancement of tumor growth by basement membrane: Modulation of growth and angiogenesis by laminin-derived synthetic peptides

Maura C. Kibbey, Keizo Yamamura, Soo Han Jun, Derrick S. Grant, and Hynda K. Kleinman

The study of human cancers has been impaired by a lack of good animal models. Human cancers are difficult to grow in culture, and the use of immunologically deficient mice has had limited success in propagating human tumors [1]. If a tumor is able to grow in a mouse, its ability to subsequently survive in culture is enhanced. We and others have found much greater take and growth of both murine and human tumor cells and primary isolates in mice when the cells are premixed with a basement membrane extract (Matrigel) and injected subcutaneously [2–5]. In addition, we have been able to further increase the growth of solid tumors by the inclusion of a synthetic angiogenic peptide derived from the laminin A chain [6]. In contrast, anti-angiogenic peptides reduce the tumor growth even when intraperitoneal injections of the peptide are initiated several days after tumor growth has begun [3]. Although these subcutaneous tumors do not frequently metastasize, the growth of such tumors is an important first step in developing animal models to study the metastatic and phenotypic properties of human cancer.

Basement membrane

The basement membrane is an extracellular matrix largely composed of collagen IV, laminin, entactin, heparan sulfate proteoglycan, and various growth factors [7]. Much that is known about basement membrane composition has been learned by utilizing a murine transplantable tumor, the EHS sarcoma, that secretes a large amount of basement membrane. Tumor cell interactions in vitro and in vivo with basement membrane and in particular with the major component laminin are well documented and demonstrate a promotion of malignant behaviour (table 1) [8,9]. Basement membrane has been found to promote nonmalignant cell differentiation and cessation of cell growth [10], whereas malignant cells become more invasive and proliferate rapidly [11]. The mechanism for this response by the malignant cells is not known but appears, in part, to involve laminin. Laminin promotes cell adhesion, migration, and collagenase IV activity, and the number of laminin

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Table 1. Effect of laminin on tumor cells

Increased collagenase IV activity
Increased adhesion
Increased migration
Laminin-adherent cells in vitro are more malignant in vivo
Intravenous coinjection of laminin promotes lung colonization
Number of laminin receptors correlates positively with malignancy

receptors per cell correlates positively with malignancy (table 1) [8,9,12,13]. As expected, laminin-adherent cells in vitro are more malignant in vivo than either laminin nonadherent cells or fibronectin-adherent cells [9]. Intravenous coinjection of laminin and tumor cells also increases the number of melanoma colonies on the surface of the lungs.

A laminin-derived synthetic peptide containing the amino acids SIKVAV (ser-ile-lys-val-ala-val) mimics some of the activities of laminin in promoting tumor cell adhesion, migration, collagenase IV secretion, and increased metastatic lung colonies [14,15]. Increased colonies in the lungs are observed even when the SIKVAV-containing peptide is injected intraperitoneally several hours after the intravenous injection of the melanoma cells [16]. Furthermore, the SIKVAV-containing peptide increases tumor volume up to threefold over that observed with basement membrane alone when coinjected subcutaneously with tumor cells and basement membrane [6]. Another laminin-derived synthetic peptide containing YIGSR (tyr-ile-gly-ser-arg) also promotes tumor cell adhesion and migration but has no effect on collagenase IV activity and reduces the number of lung colonies and size of subcutaneous tumors [17–19]. This latter peptide acts, in part, to reduce tumor growth due to its anti-angiogenic effects [20,21].

Tumor growth after subcutaneous coinjection with basement membrane

Basement membrane Matrigel, an extract isolated from the EHS tumor [22], enhances the growth of a variety of tumors (table 2). Tumor cells are premixed with Matrigel at 4°C while it is in liquid form and are injected in a final volume of 0.5 ml in a subcutaneous location. Upon contact with the body temperature of the mouse, the Matrigel polymerizes into a solid form as it warms to 37°C. Many human tumor cell lines that do not grow in mice have enhanced growth when coinjected with Matrigel. Fridman et al. injected up to 1,000,000 small cell lung carcinoma cells and were unable to obtain tumors in the absence of Matrigel, but with Matrigel only 25,000 cells were needed to yield large, rapidly growing tumors [2]. Pretlow et al. [4] found that PC3 prostatic tumor cells required 700,000 cells to form a tumor in the absence of Matrigel, but only 20 cells were required for successful tumor growth in the presence of Matrigel. Thus, not only is tumor incidence

Table 2. Subcutaneous growth of human tumor cell lines or primary isolates injected with Matrigel

Cells	Tumor growth	
	No Matrigel	With Matrigel
Lines		
Small cell lung carcinomas	−	+++
Prostatic cell lines (PC3, Tsu-pr-1)	+/−	+++
Epidermoid carcinoma KB	+	+++
Cultured renal cell carcinoma	−	+++
MCF-7 breast carcinoma	−	+++
HT1080 fibrosarcoma	−	+++
Glioblastomas	+/−	+++
A253 submandibular carcinoma	+/−	+++
Primary isolates		
Colon carcinoma	−	+++
Nelson's pituitary	N.D.	+++
Brain hemangioblastoma	N.D.	+++
Craniopharyngioma	N.D.	+++

Note: N.D., denotes not done; −, denotes no tumor growth; +/−, denotes some mice had small tumors; +, denotes small tumors; +++, denotes large tumors.

increased but also many fewer cells are required. This raises the possibility of the successful growth of needle biopsy material in this model. It should also be noted that only the tumor cells grow; normal cells such as endothelial cells, fibroblasts, etc., which may be present in patient biopsy material, are not promoted to grow in the mouse-Matrigel model [16].

All the cell lines tested to date have shown rapid growth when injected subcutaneously with Matrigel in mice [3]. These include several small cell lung carcinoma cell lines, prostate cell lines, epidermoid carcinoma KB, submandibular carcinoma A253, HT1080 fibrosarcoma, and MCF-7 breast carcinoma cells (table 2). Primary cultures of renal cell carcinoma cells also yielded tumors. Histologically, the tumors studied maintained a morphology typical of the parental tumor phenotype.

Primary isolates from patient tumors, in some but not all cases, grew more slowly than the cell lines (data not shown). This could be due to increased cell damage incurred during the isolation process. For example, colon carcinoma isolates yielded observable tumors as early as two weeks and as late as two months. If no tumors were palpable at this late time point, mice were often sacrificed. Mice injected with a patient's craniopharyngioma plus Matrigel rapidly formed tumors but then died unexpectedly while the tumors were still quite small, suggesting that metastases may have occurred. The cells from one patient with a Nelson's pituitary tumor yielded an observable tumor in less than a week following dorsal subcutaneous coinjection with Matrigel. The mouse was sacrificed after 22 days due to its

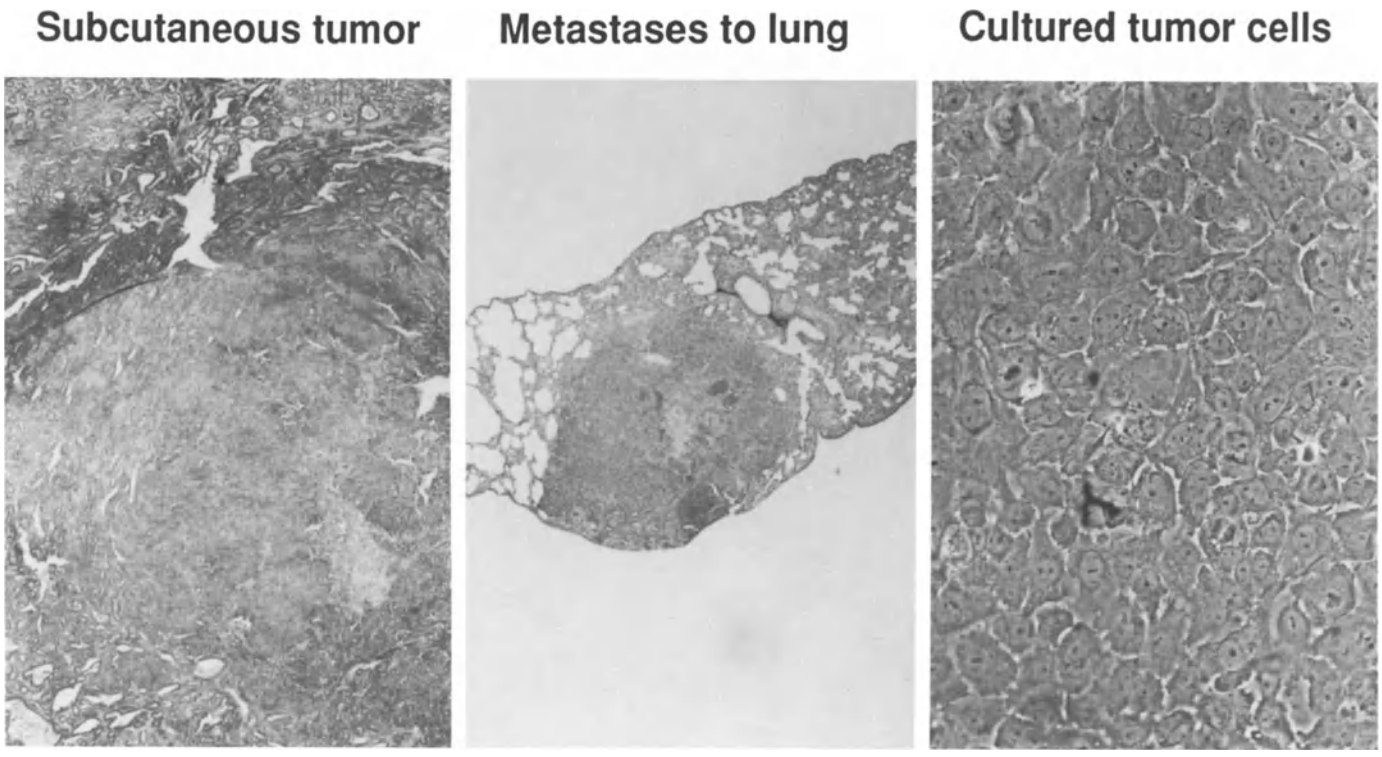

Figure 1. Histologic appearance of a Nelson's pituitary tumor after growth in athymic mice with Matrigel and subsequent cell culture. (A) tumor in the primary subcutaneous location; (B) lung metastasis; (C) cultured cells.

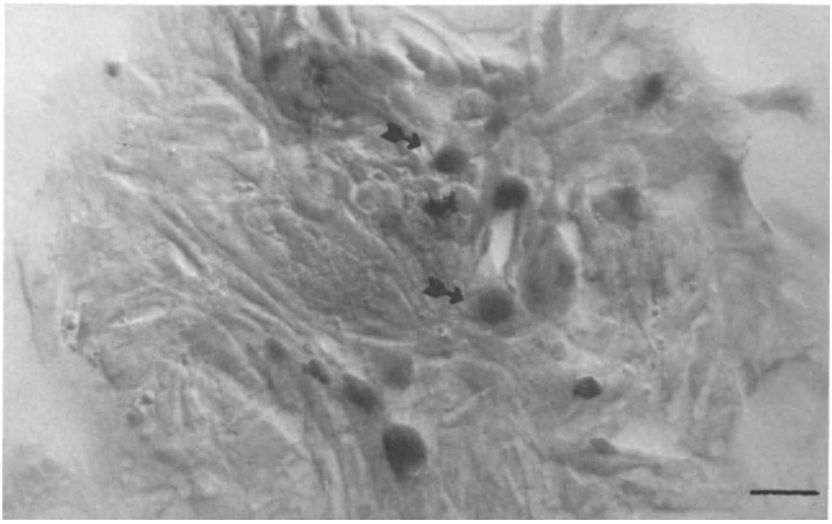

Figure 2. Mucicarmine staining of a colony of colon carcinoma cells. Patient tumor samples were grown in nude mice in the presence of Matrigel, harvested, and maintained in culture. Production of mucin was maintained by the culture cells (examples of mucin-positive cells are indicated by arrows). Cells were photographed on a Zeiss IM microscope using Hoffman modulation contrast. Magnification bar equals 30 μm.

large primary lesion (figure 1A), and due to development of a large, metastatic, ventral lesion over the ipsilateral mammary fat pad. Upon autopsy, metastatic lesions were also found in the lungs (figure 1B). These lesions were found to be ACTH positive, reminiscent of the original patient tumor. Development of this rapidly growing tumor facilitated culturing of the cells for study (figure 1C). Although after several passages these cells died, this method promoted in vitro culture of the patient tumor for clinical and basic research studies. Again, where studied, the primary isolate-derived tumors were histologically comparable to the parental tumor. Cells derived from the mouse tumors also maintained the cell phenotype. For example, a colon carcinoma in culture yielded mucin-producing cells (figure 2). We have propagated several human colon cell lines and, as expected, found some that do not produce mucin [23]. This method for growing tumor cells allows for maintenance of the differentiated phenotype of the tumor cells.

Effect of laminin-derived synthetic peptides SIKVAV and YIGSR on subcutaneous tumor growth

Laminin-derived synthetic peptides have been found to modulate the number of melanoma colonies on the surface of lungs in the Fidler B16F10 melanoma

Table 3. Effect of laminin-derived synthetic peptides on subcutaneous tumor growth

	No Matrigel	Matrigel	Matrigel + SIKVAV	Matrigel + YIGSR
B16F10[a]	+	+++	+++++	N.D.
SCLC[b]	0	+++	N.D.	+

[a] Data from Kibbey et al. [6].
[b] Data from Fridman et al. [2].
Note: +, denotes slow-growing tumors; +++, or +++++, denotes very rapidly growing, large tumors; N.D., denotes an experiment not done; 0, denotes no tumor growth.

model [15,16,19]. The SIKVAV-containing peptide increases the number of colonies, whereas the YIGSR peptide decreases the number of colonies. Similar results are obtained in the subcutaneous model. When the SIKVAV-containing peptide is coinjected with B16F10 melanoma cells and Matrigel subcutaneously in mice, a threefold increase in tumor size is obtained (table 3) [6]. In contrast, when small cell lung carcinoma cells are coinjected with Matrigel and YIGSR, tumor growth is greatly reduced [2]. Furthermore, the YIGSR is active and inhibits tumor growth even when the peptide is not coinjected with the tumor cells, but rather when daily intraperitoneal injections are begun seven days after the initial B16F10 tumor inoculations with Matrigel (figure 3). Here tumor cells were injected with Matrigel and allowed to grow for seven days until a tumor was measurable with calipers. Daily intraperitoneal injections of YIGSR (1 mg/ml) in the trimeric form (amino acid sequence CYIGSRYIGSRYIGSR) resulted in a much slower growth of the tumors. The use of YIGSR after initiation of tumor growth was also found to reduce the melanoma colonies on the surface of the lungs by approximately 80%, as reported by others [20]. These investigators initiated daily intraperitoneal injections four days after intravenous injection of the melanoma cells. These data demonstrate that it is possible to manipulate the growth of tumors with synthetic peptides either at the time of inoculation or after several days when the tumors have begun to grow. This peptide may be very important clinically, since it is active when administered systemically following tumor detection, and thus it may also inhibit undetectable micrometastases.

The synthetic peptides appear to be producing their modulatory effects on tumor growth by regulating the formation of blood vessels in the tumors. The SIKVAV peptide increases the size of tumors and histologic examination revealed a greater number of blood vessels per area than that observed with Matrigel alone or in the absence of Matrigel [6]. The SIKVAV peptide has also been found to increase angiogenesis in the chick chorioallantoic membrane assay, the chick yolk sac assay, the Fajardo disc assay in vivo mouse model, the subcutaneous Matrigel model, and in vitro with human umbilical vein endothelial cells on Matrigal [24,6]. In contrast, the tumors grown in the presence of YIGSR appear less vascularized (Fridman et al., un-

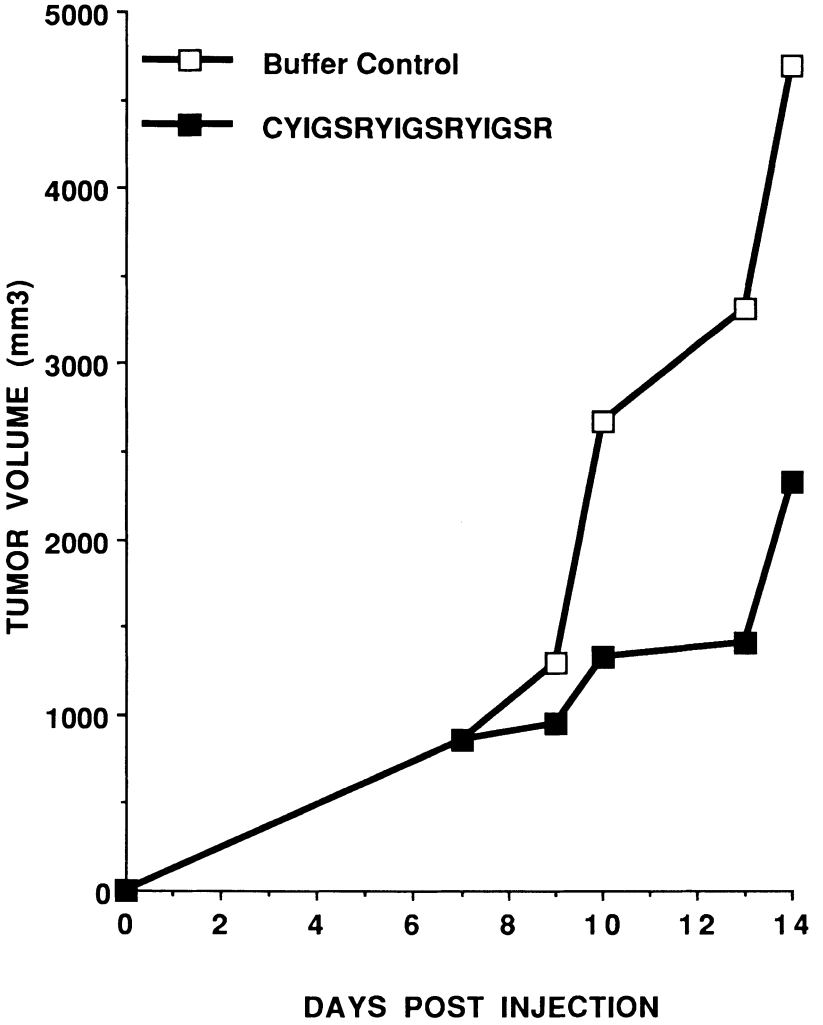

Figure 3. Effect of intraperitoneally injected CYIGSRYIGSRYIGSR peptide ('YIGSR trimer') on subcutaneous B16F10 melanoma cell growth. B16F10 cells (100,000/mouse) were mixed with Matrigel (3.5 mg/mouse) and injected into ten C57BL6N mice in a final volume of 0.5 ml/mouse. After one week, five mice began to receive daily intraperitoneal injections of endotoxin-filtered YIGSR trimer (1 mg/mouse/day) for ten days. Control mice received daily phosphate-buffered saline injections. Tumors were measured (l × w × h) with calipers.

published). YIGSR has also been found to decrease angiogenesis in the rabbit PGE_2 eye model (E. Adamson, unpublished), the chick chorioallantoic membrane assay, and in vitro with human umbilical vein endothelial cells on Matrigel [20,24,25]. These data confirm the role of angiogenesis in regulating tumor expansion [26] and demonstrate that small diffusable

peptides can influence vessel growth in vivo and modulate tumor growth. The angiogenic peptide may be useful in further boosting tumor growth and in obtaining metastatic lesions with the newly developed subcutaneous Matrigel models. The anti-angiogenic peptide has potential use as a therapeutic for human cancers.

The development of the new method of growing human tumors described here may provide an ideal model system for studying the best therapeutic forms of the peptide and methods of administration. The animal model systems described also have use in testing the efficacy of other therapeutics and perhaps the sensitivity of certain individual tumors to conventional and unconventional treatments.

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15. Fetal-like fibroblasts: Their production of migration-stimulating factor and role in tumor progression

Seth L. Schor, Anne Marie Grey, Ian Ellis, Ana M. Schor, Anthony Howell, Philip Sloan, and Ruth Murphy

Cancer pathogenesis is a multistep process involving the occurrence of an initiating genetic lesion and a series of subsequent events collectively referred to as *progression* [1,2]. In spite of the general acceptance of this model, little information is currently available regarding either the frequency at which initiating genetic lesions occur or the proportion of initiated cells that proceed on to develop into a clinically recognizable malignancy. Our assessment of the literature suggests that initiating events may be a relatively common occurrence and that only a small proportion of these cells actually go on to produce clinical tumors. Seen in this context, the various events that contribute to tumor progression play a critical role in determining the kinetics of disease development and may thereby furnish a potential target for intervention in individuals believed to be at elevated risk.

Recent studies have indicated that the gradual acquisition during tumor progression of more 'aggressive' phenotypic characteristics, such as diminished growth control [3], local invasion [4], and metastasis [5], also involve the accumulation of genetic lesions within the emerging population of (pre-) neoplastic cells. The primary role of genetic lesions in the process of cancer development is further suggested by the occurrence of well-defined hereditary syndromes characterized by increased susceptibility to specific types of cancer (e.g., carcinoma of the breast) [6]. The identification and mapping of the relevant genes is currently being pursued with great vigor [7].

Although the acquisition of genetic damage clearly plays a fundamental role in cancer pathogenesis, other mechanisms operative at higher (tissue) levels of organization also contribute to the course of disease progression. These higher-level mechanisms involve a complex network of reciprocal cell–cell interactions that modulate various aspects of tumor and stromal cell behavior. For example, tumor-induced angiogenesis is required for tumor growth and dissemination and is mediated by the interaction of tumor cells with surrounding stromal endothelial cells. Similarly, interactions between tumor cells and other stromal constituents, such as fibroblasts and matrix macromolecules, affect such potentially relevant phenomena as the local production of matrix-degrading enzymes [8] and tumor cell motility [9].

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Perhaps the most dramatic manifestation of such tissue-level mechanisms relates to the control of gene expression itself. In this regard, several studies have indicated that the appropriate host environment may actually suppress the expression of oncogenes ordinarily sufficient to result in tumor development [10,11].

Such tissue-level interactions are clearly dependent upon the nature of the participating stromal cells. Viewed in this context, it is of interest that several groups have documented the presence of aberrant stromal fibroblasts in patients with various types of both sporadic and hereditary cancers [12–18]. Our own work in this area has indicated that fibroblasts obtained from a majority of breast cancer patients resemble fetal cells in terms of their apparently persistent production of a *migration-stimulating factor* (MSF) that is not made by their normal adult counterparts. These findings provide the principal focus of this chapter, in which data are reviewed regarding 1) our current understanding of the structure of MSF, 2) its mode of action on target fibroblasts, 3) its possible physiological contribution to wound healing, and 4) how this normally beneficial function may be subverted to contribute to tumor progression. These various points are discussed in relation to a proposed epigenetic model of fibroblast function and contribution to cancer progression.

Initial identification and characterization of MSF

Several years ago we described a means of quantitating cell migration into three-dimensional matrices of type I collagen fibers [19]. In this assay system, cells are plated onto the surface of the collagen substratum and their migration down into the collagenous matrix measured by microscopic observation after a standard four-day incubation period. In our early studies, we observed that the migration of adult skin fibroblasts into the collagen matrix was dependent upon cell density, which involved a significant down-regulation of migration at confluence [20]. This study also revealed that fetal fibroblasts displayed a distinct migratory phenotype characterized by the persistence of elevated levels of migration at confluent cell densities. Subsequent work revealed that this behavioral difference between the two cell types resulted from the secretion by fetal fibroblasts of a soluble migration-stimulating factor (MSF) that was not produced by their adult counterparts [21]. Interestingly, confluent adult fibroblasts retain responsiveness to MSF, as evidenced by the significant stimulation of their migration when exposed to it.

Using this migratory response of adult fibroblasts as a convenient bioassay for monitoring MSF activity, we have developed a protocol for the purification of MSF from fetal fibroblast conditioned medium [22]. Briefly, this involves 1) an initial precipitation of MSF activity at 20% ammonium sulphate, 2) heparin-affinity chromatography (with MSF activity eluting at

0.3–0.6M NaCl), 3) FPLC gel filtration chromatography, and 4) FPLC reverse-phase chromatography. The precipitation of all MSF activity at such a low concentration of ammonium sulphate distinguishes it from the majority of other proteins in fibroblast-conditioned medium and results in its efficient early purification.

In view of the apparent instability of MSF following exposure to the organic buffers used in the final reverse-phase chromatography, we have replaced this procedure with a milder anion exchange (Mono Q) FPLC step. MSF obtained by this scheme has been characterized in terms of a number of biochemical criteria and with respect to its biological activity (Grey et al., manuscript in preparation). These results indicate that all the applied MSF activity is unbound to Mono Q and recovered in the initial column wash (figure 1). SDS-PAGE reveals that this material consists of three principal constituent bands: one with a molecular mass of approximately 119,000 kDa and a doublet with molecular masses of 33 and 43 kDa, respectively (figure 2, track A). Our data further suggest that the two lower-molecular-weight bands are derived from the degradation of the higher-molecular-mass species, and that at least one of the lower-molecular doublet proteins may sub-

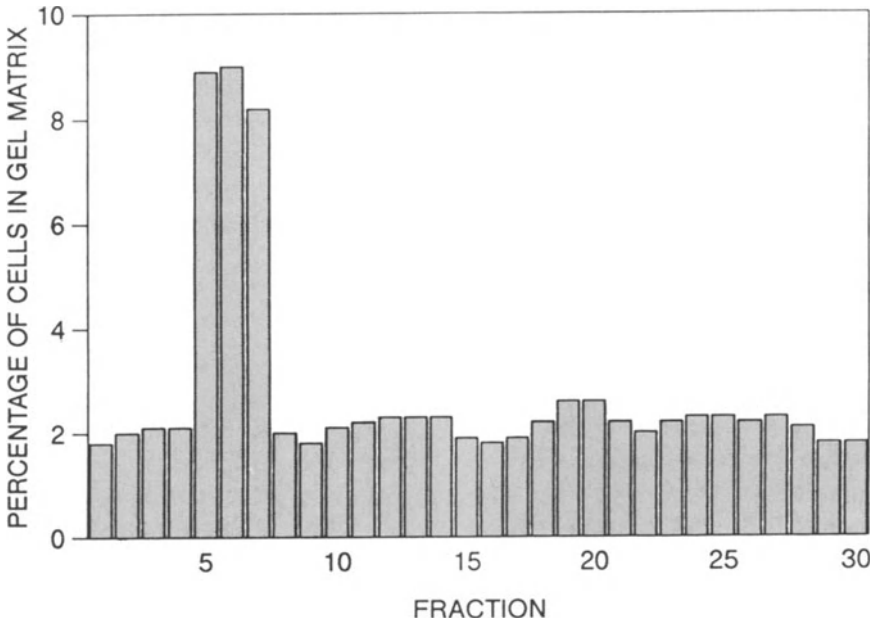

Figure 1. The elution profile of MSF activity on Mono Q anion exchange chromatography. Partially purified MSF activity following Superose 12 gel filtration chromatography was applied to a Mono Q column and eluted with a linear gradient of 0–1.0N NaCl between 4 and 24 ml, and a decreasing linear gradient of 1.0–0N NaCl between 29 and 32 ml. One-ml fractions were collected and analyzed for MSF activity using the collagen matrix migration assay.

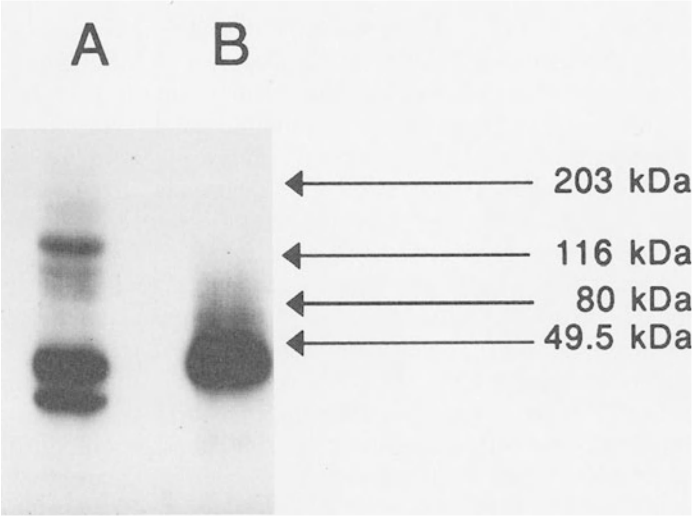

Figure 2. The composition of MSF eluted from the Mono Q anion exchange column. The peak of MSF biological activity that was recovered following anion exchange chromatography was iodinated, and the constituent proteins were visualized by SDS-PAGE and fluorography (track A). An aliquote of proteolytically generated gelatin-binding fragment of fibronectin was also iodinated and similarly analyzed (track B).

sequently break down into a biologically active molecule with a molecular mass of approximately 25 kDa.

All three protein bands in the Mono Q unbound preparation of MSF are immunoprecipitated with a polyclonal antifibronectin antibody, indicating that each one contains epitopes also present in fibronectin. In keeping with this observation, amino acid sequence analysis has indicated that the 43-kDa protein exhibits striking N-terminal sequence homology with the gelatin-binding domain of fibronectin, as follows:

– P Y G H – V T D S G V V Y G V T M *MSF*
1 5 10 15
Q P P P Y G H C V T D S G V V Y S V G M Q *fibronectin*
270 275 275 275 290

More recent data indicate that the 119-kDa protein has an apparently unique N-terminal sequence that is also present in the lower-molecular-mass molecule (Grey et al., in preparation). These observations suggest that MSF is a novel molecule containing a domain exhibiting significant structural homology with the gelatin-binding region of fibronectin.

As part of our continuing work concerned with identifying the active amino acid motif in MSF, we have compared MSF with bona fide preparations of the gelatin-binding fragment (GBF) of fibronectin in terms of a number of biological and biochemical criteria. Preparations of GBF were

produced by the limited thermolysin degradation of human plasma fibronectin and subsequent separation of the generated peptide fragments by hydroxyapatite chromatography [23]. The GBF generated by this protocol had a molecular mass of approximately 43 kDa (figure 2, track B) and amino terminus at ala_{262} (i.e., only ten amino acids removed from that of the similar molecular mass molecule in the MSF preparation).

In the first instance, the effects of these molecules on fibroblast migration were examined. Our results indicated that MSF *and* GBF stimulated the migration of confluent adult fibroblasts with a similar biphasic dose–response (figure 3); in both cases, significant migration-stimulating activity was apparent at concentrations of effector molecule as low as 1.0–10.0 pg/ml and returned to control (unstimulated) levels at concentrations greater than 25 ng/ml. These studies also indicated that native fibronectin and all its other peptide degradation products were completely devoid of migration-stimulating activity at concentrations up to 1 μg/ml (data not shown). In marked contrast to these findings, previous studies using Boyden chamber assays have indicated that 1) native fibronectin-stimulated fibroblast migration, but only when present at the relatively high concentrations of 10–100 μg/ml [24,25], and 2) similarly high concentrations of fibronectin pro-

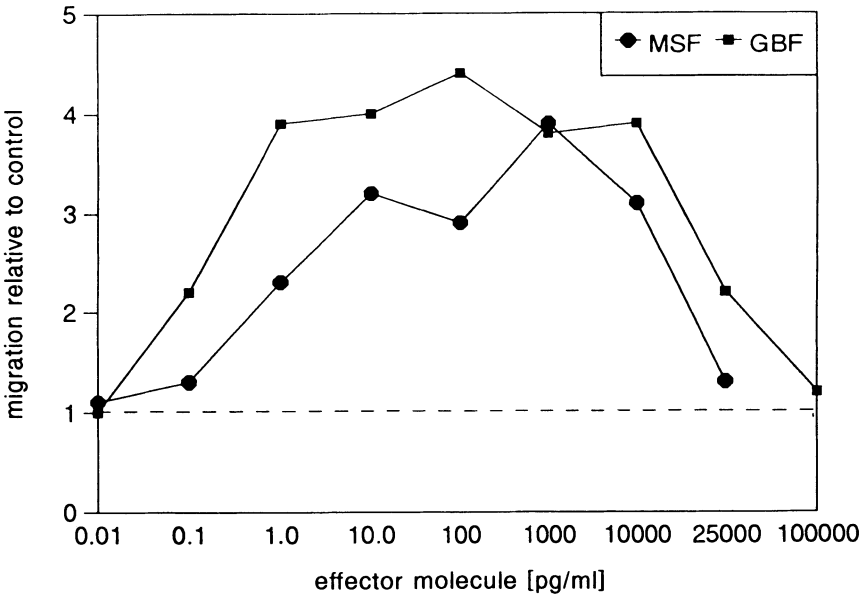

Figure 3. Dose–response of MSF and GBF on the migration of confluent adult fibroblasts. Confluent adult fibroblasts were exposed to different concentrations of MSF and the cultures incubated for four days. The percentages of cells within the collagen matrix were then determined, and these data were expressed relative to the control (dotted line). Under these confluent conditions, adult fibroblasts displayed a relatively low level of migration in control cultures (2.3 ± 0.6%).

teolytic fragments containing the central cell-binding domain could replace native fibronectin in this assay, while fragments containing the gelatin-binding domain were completely inactive [26]. The very potent migration-stimulating activity of GBF in the collagen matrix assay was most unexpected in the light of these reports and once again serves to underscore the importance of the substratum in determining cellular responsiveness to potential soluble effector molecules. The extremely low concentration at which GBF stimulates fibroblast migration in the collagen gel assay system implies that it is acting in a 'cytokine-like' fashion involving its interaction with an hitherto unrecognized cell surface receptor; our current data suggest that this is indeed that case (Schor et al., manuscript submitted for publication).

The molecular basis of the observed bell-shaped dose–response of MSF and GBF on fibroblast migration is not understood; we have postulated that it may reflect the involvement of distinct classes of cell surface receptors and/or relative receptor occupancy in determining cellular response. Similar biphasic dose–response curves have been observed with respect to the effects of other cytokines on various aspects of cell behavior, including migration [27], although the nature of the responsible mechanisms remains equally obscure.

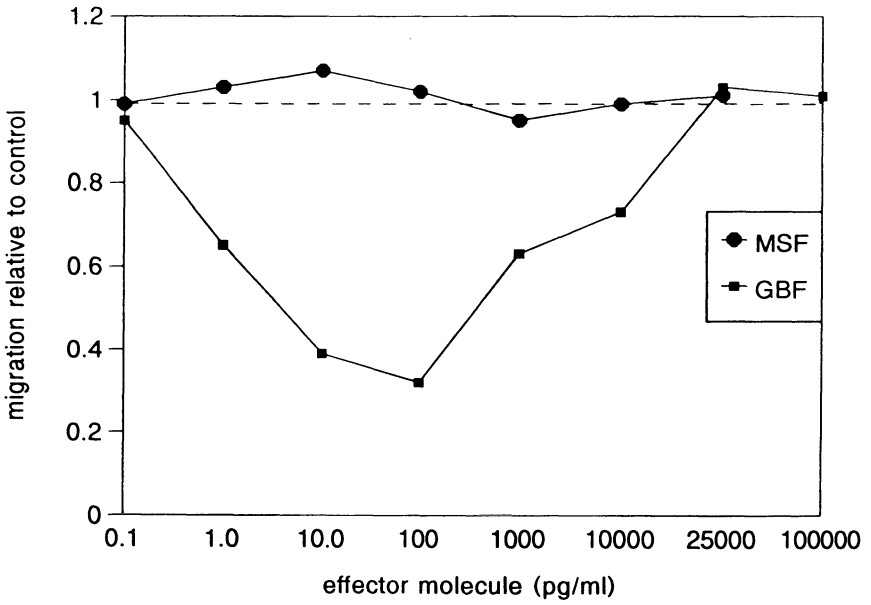

Figure 4. Dose–response of MSF and GBF on the migration of subconfluent adult fibroblasts. Subconfluent adult fibroblasts were exposed to different concentrations of MSF and GBF and the cultures incubated for four days. The percentages of cells within the collagen matrix were then determined and these data expressed relative to the control (dotted line). Under these subconfluent conditions, adult fibroblasts displayed an elevated absolute level of migration in control cultures (25.6 ± 1.6%).

In spite of this striking similarity, MSF and GBF differ from each other in terms of their respective effects upon the migration of subconfluent cells; as indicated in figure 4, MSF has no effect upon the inherently elevated migration of subconfluent skin fibroblasts, whereas GBF actually *inhibits* cell migration, again at extremely low concentrations (to be discussed in further detail in Grey et al., in preparation). This represents a significant difference in the biological activity of MSF and GBF, and supports the view that they are structurally related, but distinct molecules. The underlying mechanisms responsible for the diametrically opposed effects of GBF on the migration of confluent and subconfluent fibroblasts is currently under investigation and may ultimately be related to the role of fibronectin (fragments) in determining the alternative initiation and cessation of active cell motility that characterizes cell behavior in vivo [28].

MSF and GBF also differed in terms of their respective recognition by a recently generated rabbit polyclonal anti-MSF antibody. The antibody was raised against the Mono Q unbound preparation of MSF and purified by sequential protein A, fibronectin, and GBF-affinity chromatography. The partially purified IgG fraction of antibody obtained after protein A affinity chromatography recognized MSF, as well as native fibronectin and GBF in

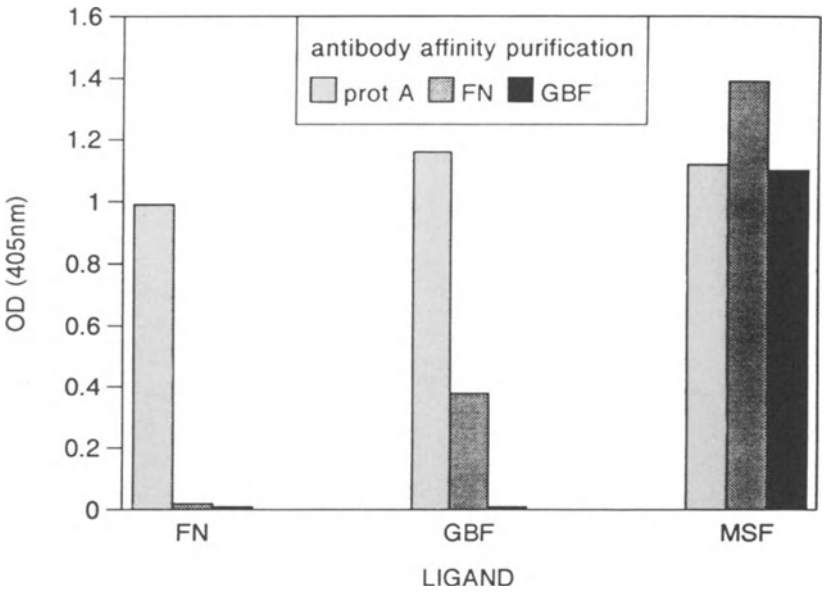

Figure 5. The recognition of fibronectin, the gelatin-binding fragment of fibronectin, and MSF by variously purified anti-MSF polyclonal antibody. Anti-MSF polyclonal antibody was purified by sequential chromatography on protein A (prot A), fibronectin (FN), and gelatin-binding fragment (GBF) affinity columns. The binding of these different antibody preparations to the different ligands was then determined by ELISA using an AP-conjugated secondary antibody. Binding is measured by the OD (405 nm).

an ELISA (figure 5); this result is consistent with the shared epitopes present in MSF and GBF. Subsequent purification of the antibody by sequential fibronectin and GBF-affinity chromatography resulted in the expected elimination of antibody recognition of these two ligands in the ELISA; such affinity-purified antibody still recognized MSF in a specific fashion, indicating that it contains epitopes *not* shared by either native fibronectin or GBF. Related experiments using partially degraded, but unfractionated, preparations of fibronectin confirmed that the fibronectin- and GBF-affinity purified anti-MSF antibody did not recognize any cryptic epitopes present in fibronectin.

Interestingly, the stimulation of fibroblast migration by MSF was completely neutralized by the fibronectin- and GBF-affinity purified anti-MSF antibody at dilutions of up to 1:500, while considerably higher concentrations of antibody (1:10 dilution) had no effect on the comparable biological activity of GBF (figure 6). Related data indicated that the activity of both MSF and GBF were completely neutralized by two monoclonal anti-

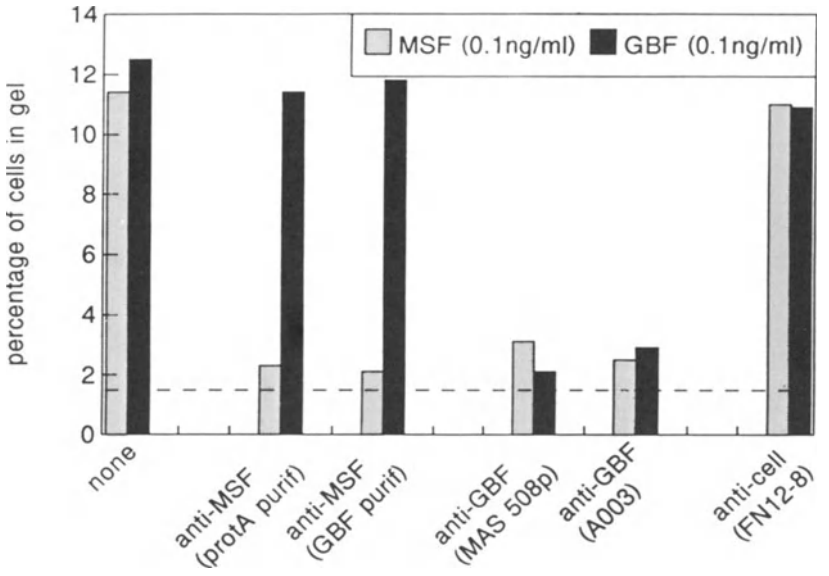

Figure 6. The effects of various antibodies on the stimulation of fibroblast migration by MSF and GBF. Fibroblasts were co-incubated with various antibodies (indicated on the *x*-axis) in the presence of either MSF or GBF in the standard migration assay. Anti-MSF polyclonal antibody was used following both the protein A (protA purif) and GBF (GBF purif) affinity chromatography steps at final dilutions 1:10. In addition, two monoclonal antibodies were used that recognize epitopes present in the gelatin-binding domain of fibronectin (MAS 508p and A003 obtained from Sera Lab, Crawley Down and Bioquote Ltd, York, respectively), as was a third that recognized an epitope present in the cell-binding domain (FN 12-8 obtained from Pierce and Warriner, Chester); all these three antibodies were used at a final concentration of 1 μg/ml.

bodies recognizing epitopes in the gelatin-binding domain, but completely unaffected by a monoclonal antibody recognizing an epitope in the cell-binding domain of fibronectin.

Mode of action of MSF

We have previously reported that MSF stimulates the synthesis of a high-molecular-weight species of hyaluronic acid (HA) [29]. HA is a linear glycosaminoglycan consisting of repeating disaccharide subunits of glucuronic acid and N-acetylglucosamine. It is a major biosynthetic product of fibroblasts and has been shown to promote the migration of a number of cell types both during embryonic development [30,31] and in vitro [32,33]. The involvement of HA in modulating cell migration in vivo appears to continue in the adult, with various lines of evidence implicating it in the regulation of cell movement in various pathological processes, such as wound healing and tumor invasion [34].

HA is a polydisperse macromolecule exhibiting significant tissue-dependent variation in molecular mass. The biological activity of HA with respect to the control of a number of physiological and pathological processes appears to be critically dependent upon molecular mass [35]. In this regard, it is important to note that our data indicate that MSF specifically stimulates the synthesis of a high-molecular-mass (greater than 10^6 kDa) size-class of HA [29].

Our data indicate that the stimulation of fibroblast migration by MSF is in fact a secondary consequence of its primary effect upon HA synthesis. This was first suggested by the observation that the stimulation of both cell migration and HA synthesis by MSF follows parallel biphasic dose–response curves (figure 7). In a related series of experiments, we noted that the stimulatory effect of MSF on cell migration was completely blocked by co-exposure of fibroblasts to *Streptomyces* hyaluronidase during the four-day duration of the assay [36]. Finally, the addition of exogenous high-molecular-mass HA to control adult fibroblasts was found to induce the stimulation of their migration in a biphasic dose-dependent fashion similar to that produced by MSF [29].

The stimulation of HA synthesis by MSF and the apparent subsequent effect of this HA on cell migration should caution us that the ascription of a biological function in the naming of a cytokine (e.g., *migration*-stimulating factor) generally reflects its activity in the particular bioassay first used in its identification rather than being a necessarily accurate description of its principal physiological function in vivo. In this regard, many well-characterized 'growth factors' (such as PDGF and EGF) also affect a variety of aspects of cell behavior in addition to proliferation, including cell migration [37]. With this multifunctionality of cytokine action in mind, it should be noted that the

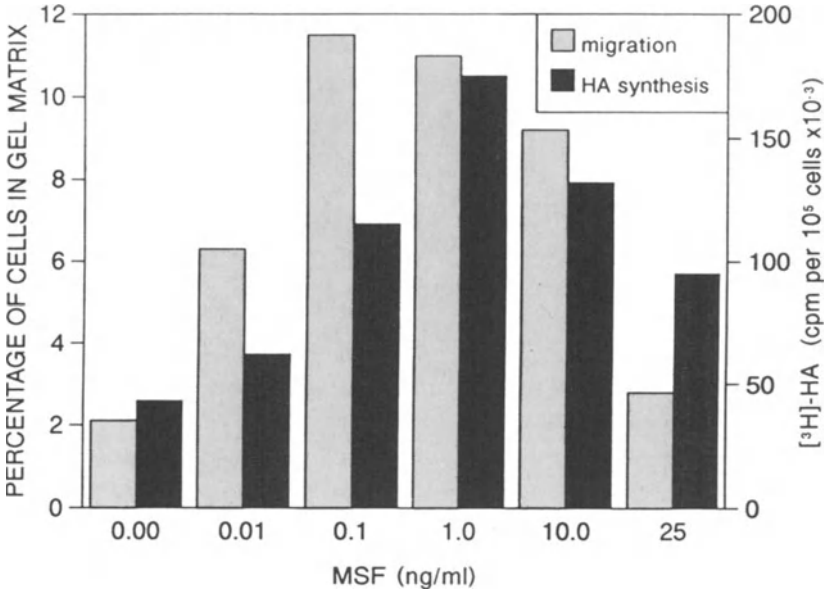

Figure 7. The dose–response of MSF on the migration of adult fibroblasts and their synthesis of hyaluronic acid (HA). Confluent cultures of adult fibroblasts on collagen gels were exposed to different concentrations of MSF. Fibroblast migration into the collagen matrix and the incorporation of ^{3}H-glucosamine into HA was determined as indicated in Ellis et al. [29].

principal biological activity of MSF in vivo may not relate to cell motility per se, but rather to some other aspect of cell function, such as HA production.

In this regard, we have recently reported the presence of MSF in 16 of 17 (94.1%) wound fluid samples [38]. The directed migration of fibroblasts into the wound site and the transient increase of HA in granulation tissue during the wound-healing response are both consistent with the involvement of MSF. Although the source of MSF in wound fluid is not known, its absence from matched serum samples collected from the same patients suggests that it is not released from degranulating platelets nor derived from a plasma transudate.

The biological activity of a particular cytokine is influenced by the presence of other cytokines in the microenvironment. In view of this inter-dependence of cytokine function, we have been particularly interested in ascertaining the interaction of MSF with other potentially relevant cytokines. In the first instance, we have reported that the stimulatory effects of MSF on both cell migration and HA biosynthesis are inhibited by TGF-β1 (figure 8) [29]. Such an apparently antagonistic effect of these two cytokines may reflect their 'balancing' role in the control of cell behavior during various physiological and pathological events, such as wound healing.

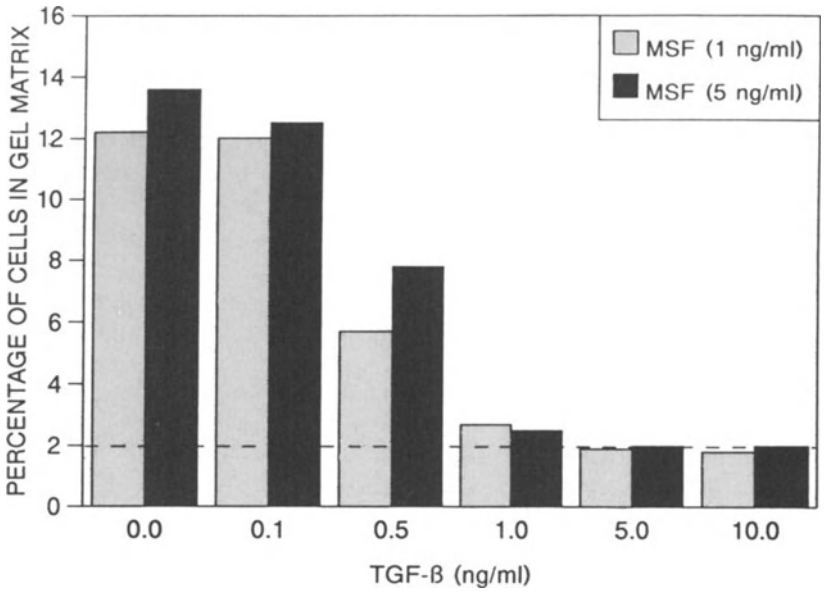

Figure 8. The effects of TGF-β on the stimulation of fibroblast migration by MSF. Confluent adult fibroblasts were incubated with 0, 1.0, and 5.0 ng/ml MSF in the presence of various concentrations of TGF-β1 (British Bio Technology, Cowley) and the percentage of cells within the collagen matrix were determined four days later. Control cells incubated in the absence of both MSF and TGF-β (dotted line) displayed a low level of migration (2.0 ± 0.2%).

The presence of MSF-secreting fibroblasts in breast cancer patients and their postulated direct contribution to disease progression

Much of our previous work has been concerned with documenting the presence of MSF-secreting ('fetal-like') fibroblasts in breast cancer patients. These studies have demonstrated that 1) tumor-derived fibroblasts obtained from approximately 50% of sporadic breast cancer patients expressed a fetal-like migratory phenotype [40], 2) paired skin fibroblasts obtained from the same individuals also expressed a fetal-like migratory phenotype, thereby indicating the systemic nature of this stromal cell abnormality [40,41], and 3) skin fibroblasts obtained from approximately 90% of patients with familial breast cancer behaved in a similar fetal-like fashion, as did greater than 50% of their unaffected first-degree relatives [42,43]. The high incidence of fibroblasts exhibiting a fetal-like migratory phenotype in the unaffected first-degree relatives of the familial patients (i.e., a population with a clearly documented elevated risk of developing breast cancer) [44] is of particular significance, since it indicates that the systemic presence of these cells is likely to precede the development of overt malignant disease.

Subsequent work indicated that the fetal-like fibroblasts obtained from

these breast cancer patients and their first-degree relatives also produce MSF [45] (see also Schor et al., in preparation). This MSF is indistinguishable from that produced by fetal fibroblasts with respect to all the biological and biochemical parameters we have investigated to date.

We have also reported that detectable levels of MSF are present in the serum of sporadic breast cancer patients [46]. In this study, serum was collected from two groups of patients; the first (untreated) group consisted of newly diagnosed patients (n = 12) from whom serum was collected both 24 hours prior to surgical resection of the primary tumor and four days postoperatively, whilst the second (treated) group consisted of patients (n = 14) at various times after tumor resection who had received adjuvant therapy. Serum samples were also collected from age-matched healthy controls with no family history of breast cancer (n = 20). Serum samples were fractionated according to our protocol for MSF and then assessed for migration-stimulating activity in our standard collagen gel assay. Our data indicate that MSF activity was present in 10 of 12 (83.3%) serum samples obtained from untreated patients prior to surgery, and 9 of 12 (75%) these same individuals four days postoperatively (figure 9). Corresponding data obtained from the treated group indicated that detectable MSF activity was

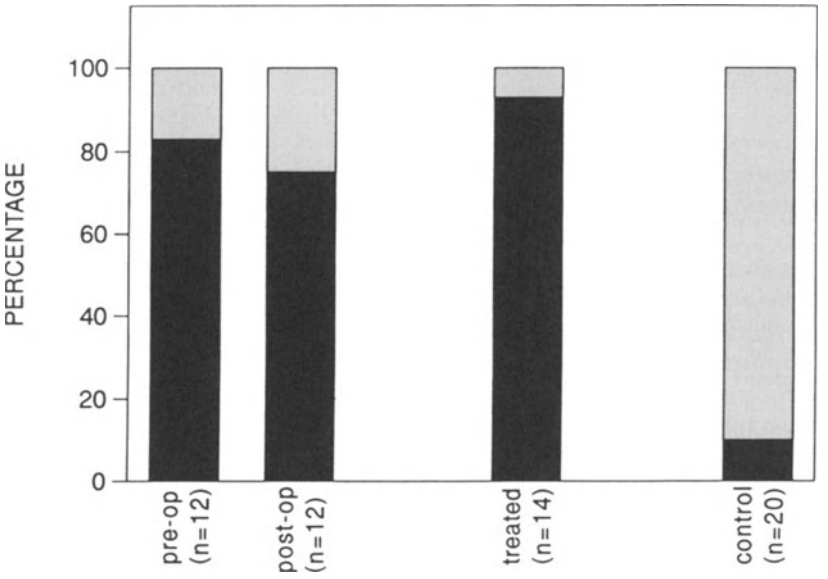

Figure 9. The presence of detectable levels of MSF in the serum of both treated and untreated breast cancer patients. The presence of MSF activity in the serum of breast cancer patients and age-matched controls was determined using the collagen matrix bioassay. The percentage of each patient group containing detectable MSF activity is indicated by the black bar and the percentage devoid of activity by the stacked grey bar. Details regarding patients and experimental procedures may be found in Picardo et al. [46].

present in 13 of 14 (93%) serum samples. In marked contrast to this relatively high incidence of MSF in the patient sera, we detected MSF activity in only 2 of 20 (10%) control sera. Biochemical characterization of the serum-derived MSF indicated that it was indistinguishable from its fibroblast-produced counterpart.

The presence of MSF in the serum of the postoperative patient group, whose members had been free of detectable residual disease for periods of up to 13 years, clearly distinguishes MSF from previously described oncofetal proteins that function as markers of tumor burden. Taken together with our previous results, the presence of MSF in the serum of these patients may reflect the systemic and persistent presence of a population of fetal-like (MSF-producing) fibroblasts.

Fetal-like fibroblasts as accelerators of cancer progression: consequence of perturbation of epithelial–stromal interactions

The detection of MSF-secreting fibroblasts in a significant proportion of breast cancer patients is consistent with numerous published reports documenting the presence of aberrant skin and tumor-derived fibroblasts in patients with a variety of both sporadic and hereditary cancers (reviewed in [47]). Such cells have been reported to express a number of phenotypic characteristics commonly associated with transformation, including colony formation in semisolid medium and reduced serum requirement for growth. In these studies, the presence of aberrant skin fibroblasts has invariably been considered to reflect the presence of an inherited (and hence systemically expressed) 'partially transforming' genetic lesion. According to this view, the aberrant behavior of these fibroblasts provides a convenient marker for the presence of this putative genetic lesion, but does not itself contribute to the course of cancer pathogenesis: expression of the inherited genetic lesion by the potential target epithelial cells population is considered to be the only mechanistically relevant event.

Our interpretation of these observations differs from this commonly held view in two important respects: we suggest that 1) the aberrant skin fibroblasts in cancer patients are not 'partially transformed,' but are instead expressing a spectrum of phenotypic characteristics displayed by normal fetal cells, and 2) the inappropriate expression of these fetal-like phenotypic characteristics by stromal fibroblasts in the adult directly contributes to the course of cancer progression by perturbing normal epithelial–stromal interactions.

With respect to the first point, it should be noted that many of the transformation-associated characteristics displayed by the aberrant cancer patient fibroblasts are also commonly expressed by fetal cells, e.g., colony formation in semisolid medium [48]. The fetal-like nature of tumor-associated fibroblasts has been more explicitly demonstrated by both Azzarone et al.

[17] and Wynford-Thomas et al. [18], who independently reported that such cells exhibit an extended lifespan in vitro that is characteristic of fetal fibroblasts (not, however, an indefinite lifespan characteristic of transformed cells). In a comprehensive study of the pathogenesis of benign prostatic hyperplasia and its subsequent malignant transformation, McNeal [49] noted the early appearance of a 'fetal-like' stroma and postulated that its interaction with the emerging population of aberrant epithelial cells was a key factor in disease progression. Tumor-associated fibroblasts have also been reported to resemble fetal cells in terms of their expression of a fetal-specific cell surface antigen [50] and production of stromelysin-3 [51].

The shift in emphasis from *partially transformed* to *fetal-like* is more than just a question of semantics. The designation *partially transformed* carries with it the implication that this is an aberrant state resulting from an expressed genetic lesion. In contrast, the term *fetal-like* implies that the particular phenotypic attributes that define this state (e.g., continued production of MSF) are inherently physiological, although their expression may be inappropriate in the adult. Inherited genetic lesions may still be responsible for the inappropriate expression of these fetal-like characteristics, but, as will be discussed in the following section, other epigenetic mechanisms may also be involved.

The second, and more fundamental, issue relates to the potential direct contribution of these fetal-like fibroblasts to disease pathogenesis as a result of their perturbation of normal epithelial–stromal interactions (as initially proposed in [47]). Such tissue interactions play a significant and well-documented role in the control of cell proliferation, migration, and differentiation during embryonic development and continue to contribute to the maintenance of a differentiated phenotype in the adult [52]. In the light of these observations, various workers have suggested that perturbation of epithelial–stromal interactions in the adult may contribute to the pathogenesis of various disease states (including cancer) characterized by abnormalities in epithelial cell proliferation and differentiation [53]. Various studies have lent experimental support to this view by indicating that interactions between tumor cells and fibroblasts contribute to the events of progression. For example, such interactions have been reported to enhance tumor metastasis in vivo [54]. These interactions are complex, and the precise effect of stromal fibroblasts on tumor cells (e.g., inhibition or stimulation of proliferation) may alter during the course of tumor progression [55].

As is the case with cell interactions during embryonic development and in the healthy adult, signaling between tumor and stromal cells involves the complex interplay of (soluble) cytokines and (insoluble) matrix macromolecules. The effects of cytokines and matrix macromolecules are mutually interdependent in the sense that 1) the specific response of cells to cytokines is modulated by the nature of the surrounding matrix, and 2) the deposition of this matrix is modulated by the action of cytokines [56]. Seen in this light,

this cytokine–matrix interdependence is an extension of the notion of *dynamic reciprocity* first proposed by Bissell et al. [57] to describe the complex nature of cell–matrix interactions. Our recent data concerning the potent effect of the gelatin-binding fragment of fibronectin on cell migration (figures 3 and 4) further indicate that soluble degradation products of matrix macromolecules may exhibit unsuspected 'cytokine-like' biological activities not shared by the native matrix molecule; functioning in this capacity, the matrix may act as an inactive precursor of soluble cytokines that are released by the action of locally produced degradative enzymes.

Interactions between tumor cells and fibroblasts have been reported to modulate their respective production of both cytokines and matrix macromolecules in a bidirectional fashion [58]. These interactions may involve positive feedback loops that may result in an expansion in cell number and/or amplification of signal molecule synthesis. For example, Cullen et al. [59] reported that mammary carcinoma cells synthesize PDGF, which stimulates fibroblast proliferation and synthesis of IGF I and II; interestingly, the fibroblast-produced IGF in turn stimulates mammary carcinoma cell proliferation and synthesis of PDGF.

We have previously speculated that the apparently persistent fetal-like fibroblasts of cancer patients may contribute to the course of disease progression by creating a milieu that promotes the clonal expansion and invasive behavior of the emerging neoplastic cell population. Our data suggest that the MSF produced by these fibroblasts may function in this capacity, perhaps as a consequence of its effect on HA synthesis. In this regard, various studies have noted elevated levels of HA associated with the stroma of different types of tumors and have demonstrated that this is correlated with more aggressive invasive behavior [60]. HA has also been reported to modulate a number of processes of potential relevance to tumor progression, including the proliferation and differentiation of mammary epithelial cells [61] and angiogenesis [35]. According to this model, MSF functions as an autocrine regulator of fibroblast matrix synthesis, which in turn affects epithelial cell behavior. Our recent data suggest that MSF may also exert a direct paracrine effect upon gene expression by target mammary epithelial cells (A. Schor et al., in preparation).

The postulated involvement of MSF in both cancer pathogenesis and wound healing may be relevant to the observation of Dvorak [62] that the tumor–host interface resembles a 'wound that does not heal.' In this context, the same effects of MSF that contribute positively to wound healing when expressed *locally* and in a *transient* manner may facilitate cancer progression when they are *systemic* and/or *prolonged* in nature.

Tumor progression is an indolent process in which many decades may elapse between inception of the initiating genetic lesion and the emergence of a clinically recognizable malignancy. Information regarding the proportion of 'initiated' cells that eventually go on to develop into a tumor are not generally available, although recent data suggest that this figure may indeed

be quite low. For example, Nielsen et al. [63] have documented the presence of microfoci of carcinoma in situ in the breasts of apparently healthy women who died in road traffic accidents; this study revealed that greater than 40% of women over the age of 40 had such histologically discernable lesions, although clearly only a relatively small proportion of these will proceed on to develop into an overt malignancy *during the lifespan of the individual.* These observations suggest that factors that may alter the kinetics of progression may actually play an important, and perhaps decisive, role in determining the probability of disease inception.

It is in this postulated role of an *accelerator* of cancer progression that we view the contribution of fetal-like (MSF-secreting) fibroblasts to disease pathogenesis. We have intentionally avoided the word *promoter* in describing the mode of action of fetal-like fibroblasts in view of the rigorous (and distinct) definition of this term in the carcinogenic literature [64]. According to the model, we propose that these fetal-like fibroblasts contribute to the creation of an environment that is conducive to the clonal expansion of the evolving neoplastic cell population. The question of whether this involves permissive or inductive mechanisms — and other fundamental questions relating to mechanism — needs to be the focus of future studies.

The presence and potential physiological function of MSF-secreting fibroblasts in the healthy adult: a clonal modulation model to account for their apparent increased number in cancer patients

In spite of their ease of culture in vitro, fibroblasts remain a relatively poorly defined population of cells with no unique cell-specific markers. We have previously reviewed the literature indicating that cells commonly regarded as fibroblasts are a phenotypically heterogenous population displaying both inter- and intrasite heterogeneity in tissue distribution [65].

This heterogeneity in fibroblast phenotype has significant implications regarding our understanding of the origin of MSF-secreting (fetal-like) fibroblasts in breast cancer patients. According to the commonly accepted germ-line model (discussed above), these fibroblasts are considered to arise from the inheritance of a partially transforming genetic lesion. As part of our alternative interpretation of the role of these fibroblasts in cancer pathogenesis, we have proposed a *clonal modulation* model to account for their presence in cancer patients [65]. According to this model, we suggest that 1) distinct subpopulations of MSF-secreting fibroblasts exist in the healthy adult, where they may function in processes such as wound healing, 2) there is a prolonged and systemic elevation in the relative number of these fibroblasts in cancer patients, and 3) the factors leading to this disruption in relative fibroblast phenotypic balance may involve both internal signals (e.g., as might be produced by the emerging population of neoplastic cells themselves) or environmental factors. According to this epigenetic

model, the MSF-secreting fibroblasts detected in cancer patients are not considered to be intrinsically aberrant, but rather an expanded subpopulation of cells also present in the healthy adult.

Our recent data clearly confirm that subpopulations of MSF-secreting fibroblasts are in fact present in the adult and that these display both inter- and intrasite heterogeneity with respect to their tissue distribution. For example, fibroblasts obtained from 15 of 20 (75%) oral mucosal biopsies produced detectable amounts of MSF compared to only 2 of 20 (10%) paired forearm skin fibroblasts obtained from the same individuals (Picardo et al., in preparation). Interestingly, wound healing in the oral mucosa is clinically distinguished from dermal healing in terms of both its rapidity and lack of scar formation. It is possible that the presence of these MSF-producing fibroblasts contribute to this regenerative and characteristically fetal-like mode of wound healing.

Related studies have revealed the existence of intrasite heterogeneity in the oral mucosa with respect to the tissue distribution of MSF-producing fibroblasts. This involved the separation of gingival lamina propria (connective tissue) from its overlying epithelium by exposure to trypsin and the subsequent microdissection of the lamina propria to allow the selective culture of fibroblasts derived from the tips of the papillae and deeper reticular tissue. Only fibroblasts derived from the papillae produced MSF (Irwin et al., in preparation). Prolonged subculture of papillary fibroblasts resulted in their cessation in MSF production and their adoption of a reticular fibroblast phenotype. Staining of gingival tissue with affinity-purified anti-MSF antibody confirmed the preferential localization of MSF in the papillae (figures 10A and 10B); interestingly, this particular pattern of MSF distribution is identical to that previously described for tenascin [66], another fetal-associated fibroblast product [67].

We have also observed intrasite heterogeneity with respect to the distribution of MSF-secreting fibroblasts in the normal breast. Intra- and interlobular fibroblasts were isolated by controlled enzymatic digestion and differential sedimentation from five specimens of normal breast obtained from patients undergoing reduction mammoplasty and five specimens of histologically normal breast adjacent to a carcinoma. Interlobular fibroblasts obtained from 5 of 5 (100%) reduction mammoplasty specimens were found to produce MSF [68] (see also A. Schor et al., in preparation); in contrast, none (0 of 5) of the intralobular fibroblasts produced detectable levels of MSF. Such a clearly defined intrasite heterogeneity in the distribution of MSF-secreting fibroblasts within the normal breast was again confirmed by immunolocalization with the affinity-purified anti-MSF antibody (figure 10C). Interestingly, fibroblasts obtained from histologically normal breast adjacent to a carcinoma displayed a different tissue distribution of MSF-secreting cells; in these samples, 5 of 5 (100%) of *both* inter- and intralobular fibroblasts produced MSF when cultured in vitro. These initial data suggest that histologically normal breast adjacent to a carcinoma may in fact be distinct

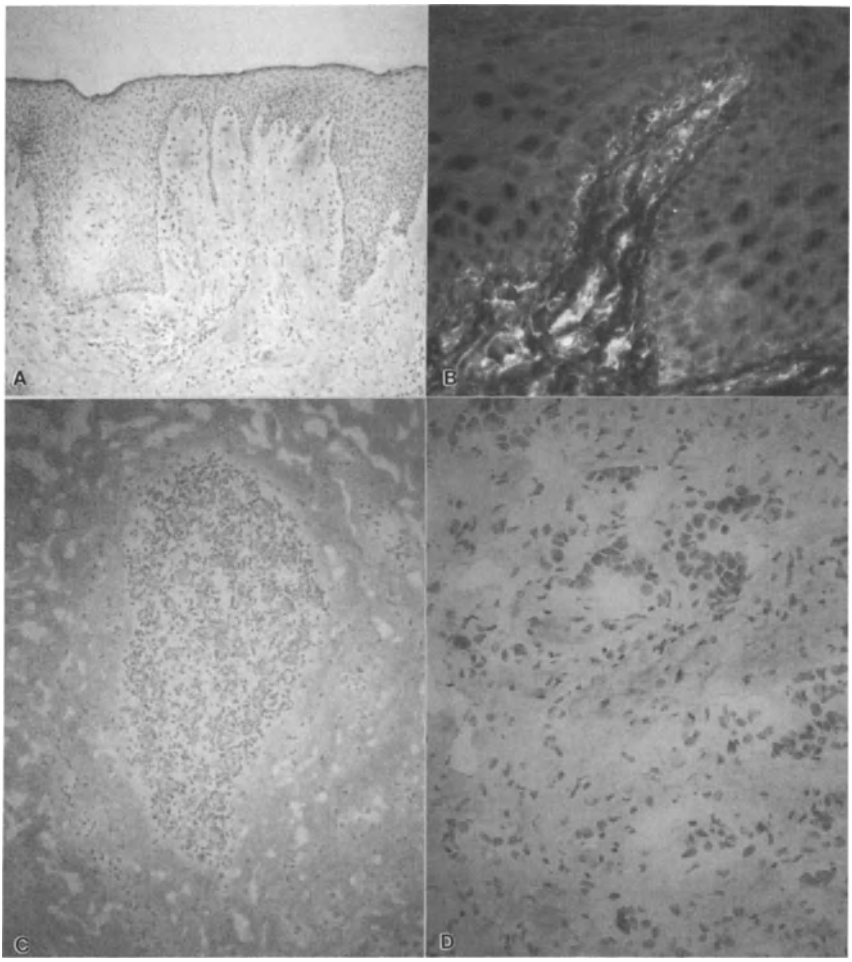

Figure 10. Immunolocalization of MSF. (A) Immunoperoxidase localization of MSF in the papillae of normal human gingiva. (×175). (B) Higher magnification of immunofluorescent localization of MSF in the papillae of normal human gingiva. (×250). (C) Immunoperixidase localization of MSF in the interlobular stroma of normal human breast. (×175). (D) Focal immunoperoxidase localization of MSF in the stroma associated with invasive carcinoma of the breast. (×400).

from similarly appearing tissue in the disease-free breast. We may further speculate that the close proximity of MSF-secreting intralobular fibroblasts and epithelial cells in the diseased breast may have both preceded and contributed to the eventual emergence of a clinically recognizable malignancy.

Various studies have suggested that the fetal-like fibroblasts present in the stroma associated with carcinomas are in fact a subset of the total

fibroblast population. For example, Chambon and colleagues have recently reported that only a proportion of fibroblasts in the stroma of breast cancers resemble fetal cells in terms of their production of stromelysin-3, a novel protease apparently not produced by normal adult fibroblasts [51]. In vitro studies by Dabbous et al. [69] similarly revealed the existence of signficant clonal heterogeneity in the production of proteases by tumor-associated fibroblasts in response to inductive signals from co-cultured carcinoma cells. Our own initial data clearly indicate the presence of a minority subpopulation of MSF-secreting fibroblasts within the stroma of mammary carcinoma (figure 10D).

Nothing is known about the signals that may lead to the postulated systemic clonal expansion of MSF-producing fibroblasts. These may involve the action of soluble factors produced by the emerging population of preneoplastic cells (perhaps even before these are histologically recognizable) with the extant subpopulation of MSF-producing fibroblasts in a positively iterative feedback loop similar to that previously discussed. Alternatively, locally produced toxins (as in the gut) or environmental factors may induce a wound-healing response characterized by the systemic expansion of MSF-producing clones.

The essential discriminating feature of this clonal modulation model is that epigenetic, rather than genetic, mechanisms are postulated to account for the presence of fetal-like fibroblasts in cancer patients. These epigenetic mechanisms rely on continuing cell–cell interactions to regulate the postulated dynamic balance of phenotype expression within the fibroblast population.

Conclusions and prospectives for future studies

Our present data suggest that MSF is a novel gene product containing a region of amino acid sequence homology with the gelatin-binding domain of fibronectin. Ongoing studies are concerned with 1) the further molecular characterization of MSF, including determination of its complete amino acid sequence, 2) characterizing the cell surface receptor for MSF, and 3) quantitating the serum levels of MSF in breast cancer patients and correlating these with various clinically relevant parameters relating to risk. This information may provide further insight into the involvement of MSF in the complex process of cancer progression and ultimately lead to the development of novel therapeutic modalities targeted at normalizing epithelial–stromal interactions.

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16. Urokinase plasminogen activator (uPA) and its type 1 inhibitor (PAI-1): Regulators of proteolysis during cancer invasion and prognostic parameters in breast cancer

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In order to invade and spread, cancer cells must degrade extracellular matrix proteins. This degradation is catalyzed by the concerted action of several enzymes, including metalloproteases such as interstitial collagenases, type IV collagenases and stromelysins [1], and serine proteases such as plasmin [2]. Plasmin is formed from its precursor, plasminogen, by two activators: tissue-type plasminogen activator (tPA), which is involved in thrombolysis [3], and urokinase-type plasminogen activator (uPA), which plays a central role in tissue remodeling, including cancer invasion [2]. The activation of plasminogen is regulated by two specific plasminogen activator inhibitors (PAI-1 and PAI-2) [4]. Both uPA and PAI-1 are present in breast cancer tissue, and it was recently found that high levels of uPA [5–8] and PAI-1 [8,9] in breast cancer tissue are each associated with poor prognosis.

The urokinase pathway of plasminogen activation

Urokinase-type plasminogen activator

The biochemistry of uPA has been reviewed previously [2]. It is a serine protease, which is synthesized as an approximately 50-kD glycosylated single polypeptide chain proenzyme, pro-uPA, that is virtually catalytically inactive. The human uPA gene is located on chromosome 10 and is transcribed into a 2.5-kb-long mRNA [10]. Pro-uPA is converted to active uPA, consisting of two polypeptide chains (A and B) held together by a disulfide bond, the A-chain arising from the amino-terminal part of pro-uPA, and the B-chain arising from the carboxy-terminal part. The A-chain consists of two structural domains, a growth factor domain with homology to EGF, and a kringle domain [11]. The B-chain is homologous to the catalytic part of other serine proteases, such as trypsin, chymotrypsin, and plasmin. Two-chain uPA can, by the metalloproteinase matrilysin (or PUMP-1) [12], be converted into a 33-kDa catalytic active form of uPA consisting of the B-chain and the

R. Dickson and M. Lippman (eds.), Mammary Tumorigenesis and Malignant Progression.

carboxy-terminal part of the A-chain (low-molecular-weight uPA), and a 17-kDa noncatalytic fragment consisting of the N-terminal part of the A-chain.

uPA cleaves a single peptide bond in plasminogen, converting it to plasmin, that degrades a broad spectrum of proteins, including fibronectin, fibrin, and laminin (for a review, see [2]). In addition, plasmin activates latent forms of some metalloproteases [13] and affects various growth factor systems, e.g., by activating latent TGF-β [14,15] and dissociating IGF-I from its binding protein [16] and bFGF from the surface of some cell types [17].

Many cytokines and hormones control the expression of uPA in a cell-specific way (see [18]).

Urokinase-type plasminogen activator receptor

A specific cell surface receptor for uPA (uPAR) was first detected by a saturable binding of uPA to monocytes and monocyte-like cells [19] and has since been found on many types of cultured cancer cells [20,21]. Human uPAR is a single polypeptide-chain, highly glycosylated protein with a molecular weight of 55–60 kDa [22,23]. It is translated from a 1.4-kb mRNA [23], encoded by a single gene located on chromosome 19 [24]. It consists of three homologous domains. The amino-terminal domain (domain 1) contains the ligand-binding region [25], which binds to the EGF-like domain in the uPA molecule [26]. uPAR is carboxy-terminally anchored to the cell surface by a glycosyl-phosphatidylinositol moiety [27]. A possible function of this lipid anchor is to facilitate movement of uPAR on the cell membrane. uPAR can be cleaved by uPA and plasmin, releasing domain 1 and leaving domains 2 and 3 on the cell surface [28].

uPAR binds both active uPA and pro-uPA with a high affinity (K_d 10^{-9}–10^{-11} M) that depends on the cell type. Pro-uPA can be activated when it is receptor bound, and receptor-bound uPA is catalytically active [20,29]. Binding of pro-uPA to uPAR and concomitant cell surface binding of plasminogen strongly enhances plasmin generation [29,30], and the surface of uPAR-expressing cells are preferential sites for plasminogen activation under physiological conditions [31].

The activation of receptor-bound pro-uPA is efficiently catalyzed by surface-bound plasmin, leading to a strong amplification of the overall plasminogen activation reaction [29]. Several other proteolytic enzymes, including plasma kallikrein [32] and cathepsin B [33], can activate pro-uPA. The physiological relevance of these latter enzymes in pro-uPA activation remains to be determined, and it is still not known how the uPA pathway of plasminogen activation is initiated in vivo.

uPAR synthesis is regulated by cytokines such as TGF-β1, TGF-β2, EGF, and the tumor-promotor phorbol myristate acetate. This regulation has in some cases been traced back to the transcriptional level, but also changes in the stability of uPAR mRNA play a role [34,35]; L. Lund, personal communication).

Type 1 and 2 plasminogen activator inhibitor

uPA activity is controlled by two specific plasminogen activator inhibitors, PAI-1 and PAI-2 (for a review, see [4]). These molecules are products of different genes, located on chromosome 7 and 18, respectively. They are both glycoproteins with a molecular weight of approximately 50 kDa, and both belong to the serine protease inhibitor (serpin) family. PAI-1 and PAI-2 differ in their relative ability to react with uPA and tPA and also in their immunological reactivity. PAI-1 autoinactivates into a latent form but is protected from this inactivation by binding to vitronectin [36]. uPA and t-PA are also inhibited by protease-nexin 1, which in contrast to PAI-1 and PAI-2, also inhibits other trypsin-like proteases such as plasmin and thrombin [37]. The inhibitors bind to the catalytic B-chain of active uPA. They do not react with pro-uPA. PAI-1 inhibits receptor-bound uPA nearly as efficiently as uPA in solution [29]. Several cell types internalize and degrade complexes between uPA and PAI-1 or PAI-2 [38–40]. In some cases this internalization appears to be dependent on binding to the uPA receptor [39], and recent reports indicate that the α-2-macroglobulin receptor in some cell types also plays a role in internalization of uPA/PAI-1 complexes [41]. Expression of both PAI-1 and PAI-2 is regulated by a variety of cytokines, growth factors, and hormones [4]. The regulation of the various components of the uPA system appears to be independent of each other [4,18,34,35].

Role of the urokinase plasminogen activation system in cancer cell invasion and metastasis

The uPA pathway of plasminogen activation contributes to extracellular matrix dissolution in a number of processes involving tissue degradation, such as trophoblast invasion [42,43], postlactational involution of the mammary gland [44], rupture of the follicular wall during ovulation [42], and wound healing [45,46] (for a review, see [2]).

A variety of findings strongly suggest a similar role of the uPA-system in cancer cell invasion. Histochemical studies of the occurrence and localization of the various components of this system have shown that both uPA and uPAR are expressed at invasive foci in most experimental and human cancers that have so far been investigated. These studies have been performed both at the protein level by immunohistochemistry, and at the mRNA level by in situ hybridization. Because of the strong amplification of the proteolytic activity that is characteristic for the plasminogen activation cascade, uPA and uPAR are of very low abundancy in tissues. Special care must therefore be exercised in design and interpretation of such studies.

In the highly invasive murine Lewis lung carcinoma, uPA protein [47] and mRNA (P. Kristensen, personal communication) are consistently expressed by the cancer cells at invasive foci. Through the use of a recently isolated

cDNA for mouse uPAR [48], it has been found that also uPAR mRNA in this experimental carcinoma is expressed by the invading cancer cells (J. Eriksen, personal communication). PAI-1 protein is not found in the invasive areas of the Lewis lung carcinoma but is expressed by the cancer cells in noninvasive areas, suggesting that this inhibitor plays a role in protecting the tumor tissue against the proteolytic degradation [49].

Of human cancers, colon adenocarcinomas have been most intensively studied for expression of components of the plasminogen activation system. Both uPA and uPAR are consistently present at invasive foci, but surprisingly, uPA protein [50] and mRNA [51] are not found in the cancer cells but in fibroblast-like stromal cells located adjacent to the invading cancer cells.[1] uPAR mRNA [51] and protein (C. Pyke, unpublished results) are located in cancer cells and in tumor infiltrating macrophages. Therefore, in this type of cancer the malignant cells and the macrophages can presumably bind and utilize uPA released from the fibroblast-like cells. PAI-1 mRNA is in colon adenocarcinomas expressed by endothelial cells in the tumor stroma [52], while there is no PAI-1 expression in the surrounding normal tissue, suggesting that PAI-1 also in this type of cancer plays a role in protecting the tumor tissue against degradation.

In human squamous skin cancer, both uPA and uPAR mRNA are expressed by the invading cancer cells [53,54]. In ductal mammary carcinomas, uPAR immunoreactivity is located in macrophages infiltrating the invasive foci [55], while uPA mRNA is found in adjacent fibroblast-like cells, and in some cases also in the cancer cells [53,56]. PAI-1 has been detected by immunohistochemistry in endothelial cells, cancer cells, and some nonmalignant epithelial cells in breast cancer [57], while PAI-1 mRNA in a recent in situ hybridization study was found to be confined to endothelial cells in breast tumor stroma (C. Pyke, unpublished results).

Apart from showing a consistent expression of uPA and uPAR at invasive foci, the above-discussed studies also show that some of the components of the uPA system are expressed by the stromal cells during cancer invasion.

A similar stromal cell expression has recently been found for several metalloproteases believed to be involved in cancer invasion [58–62], and a picture is now emerging of the stromal cells often being actively involved in the invasive process (for a recent review, see [63]).

Another line of evidence supporting the assumption of a role of uPA in cancer invasion comes from studies showing inhibition of invasion or metastasis in model systems by specific inhibition of uPA activity. Such studies have been performed with in vitro systems measuring penetration of amniotic membranes [64] and penetration or degradation of extracellular matrixes [65–68]. Extensive studies on inhibition of invasion and metastasis in vivo have been performed by Ossowski and co-workers. They found that inhibition of uPA by anticatalytic antibodies inhibits invasion and metastasis of human cancer cells transplanted onto the chorioallantoic membrane of chicken embryos [69,70], and also inhibits local invasion (but not metastasis)

of human cancer xenografts in nude mice [71]. Several of these studies suggest that receptor binding of uPA is crucial for the invasion in the respective model systems [67,70], and a similar conclusion has been drawn from studies of matrix degradation by co-cultivation of genetically engineered mouse cells producing either human uPA or uPAR [72], or by co-transplantation of such cells onto chicken chorionallantoic membranes [73].

Prognostic significance of uPA and PAI-1 in breast cancer

The first study that related uPA content in breast cancer tissue to patient prognosis [5] measured uPA levels by assaying the enzyme activity present in the tumor extracts and demonstrated that high activity was significantly associated with shorter disease-free interval. Subsequent studies measuring the uPA content by ELISA showed that high levels of uPA immunoreactivity was not only associated with shorter relapse-free survival but also strongly associated with short overall survival [6–9].

Jänicke et al. [7] thus found in a study of 115 patients with a medium observation time of 12.5 months that patients with high uPA levels had significantly shorter disease-free survival, the relative risk being 21.1 (95% confidence interval 2.6–174.6). This association was significant in both node-negative and node-positive patients. Similarly, Duffy et al. [6] found a significant correlation between survival and uPA-level in 166 breast cancer patients with a medium observation time of 34 months, the relative risk being 11.3 (95% confidence interval 1.22–99.0).

These two studies were performed with detergent extracts of mammary cancer tissue. These extracts contain more uPA than those performed with detergent-free buffers, such as those used for routine preparation of cytosols for steroid hormone receptors [74]. By the use of a combination of one polyclonal antibody preparation and three monoclonal antibodies, an ELISA was constructed that readily detects uPA immunoreactivity in cytosols. Although the cytosols only contain about 12% of the optimally extractable uPA immunoreactivity, there is a close correlation between the uPA in the cytosols and the maximally extractable amount [74]. With this uPA ELISA, a retrospective study was performed on stored cytosols from 190 pre- and postmenopausal high-risk patients who were enrolled by the Danish Breast Cancer Group and had a medium observation time of 8.5 years ([8]; Grøndahl-Hansen, unpublished results). In this study, high cytosolic uPA was significantly associated with short overall survival in both pre- and postmenopausal patients, the relative risk being 2.0 (95% confidence intervals 1.1–3.7) in the premenopausal women.

It is not known whether the lesser strength of uPA level as a prognostic parameter in this latter study reflects that the uPA level in detergent extracts is a stronger prognostic parameter than the level in cytosolic extracts. The differences may also be related to variations in the groups of

patients studied, or that the two studies with detergent extracts used cut-off points between low and high uPA level selected to give the highest relative risk, while the study of uPA in cytosols used the median uPA value as a cut-off point.

Also, high PAI-1 level in mammary cancer tissue, as determined by ELISA, appears to be associated with poor prognosis [8,9]. Jänicke et al. [9] thus found in a study of 113 patients with a medium observation time of 25 months that patients with high PAI-1-level in their primary tumor had a significantly shorter relapse-free survival than patients with low PAI-1-level, the relative risk being 2.8 (95% confidence interval 0.98–8.3). In the Danish study of 190 high risk breast cancer patients ([8]; Grøndahl-Hansen, unpublished results), high PAI-1 level in cytosolic extracts was significantly correlated to short overall survival and short relapse-free survival in both pre- and postmenopausal patients, the relative risk with respect to overall survival being 2.9 (95% confidence interval 1.5–5.8) in postmenopausal women.

There is a positive correlation between uPA and PAI-1 levels in breast cancer tissue ([8,9,57]; Grøndahl-Hansen, unpublished data), while the two parameters in the various studies are not or are only weakly associated with estrogen and progesteron receptor level. In multivariate analyses including established prognostic parameters, such as number of tumor positive lymph nodes, tumor size, and estrogen and progesteron receptor levels, levels of either uPA or PAI-1 are found to be independent and statistically significant prognostic parameters in most patient groups studied [6,8,9]. Determination of uPA- and PAI-levels thus appears to add significant prognostic information to that obtained by the established parameters. It should be noted that this information, at least regarding PAI-1 levels, can be obtained from cytosols routinely prepared for steroid hormone receptor analysis.

The association between high uPA level and poor prognosis is in good agreement with the supposed function of uPA in cancer invasion. The association between high PAI-1 level and poor prognosis may be related to the above-discussed findings of PAI-1 mRNA being expressed in the tumor stroma and not in the surrounding normal tissue, suggesting a function of PAI-1 in protecting the tumor against self-degradation.

The crucial role of the uPA receptor, uPAR, in cell surface plasminogen activation (discussed above) suggests that also determination of the uPAR level in breast cancer tissue may be of prognostic value. Monoclonal antibodies useful for detection of uPAR by immunohistochemistry and Western blotting have recently been developed [30], but an ELISA sensitive enough to detect uPAR in extracts of breast cancer tissue remains to be established.

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Notes

1. This lack of uPA in the colon cancer cells in vivo is in contrast to its presence in some cultured cell lines derived from colon cancers [75,76]. It is, however, well established that with respect to expression of components of the plasminogen activation system, cultured cells are not always representative of the cells in the intact organism from which they are derived [2].

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V

Rodent Model Systems of Breast Cancer

17. Molecular mechanisms of chemical carcinogenesis in rodent models

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Animal models provide an invaluable tool for studying carcinogenesis. They are particularly useful in the field of mammary biology because of the cryptic nature of the etiology in human breast cancer tumorigenesis. Animal models can be used to cope with the complexity of the physiological disease or as an assay system for manipulations performed *in vitro*. Our discussion will be limited to studies performed in mice and rats, although other animal model systems have also contributed significantly to our understanding of mammary gland tumorigenesis.

The stages of tumorigenesis were originally defined in the skin carcinogenesis model. This model has physiological states that can be easily identified and manipulated. The three broadly defined events in tumor formation are initiation, promotion, and progression [1]. During initiation, carcinogens form adducts with DNA and cause mutations in oncogenes and/or tumor-suppressor genes that become fixed upon mitosis. During promotion, the initiated cells are stimulated to divide by mechanical, hormonal, or genetic factors and form preneoplasias and benign tumors. This stage can occur long after the cells have been initiated and can be reversed if the promotional factors are removed. Progression to a malignant phenotype with acquisition of metastatic capabilities is the final stage of tumorigenesis. This stage is believed to be brought on by the increasing genetic instability of the tumor leading to mutations at key genetic sites.

Rodents models of mammary cancer have some of the same advantages as the skin carcinogenesis models [2]. They present identifiable states of preneoplasia and neoplasia of the mammary gland. Therefore, genetic events can be analyzed in terms of when they occur during the tumorigenic process and whether their appearance is sufficient for the maintenance of the phenotype.

The mouse provides a convenient model for carcinogenesis because the morphology of the glandular structures can be easily categorized into preneoplastic and neoplastic states. The animals can be hormonally manipulated by pituitary implants to form transformed cell types in the presence of carcinogens [3,4]. Alternatively, the tissues can be treated in culture and

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returned to syngeneic animals for the analysis of the physiological effect [5]. In either case the endpoint for analysis is the same. Mouse mammary glands form areas of hyperplasia during tumorigenesis that arise from the lobular alveolar structures (hyperplastic alveolar nodules — HANs) or the ducts (ductal hyperplasia — DH) [6–8]. HANs and DHs are immortal when transplanted to syngeneic mice and have an increased rate of tumor formation [2,4]. The appearance of these structures provides good measures of the effectiveness of the carcinogen treatment.

The mouse mammary gland is not entirely ideal as an assay system for chemical carcinogenesis. The predominant etiological agent for mammary tumorigenesis in several strains of mice is the mouse mammary tumor virus (MMTV). The participation of this virus in carcinogenesis may complicate the interpretation of data, although the evidence indicates that the two modes of carcinogenesis are distinct and not synergistic [9,10]. Additionally, the mammary gland development of mice free of exogenous MMTV varies from that of the human in terms of the timing of the hormonal influences. The ductal morphology that is generally indicative of a gland in the absence of hormone stimulation continues to exist through puberty [11]. Extensive alveolar development occurs only after pregnancy, thus limiting the number of target cells available to chemical carcinogens in a virgin mouse.

The rat mammary model addresses some of the shortcomings of the mouse model. There are no viral agents commonly associated with tumorigenesis, and the development of the mammary gland resembles that of human breast [2]. The suitability of the rat as a model for human breast tumorigenesis lies in the ease with which hormone-dependent tumors can be generated by carcinogens. Due to the alveolar development of the rat mammary gland, areas of hyperplasia are more difficult to identify. However, alveolar and ductal hyperplasia also occur in these animals [2,12]. By examining preneoplastic, neoplastic, and metastatic tissues, researchers can delineate the genetic alteration occurring during tumorigenesis.

The administration of carcinogens to rodents under the appropriate hormonal conditions leads to the development of preneoplasias as well as frank tumors. In this chapter, we will examine the ways in which animal models have given us insight into the specific molecular alterations driving tumor initiation, promotion, and progression.

Initiation

The most effective carcinogens for eliciting mammary cancer are *N*-nitroso-*N*-methylurea (NMU) and polycyclic hydrocarbons, such as 7,12-dimethylbenz[a]anthracene (DMBA). The mechanism of action of NMU and DMBA adds to the usefulness of the model. NMU is a direct-acting alkylating carcinogen that is active at physiological pH for about half an hour [13] and as short as eight minutes in cell culture [14]. Therefore

any consequences of NMU action will occur very early in the tumorigenic process. DMBA is an indirect carcinogen, but mammary epithelial cells are capable of metabolizing it to the active proximal carcinogens [15]. Therefore, the presence of mammary epithelial cells alone is sufficient for the activation of both of these carcinogens. They can be used both *in vivo* and *in vitro* to isolate different stages of tumorigenesis.

The initial event in mammary carcinogenesis occurs at the genetic level. The type of carcinogen used dictates the mutation that appears in the lesion. For example, NMU mutates DNA by methylating guanine residues at the O^6 and N^7 positions. Although most of these lesions are repaired by O^6-methylguanine DNA methyltransferase, some mutations escape repair, resulting in O^6-methylguanine adducts. The cell misreads the unrepaired guanine, leading to G to A transitions [16]. G to A transitions have been established as the major mutagenic lesion caused by NMU [17,18].

The mechanism of DMBA action centers on forming adducts with both dG and dA nucleotides [19]. The mutations generated by the formation of these adducts predict the transition of dG or dA residues to alternative nucleotides. Several studies have identified DNA adduct formation in mammary epithelial cells when animals are administered DMBA [20–22], indicating that this mechanism is active in mammary cells.

When carcinogens are administered to mammary cells, specific genetic targets for tumor initiation can be identified. The *ras* oncogene activation is closely linked to mammary carcinogenesis in animal models [23] and to carcinogenesis in some non-mammary human tumors [24,25]. A single administration of NMU or DMBA to a susceptible rodent can cause a point mutation in the 12th, 13th, or 61st *ras* codon, which is sufficient for activation of the oncogene [26–29].

The *ras* oncogene codes for p21. p21 is a plasma-membrane-associated protein that binds GTP and GDP with high affinity and possesses a GTPase activity. The G to A transition in Ha-*ras* changes the amino acid from a glycine to a glutamic acid residue. Substitution of glycine by other amino acids, with the exception of proline, gives rise to an activated p21. Mutation in the *ras* gene result in two kinds of alterations: mutations that reduce the affinity of GDP and GTP and those that abolish the intrinsic GTPase activity. Loss of the intrinsic hydrolytic activity leads to loss of self-regulation by hydrolysis of GTP, resulting in a *ras* protein that is constitutively activated [30,31].

The first detectable mutation in approximately 90% of the NMU-induced rat mammary tumors is a single G to A point mutation in the 12th codon of the Ha-*ras* oncogene [28]. The G to A transition supports the idea that NMU is directly responsible for the mutation, because it is characteristic of the genetic change observed in DNA as a consequence of exposure to NMU [29,32,33]. The first detectable mutation in 70% to 80% of the DMBA-induced tumors derived from rats [29] or mouse hyperplastic outgrowth line UCD/D1 [27] is an A to T transversion in the 61st codon of Ha-*ras*. The

A to T transversions, as well as the A to G transitions that are occasionally detected, conform to the expected mutations generated by DMBA.

Ha-*ras* is not the only activating mutation detected in NMU-induced mammary tumors from rats and mice. Ki-*ras* carries a G to A mutation in the 12th codon of about 50% of rat mammary tumors in rats exposed to NMU during infancy [32,34,35] and in the 12th codon of about 17% of pituitary isografted mice [36]. No investigations have uncovered mutations in N-*ras* in carcinogen-treated mammary glands.

Point mutations in *ras* are the most common mutations attributable to carcinogen exposure of the mammary gland found to date. Several investigators have attempted to establish the role of point-mutated *ras* genes as initiators of tumorigenesis. One avenue of investigation to achieve this end is the assessment of the number of cells carrying the activating *ras* mutation in the rat mammary gland shortly after exposure to NMU. Researchers have developed a clonogenic assay for determining the number of cells initiated during tumorigenesis [37]. Estimations were made by exposing the mammary gland *in situ* to NMU or DMBA, maintaining the glands in culture and using a limiting dilution transplantation. They find that 1 in 43,000 cells are mutated three weeks after exposure to NMU and that 1 in 5 of these events consists of a point mutation in the *ras* oncogene [38]. Mutations can be detected as early as two weeks after NMU exposure as determined by PCR amplification of the mutated Ha-*ras* [35,39] and limiting dilution transplantation assay [38]. However, other researchers using mismatched PCR technology contend that there are pre-existing *ras* mutations in the normal cells and that carcinogen exposure merely selects for the cells with mutation (Zarbl, unpublished). While the direct role of NMU on Ha-*ras* is open to question, it is clear that Ha-*ras* confers a selective advantage of tumorigenesis on cells carrying the mutation. Perhaps the best evidence of *ras* involvement in the initiation of mammary tumors is that when activated *ras* genes are transfected into mammary cells and then transplanted to nude mice, the cells give rise to precancerous lesions [40]. However, this same evidence indicates that *ras* activation is not sufficient for complete tumorigenesis.

The physiological state of an animal during exposure to carcinogens can determine the type of lesions and genetic alterations that occur. Studies have been performed to explore the possibility that the increased propensity for activation of Ha-*ras* instead of N-*ras* and to a lesser extent Ki-*ras* by NMU in some physiological states is due to preferential methylation. Rats were administered NMU, killed four hours later, and assayed for methylation [41]. The methylation levels were significantly higher for Ha-*ras* when compared to Ki-*ras* and N-*ras*. This could be due to the increased expression of Ha-*ras* as compared to Ki- or N-*ras* in 50-day-old rats. These results support the theory that carcinogens are more likely to mutate active genes.

Some lines of investigation indicate that the type of fatty acid present at the time of carcinogen exposure can influence the ability of mouse HANs to undergo initiation [42]. ω-6 fatty acids are extensively implicated in

mammary tumorigenesis [43–46]. The presence of ω-6 fatty acids in the culture medium of mammary cells results in an increase in the expression of Ha-*ras* p21 [42]. However, both low-fat and high-fat diets decrease the amount of the mutagenic product, 5-hydroxymethyluracil [47], and investigations into the number of cells actually initiated in the presence or absence of excess fatty acid have not uncovered a detectable difference in Ha-*ras* [48]. These observations leave the role of fatty acids in the initiation stage of tumorigenesis open to debate.

The hormonal status of the animal at the time of carcinogen exposure has a profound effect on both the sorts of lesions generated and the genetic alterations they harbor. The stage of the rat's estrous cycle at the time of carcinogen exposure can dictate the type and the number of lesions [49–51]. Late proestrus and estrus are times when circulating progesterone (Pg) and prolactin (Prl) levels are highest. Rats administered NMU during this period develop a greater number of terminal endbuds, which are the precursors to intraductal hyperplasia and HANs [52]. Intraductal hyperplasias harbor the majority of Ha-*ras* mutations [53] and are precursors to adenocarcinomas. However, the percentage of tumors with activating Ha-*ras* mutations is lowest at 15% when the carcinogen is administered during estrus [54]. NMU administration during metestrus, when serum estrogen and Pg levels are lowest, yields the highest percentage of tumors carrying the Ha-*ras* mutation (71%). This evidence argues that increased mitogenic signals at the time of carcinogen administration lead to an increase in the complexity of the initiating events at the genetic level as well as in the number of cell types affected.

The hormonal influences of carcinogenesis in the mouse are easier to delineate because the mouse mammary epithelial cells can be initiated *in vitro*. Both NMU and DMBA can initiate mouse cells in culture and give rise to characteristic precancerous lesions depending on the milieu of the culture medium at the time of exposure [55,56]. The histology is assayed following transplantation of the initiated cells into syngeneic mice. Mouse mammary tumor epithelial cells cultured in medium containing EGF and treated with DMBA [55] give rise to predominantly DHs. These observation reflect the findings *in vivo* for mice treated with DMBA where the predominant morphological change is DHs followed by the development of squamous metaplasia tumors [57,58]. When mouse mammary epithelial cells are grown in the presence of Pg and Prl and treated with DMBA [55] or NMU [56], the primary lesions in the transplanted animals are HANs. These HANs produced tumors in subsequent transplant generations. The predominant lesions in mice given pituitary isografts, which increases circulating Prl levels, are HANs, keratinizing nodules, and squamous metaplasia tumors in both DMBA [4] and NMU [55] induction protocols.

Examination of the lesions induced by *in vitro* exposure to NMU revealed that only the HANs were initiated by a G to A mutation at codon 12 of Ki-*ras*. Seventy-five percent of the HAN lines generated carried the mutation,

and all of the tumors arising from these HANs carry the identical mutation [59]. Furthermore, 17% of the tumors generated *in vivo* in pituitary-isografted mice carry the same mutation [55]. The authors hypothesize that the reason for the discrepancy between the percentage of NMU-induced tumors carrying mutated Ki-*ras* in the *in vivo* and *in vitro* protocols is due to greater complexity of the hormonal background *in vivo* at the time of initiation. The relative simplicity of the cell culture system selects for the initiation of fewer types of tumors.

This gain in the molecular complexity of carcinogen-induced tumors can also be achieved by initiating a greater number of cells. Increasing the NMU dosage in Wistar–Furth rats gives increasing tumor number, however, the proportion of tumors with Ha-*ras* activation is reduced from about 80% at 20 mg NMU/kg body weight to 40% at 50 mg NMU/kg body weight [60]. Presumably this is occurring because alternate genes are being activated in an increasing number of stem cells by the additional amounts of NMU.

Animal models have demonstrated that the activation of specific oncogenes leads to transformed phenotypes. Ha-*ras* and Ki-*ras* are specifically implicated in this process in several mammary model systems. There is no doubt that activation of the *ras* genes is one of the first genetic lesions associated with the majority of carcinogen-induced mammary cancers.

Promotion

Molecular events occurring at the promotional stage of carcinogenesis are less well characterized in the mammary gland. This is due in part to the definition of promotion. The promotional stage of tumorigenesis relies on the hormonal state of the animal and the proliferative ability of the initiated cells. Therefore, factors operating at this stage of tumorigenesis are generally characterized as epigenetic, however, they must ultimately act at the genetic level to generate changes leading to malignant tumors. In fact, many of the mediators of tumor types that operate at the initiation stage are also functional at the promotional stage of tumorigenesis.

Mutational activation of *ras* genes is not sufficient for tumorigenesis. Transfection studies have demonstrated that genes such as *myc* and *p53* can cooperate with *ras* in tumor formation [61–64]. Additionally, when transfected into mammary epithelial cells, the Ha-*ras* genes mediate the formation of lobular alveolar nodules that are mortal and do not form tumors [40]. Therefore, additional changes are required for complete tumorigenesis.

The genotypic differences between rats strains have provided an insight into the additional genetic alterations required for mammary tumorigenesis. Studies confined to inbred strains of rats demonstrate that susceptibility to NMU or polycyclic hydrocarbon exposure can be differentiated by rat strain [65–67]. Through cross-breeding of the susceptible and resistant strains,

the means for resistance was identified as an autosomally dominant gene passed in Mendelian fashion by the resistant strains [65,66,68–70]. Cross-transplantation studies have localized the activity of the gene to the epithelial cells [66,68,69,71].

Studies in these rat strains have also revealed that the mutation of *ras* alone is insufficient for tumorigenesis. The Copenhagen and Fischer F344 rats, when exposed to the same NMU carcinogenesis protocol as the Buf/N or Sprague–Dawley rats, do not develop mammary tumors, although the fraction of cells containing the Ha-*ras* mutation increases by 10- to 100-fold [72]. This resistance to transformation is believed to be due to an inability of the cells carrying the mutated Ha-*ras* gene to undergo clonal expansion resulting from the inheritance of an autosomally dominant gene called mammary carcinoma suppressor gene (*mcs*). The tumorigenicity of v-Ha-*ras* when it was transfected into mammary cells of Copenhagen rats can overcome the suppressor effect of *mcs*. However, the tumors that appeared were less aggressive tumors than those arising in Sprague–Dawley rats under the same protocol [73].

Our studies examining the role of hormones in the carcinogenesis of the mammary gland have demonstrated that initiated cells require the presence of estrogen for tumor formation. Ovariectomy prior to or following NMU administration lowers tumor incidence to negligible levels. Subsequent replacement with estrogen returns the tumor incidence to former levels with 50% of the tumors harboring activated *ras* genes [34]. These results illustrate the requirement of estrogen-induced differentiation of mammary epithelial cells to trigger neoplastic development. Moreover, it emphasizes the point that the initiation events involving Ha-*ras* oncogene activation are not able to exert their tumorigenic properties until the harboring cells became engaged in hormone-mediated differentiation.

There are at least two ways in which estrogen could be performing its role in promotion. The generally accepted function of estrogen is to expand the population of activated cells. Additionally, some studies have shown that the metabolites of estrogen can cause genotoxic damage to DMBA-treated mammary epithelial cells [74], thereby causing further mutations in the susceptible cells.

Hormones other than estrogen play a role in tumor progression. When tumor cells that have been initiated in culture or in syngeneic animals are grown in animals carrying pituitary implants, the number of tumors and the molecular characteristics are distinct from the tumors grown in intact animals [36,60]. When rats are given pituitary implants, the tumor number per animal increases, but the percentage of tumors with Ha-*ras* activation decreases from 30% to 15%. These data indicate that overproduction of prolactin can select for cells that were not necessarily initiated by *ras* activation. Jahn et al. [75] have detected elevated levels of prolactin receptor and insulin-like growth factor (IGF-1) receptor in DMBA-induced tumors in Sprague–Dawley rats. They hypothesize that the elevated levels of these

receptor help to increase, directly or indirectly, cellular proliferation in the later stages of tumorigenesis [75].

Identification of the nonestrogen-related hormones and growth factors involved in promotion of *ras*-oncogene-harboring cells will provide additional clues into the molecular pathways by which promotion of mammary tumorigenesis occurs. Although the *in vivo* mammary tumor model system can be used for this purpose, it is difficult to exclude the effects of endogenous hormone interactions. To determine the nature of the factors regulating the histological types of lesions, the cellular origin of the lesions, and the physiological influences on them, we are exploring the utility of a combination of *in vitro* growth of carcinogen-exposed mammary epithelial cells under defined culture conditions followed by transplantation into female nude mice or isogenic rats. We are testing the effects on tumorigenicity of a variety of growth factors, such as epidermal growth factor, tumor growth factor-α, tumor growth factor-β, activin, and/or inhibin.

Rats and mice provide an *in vivo* system for assaying the effect of dietary factors on chemical carcinogenesis. One of the more extensive areas of study involves dietary fats. It has been shown that the composition of dietary fat can influence the promotional phase of chemical carcinogenesis. Fats are effective in increasing tumor incidence in terms of susceptibility to DMBA or NMU in rats [46,76–78] as well as spontaneous generation of mouse tumors [79]. Fats containing linoleate, an ω-6 fatty acid, are particularly effective at eliciting tumor promotion [43,45], growth [80], and metastasis [81]. However, fatty acids from the ω-3 family, such as eicosapentaenoic acid (EPA), tend to inhibit mammary tumorigenesis [43,80,82].

Attempts have been made to measure the effect of the fatty acids on the expression of known oncogenes. Telang and co-workers [42] found a three–fold increase in the expression of Ha-*ras* when murine HANs are cultured in the presence of linoleate. However, when the HANs are cultured in the presence of EPA, there is a three–fold decrease in Ha-*ras* expression. When MCF-7 cells are transfected with v-Ha-*ras*, the HER-2/*neu* expression increases in the presence of EPA [83]. Studies such as these indicate that there is a connection between fatty acid content and oncogene expression.

Fiber content [84], selenium [85], and ethanol [86] are a few of the other dietary factors that are postulated to influence chemical carcinogenesis in animal models. A direct link between these factors and genetic alterations has not been established, although there are some indications that caloric intake may modulate Ha-*ras* and c-*fos* expression [87].

In summary, animal models are necessary in order to define the factors involved in tumor promotion. They provide a physiological endpoint for analysis. However, these factors have a relatively small effect at the molecular level, except for the induction of genes required for cellular expansion and some modulation of Ha-*ras* gene expression.

Progression

The study of progression in the rodent mammary model relies heavily on detecting the accumulation of changes in the genetic profile of the tumors. Animal models are particularly suited to progression analysis because alterations in the same tumor can be simultaneously followed and analyzed [88–90]. The endpoints for analysis of tumor progression are the estrogen responsiveness of the tumors and degree of metastatic potential.

Human tumors lacking estrogen or progesterone receptors are classified as more aggressive and are indicative of the metastatic potential [91–93]. Estrogen nonresponsiveness also provides a useful parameter in the analysis of tumor progression in rodent models. The presence of a single mutant Ha-*ras* oncogene in the presence of its wild-type counterpart is insufficient for yielding the hormone-independent phenotype [94]. This indicates that further mutations are required before tumors attain hormone independence.

Two mechanisms for cancer progression are changes in gene dosage and additional mutations in specific alternate genes. Current information indicates that both mechanisms operate in rodent tumor promotion and progression. The notion that genetic alterations are responsible for tumor progression is supported by the observation that the metastatic phenotype of rat mammary epithelial cells can be induced by transfecting nonmetastatic cell lines with DNA from metastatic cell lines [95]. The specific genes responsible for increasing the metastatic capabilities of the cell lines were not identified in that neither EJ-*ras*-1 nor Polyoma Large T Antigen was able to compensate for the entire genomic DNA. The question that remains to be answered is which, if any, of the oncogenes or tumor-suppressor genes known to date are necessary and/or sufficient for tumor progression. Research in rats and mice indicate that this question is put much too simply. It may be that different genes are fulfilling the same function so that there is no single critical gene, but an array of putative targets.

An extension of the carcinogen-induced animal model can be used to gain an understanding of the molecular mechanisms of tumor progression. Tumor progression can be monitored in this system by serially transplanting tumors in syngeneic animals [88,89]. Each generation of tumor can be assayed through histological as well as genetic parameters.

Allelic deletion of Ha-*ras* with the subsequent duplication of the mutant allele has been recognized as an mode of activation in other tumor models such as the skin [19,96]. The allelic deletion of Ha-*ras* may be due to a linkage with a nearby tumor-suppressor gene [88]. In fact, Aldaz et al. [88] have performed cytogenetic studies on several hormone-dependent and hormone-independent tumor lines and found that there is a bias in duplication of rat chromosome 1, which harbors the Ha-*ras* gene, as tumors progress. A specific amplification of the mutant Ha-*ras* alleles is observed with increasing tumor aggressiveness in two separate rat progression models (Aldaz, submitted for publication; our unpublished observations). The

amplification of mutant *ras* during tumor progression is often accompanied by loss of the wild-type Ha-*ras* allele. These observations suggest that the mutated Ha-*ras* is functioning in tumor progression.

The acquisition of the metastatic phenotype is at least partially dependent on the mutation or deletion of known oncogenes and tumor-suppressor genes. The role of Ha-*ras* in this process has been explored by several investigators. Transfection of Ha-*ras* to human MCF-7 cells does not lead to a more metastatic phenotype than the control transfected cell lines in nude mice [97]. However, there is some room for interpretation of these results, since both the Ha-*ras* and control transfected cell lines display increased metastatic potential in response to estrogen.

It is possible that Ha-*ras* can play a role in the early conversion of tumors to metastatic phenotype. Ha-*ras* transfection into the nonmetastatic mouse mammary adenocarcinoma cell line, SP1, yielded metastatic tumors when transplanted into animals [98]. However, when these tumors were harvested and assayed for the presence of Ha-*ras*, some tumors with metastatic phenotypes no longer carried the gene. Both Ha-*ras* transfected clones and revertants were metastatic when reintroduced to the animals. This suggests the role of Ha-*ras* may be to mediate the activation or deactivation of another relevant gene or set of genes that in turn plays a direct role in the metastatic phenotype.

Transfection of nonmetastatic DMBA-induced rat mammary cells with v-Ha-*ras* confers increased metastatic ability to its transfectants [99–101]. This phenotype is associated with an increase in Ha-*ras* expression [101] and in genetic instability [100]. When cells with the metastatic phenotype are fused with the parental nonmetastasizing cells, the metastatic phenotype is lost [99], although the Ha-*ras* expression is elevated. The fusion studies indicate that the elevated level of Ha-*ras* expression is not sufficient for conferring the metastatic phenotype and suggest that the critical event may involve the loss of tumor-suppressor genes.

The status of Ha-*ras* in advanced tumors is by no means the only measure of genetic alteration. The relevance and function of several putative markers for progression in breast cancer can be established through animal models. Some examples of these genes are *nm*23, which has down-regulated expression in metastatic but not nonmetastatic NMU-induced rat tumors [102]; *Prad*-1, which is overexpressed in 20% of breast tumors [103,104]; *p53*, a tumor-suppressor gene that is altered in 40% of breast cancers [105]; and HER2/*neu*, which is amplified in up to 40% of human tumors [105].

Prad-1 and *p53* are two genes that may have prognostic value in human disease. We have screened 18 lines of invasive and hormone-dependent tumors and have found only one point mutation in *p53*. This mutation was a G to A transition that occurred in the second passage of the tumor line and was maintained in subsequent passages. This shows that while *p53* mutations occur during the progression of NMU-induced rat mammary tumors, they are not essential to the progression of these tumors. However, we have

detected *Prad*-1 amplification in later passages of hormone-independent and metastatic tumors by differential PCR analysis, indicating that the role of some of the putative markers for advanced disease in humans can be elucidated through the use of animal models. We are pursuing investigations to confirm these findings.

HER-2/*neu* amplification appears to be associated with advanced breast disease in humans [105–108] and may be indicative of a poorer prognosis [105,109–111]. Using Southern blot analysis of the transplanted NMU-induced mammary tumors, we have detected rearrangement of the *neu* oncogene in 2 of 10 locally invasive tumors, 5 of 10 hormone-independent tumors, and 6 of 6 metastases. This rearrangement is absent in all of the normal mammary tissue and primary tumors tested (our unpublished observations). Furthermore, we find that the mutated *neu* in these tumors is accompanied by complete loss of the wild-type alleles. We are in the process of determining the precise nature of the alteration in *neu*. We have narrowed the location of mutational site to the vicinity of the transmembrane domain. There is precedent that mutations in this area are capable of activating *neu*. Overexpression of the *neu* protein, p185, without gene amplification has been reported in a human breast tumor [112]. This overexpression has been hypothesized to be the result of a T to A transition in the transmembrane region of the HER-2/*neu* at codon 661, replacing the amino acid isoleucine with asparagine. Rat *neu* genes isolated from *N*-ethyl-*N*-nitrosourea (ENU)-induced schwannomas harbor T to A transitions at codon 664 that results in the replacement of valine with glutamic acid in the transmembrane domain [113,114] and confers metastatic properties to *neu*-transfected 3T3 cells [115]. Whereas the hormone-independent or metastatic tumors show distinct shifts, the transition of amino acid 664 does not lead to any change in the profile of DNA fragments in Southern analysis compared to the wild-type *neu* gene. This indicates that a different mutation/rearrangement is operational in our system during the progression of mammary carcinogenesis.

Conclusion

Animal models are instrumental in studying mammary molecular carcinogenesis. They provide systems that can be manipulated to render the physiological form and structure for each phase in the tumorigenic process. In addition to identifying genes that play pivotal roles in tumorigenesis, researchers use these models to understand the mechanisms through which tumorigenesis occurs. The complexity of the genetic events involved in the tumorigenic process is becoming increasingly apparent as the roster of oncogenes and tumor-suppressor genes that participate in tumorigenesis grows. Eventually these investigations will be significantly enhanced by newly developed techniques, such as fluorescent in situ hybridization (FISH) and competitive genomic hybridization (CGH), which allow for examination

of specific alterations while scanning the entire genome. However, animal studies also show that understanding the role of epigenetic factors in tumorigenesis is just as essential to solving the puzzle of mammary tumorigenesis as is the understanding the genetic events. Epigenetic factors moderate not only the type of lesions generated by carcinogens but also the genetic changes that occur during mammary tumorigenesis.

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18. Fgf-3, an oncogene in murine breast cancer

Clive Dickson and Vera Fantl

Cancer originates from cells that have acquired a succession of genetic changes that progressively enable them to proliferate outside the constraints of normal growth control. The earliest changes are considered to impinge on very few or even single cells, providing them with a growth advantage that facilitates their clonal expansion, and leads to the production of a significant population of cells that can act as a target for secondary mutations. Thus genetic alterations that have been envisaged may result in the inappropriate induction of potent cell signaling molecules that function as growth factors and/or promote neovascularization, the loss of functions that normally inhibit cell proliferation, and the induction of enzymes that can affect tissue remodeling. The basis for this conceptual framework, and the identification of candidate gene products involved in neoplastic transformation, has arisen in large part from the study of tumors in animal systems.

Breast cancer in mice

A major model for the study of breast cancer has been the use of mouse strains that naturally develop a high incidence of breast carcinoma (see [1]). In such mice, mammary tumors are caused by a congenital infection with the mouse mammary tumor virus (MMTV). The potential for carcinogenesis by MMTV occurs during part of the normal viral life cycle, at a point when viral genomic DNA becomes integrated into the host cell chromosome (reviewed in [2,3]). Like all retroviruses, MMTV is an obligate insertional mutagen. Integration of viral DNA into the chromosome occurs effectively at random sites, and in the majority of cases has no harmful effect on the host cell. However, on rare occasions, insertion may alter the regulation of a particular gene, and in some instances may also change its structure and pervert its function (see table 1 and [4–8]). Thus, tumor cells, unlike other infected cells of the mammary gland, harbor insertions close to candidate oncogenes. This situation provides a means to identify the putative on-

R. Dickson and M. Lippman (eds.), Mammary Tumorigenesis and Malignant Progression.

Table 1. MMTV integration loci

Integration site	Gene designation	Chromosome asignment	Gene family	References number
int-1	*Wnt-1*	15	Wnt	5, 57
int-2	*Fgf-3*	7	FGF	57
int-3	*Int-3*	17	Notch	6
int-4	*Wnt-3*	11	Wnt	7
hst-1	*Fgf-4*	7	FGF	58
int-5	?	9	?	56

cogene, by virtue of its position adjacent to the newly acquired proviral element. For reasons that are still obscure, only a subset of the potential oncogenes appear to be activated by a particular virus such as MMTV. For example, the *c-myc* gene, is a frequent target for murine leukemia viruses, but there are no reports of its perturbation by MMTV. Nevertheless, when *c-myc* is used as a transgene under the control of the MMTV long terminal repeat (LTR), it can function as an oncogene in this tissue and induce hyperplastic lesions of the mammary gland [9–11].

Several previously unknown genes have been identified as common integration sites for MMTV in breast tumors (table 1), two of which, *Wnt-1* (formerly *int-1*) and *Fgf-3* (formerly *int-2*), are the most frequently involved (reviewed in [12]). Although they are members of different multigene families, the proteins encoded by these genes share some common features. Both are small secreted glycoproteins expressed principally during embryogenesis each in a distinct spatial and temporal pattern [13–18]. *Wnt-1* was later recognized as the homolog of the Drosophila gene *wingless*, which is involved in segment polarity in the developing embryo, reinforcing the idea that the gene functions in pattern formation during development [19]. *Fgf-3*, as its name implies, was found to be related to the fibroblast growth factor family [20], members of which have variously been shown to induce cellular proliferation, migration, and differentiation (reviewed in [21]). Hence, from the distribution and inferred properties of these proteins, it appears that both Wnt-1 and Fgf-3 are important signaling molecules in embryogensis, and that their aberrant expression in the mammary gland contributes to tumor induction.

Further investigation of the properties of Fgf-3 have shown that the secreted protein has a strong affinity for glycosaminoglycan-containing molecules in the extracellular matrix and on the cell surface [22]. The binding of Fgf-3 to glycosaminoglycans is a property shared with other FGFs; for Fgf-2, it is known to be an essential step for effective signal transduction by high-affinity cell surface tyrosine kinase receptors (see [23]). In general, FGFs are potent mitogens, but this property has not been reliably demonstrated for Fgf-3 [24,25], although high expression of Fgf-3 in cell cultures can cause morphological transformation [26,27]. However,

compared to *Fgf-4* and *Fgf-5*, transformation by *Fgf-3* is considerably less efficient. Interestingly, similar extracellular matrix-binding properties and a restricted transforming capacity have also been reported for the *Wnt-1* gene products [28–32]. These in vitro studies have not identified clear roles for either Fgf-3 or Wnt-1 in mammary carcinogenesis. It may be that in vitro systems cannot reflect accurately enough the conditions in the mammary gland, where cells are continually exposed to fluctuating concentrations of hormones and other factors. Thus, changes in the cellular environment may influence the ability of the mammary gland to respond to these factors. Moreover, the observation that many mouse mammary tumors show insertions at both *Wnt-1* and *Fgf-3* suggests that despite their similar properties in cell culture, these gene products may act in concert, disrupting quite different growth and/or differentiation pathways in the tumor cell [33,34]. To better understand the roles of these genes in tumorigenesis, we and others have developed transgenic mice that constitutively express the genes ectopically in the mammary gland [35–37]. For the remainder of this chapter, the study of *Fgf-3* transgenic mice will be used as an example of this approach, although reference will be made to *Wnt-1* transgenic mice for comparative purposes.

Production of Fgf-3 transgenic mice

Ectopic Fgf-3 expression in the mammary gland entails the introduction of the gene permanently into the germline under the control of regulatory elements that function efficiently in mammary tissue. For the majority of studies to date, the LTR region of MMTV that encompasses the viral promoter and enhancer elements has been used (reviewed in [38]). Several transgenic mouse lines have been obtained that by means of the MMTV LTR express *Fgf-3* in the mammary gland. These lines either harbor the acquired *Fgf-3* sequences as a cDNA [37] or as a genomic element [59] (see figure 1). Both types of transgene configuration give similar phenotypes with varying degrees of severity. In two of the transgenic lines described, a truncated viral promoter unit was used, resulting in a change in tissue-specific expression ([37]; see also below). Although the MMTV LTR is usually directly coupled to the coding region of the transgene, a novel system using this promoter for targeting *Fgf-3* expression to the mammary gland has been described by Ornitz et al. [36]. In this instance, a binary transgenic system was developed where the *Fgf-3* gene was introduced as a transgene under the control of a yeast-specific promoter element. This transgene can only be expressed in this strain when it is genetically crossed with another transgenic line expressing the yeast-activating transcription factor. With the latter under the control of the MMTV promoter, F1 animals specifically express *Fgf-3* in tissues in which the MMTV promoter is active.

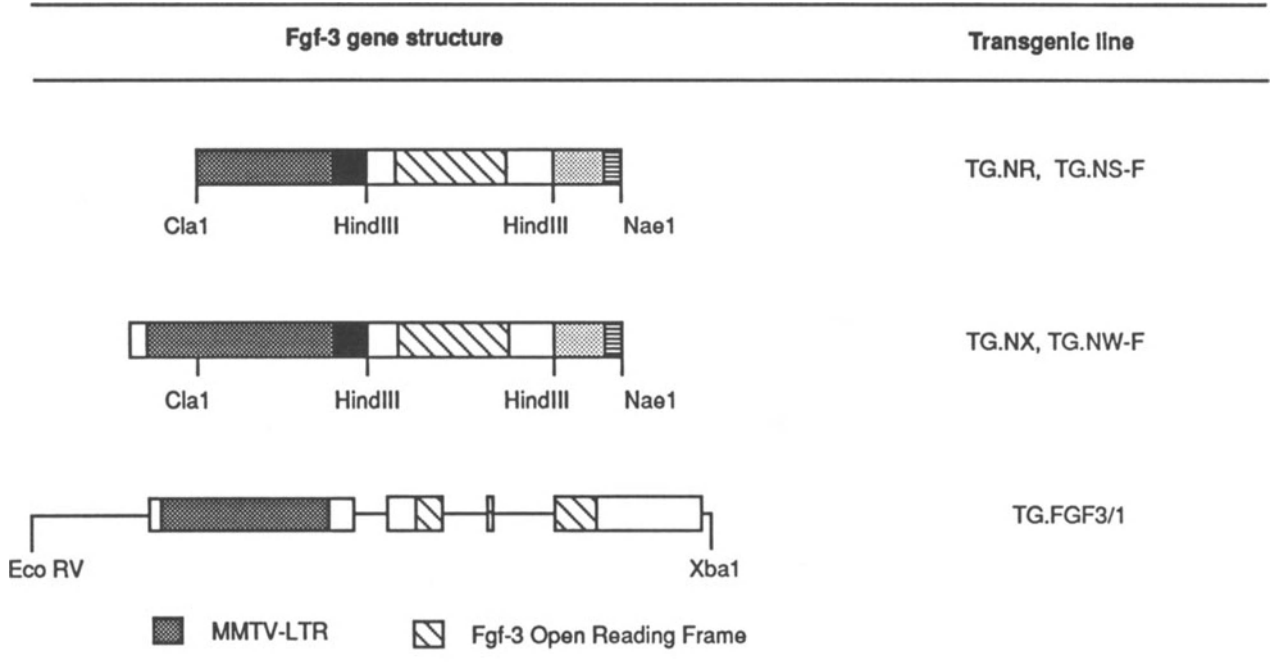

Figure 1. Structure of the MMTV-LTR/Fgf-3 hybrid genes used to create the transgenic mouse lines as indicated. The information for generating transgenic lines TG.NR, TG.NS-F, TG.NX, and TG.NW-f was taken from [37], and line TG.FGF3/1 is from unpublished data of G. Stamp, R. Poulsom, and authors.

Tissue specificity of transgene expression

Normal expression of the *Fgf-3* gene is primarily restricted to specific sites in the embryo, although very low levels can be detected in adult brain and testis [16,17,59]. The introduction into mice of further copies of *Fgf-3* as a transgene under the control of the MMTV promoter extends this transcription pattern to tissues previously reported to express the viral sequences [39] and reviewed in [40]. These include the salivary gland and the sex accessory glands of male mice, particularly the seminal vesicles, and epididymus. However, as discussed above, the major ectopic site of synthesis is the mammary gland, and during pregnancy the levels of expression are greatly enhanced. Using sensitive detection techniques such as RNase protection and the polymerase chain reaction on reverse transcribed tissue RNA, expression is often found in spleen, thymus, gut, and lung. Interestingly, the transgenic animals (TG.NR and TG.NS-F) expressing the *Fgf-3* transgene from the truncated viral promoter show a high level of expression in the prostate, with adverse effects as described below [37].

Consequences of transgene expression

In all transgenic mouse lines examined, ectopic expression of *Fgf-3* causes abnormal growth of the mammary epithelium in breeding females. The lesions range from hypertrophy with retention of well-differentiated structures to gross hyperplasia as well as frank neoplasia [36,37,59]. A detailed histological analysis of the lesions from line TG.FGF3/1 (see figure 1), showed that hyperplastic cells could to varying degrees invade the dermis, causing loss of fur due to disruption of hair follicles ([59]; see also figure 2). The degree of mammary hyperplasia was usually more apparent with successive pregnancies. However, despite the large mass of hyperplastic gland, tumors appeared infrequently, and as a late event. Histological examination of mammary lesions showed patterns of proliferation resembling ductal hyperplasias, papillocystic tumors, and solid nodular aggregates (figure 3A). In some transgenic lines, *Fgf-3* transcripts have also been detected in mammary glands of virgin mice, although palpable lesions were rarely detected [36,41,59]. However, histological examination of apparently normal-looking glands showed areas of microscopic hyperplasia (figure 3C). At a low frequency, virgin females may develop late-arising mammary tumors that appear to be indistinguishable from those arising in multiparous females [59].

Expression of the transgene has been further assessed in mammary glands of virgin and multiparous female mice using in situ hybridization to localize precisely the sites of synthesis [59]. *Fgf-3* RNA was detected in the epithelial cells of the mammary gland, but expression was primarily restricted to a subset of cells forming tubular-like structures and only present at very low

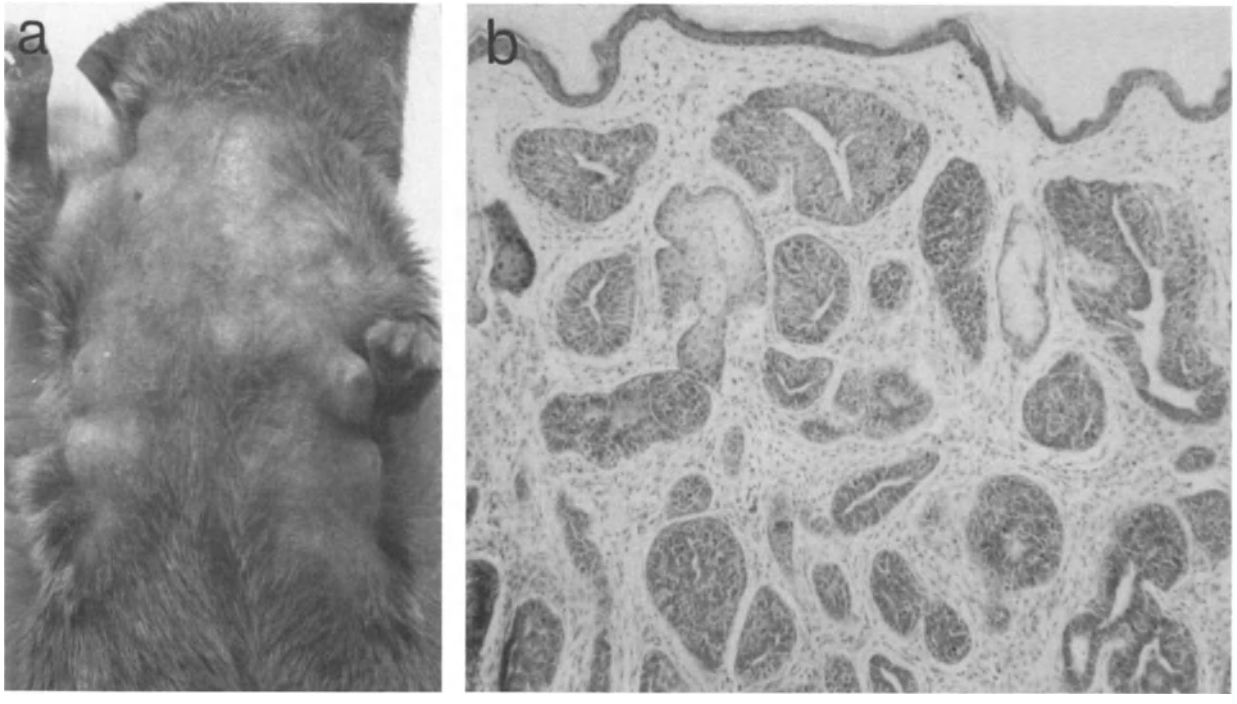

Figure 2. (A) Multiparous transgenic (TG-FGF3/1) mouse; and (B) histological section, showing solid nodular/acinar proliferation invading the dermis, resulting in the hair loss shown in (A).

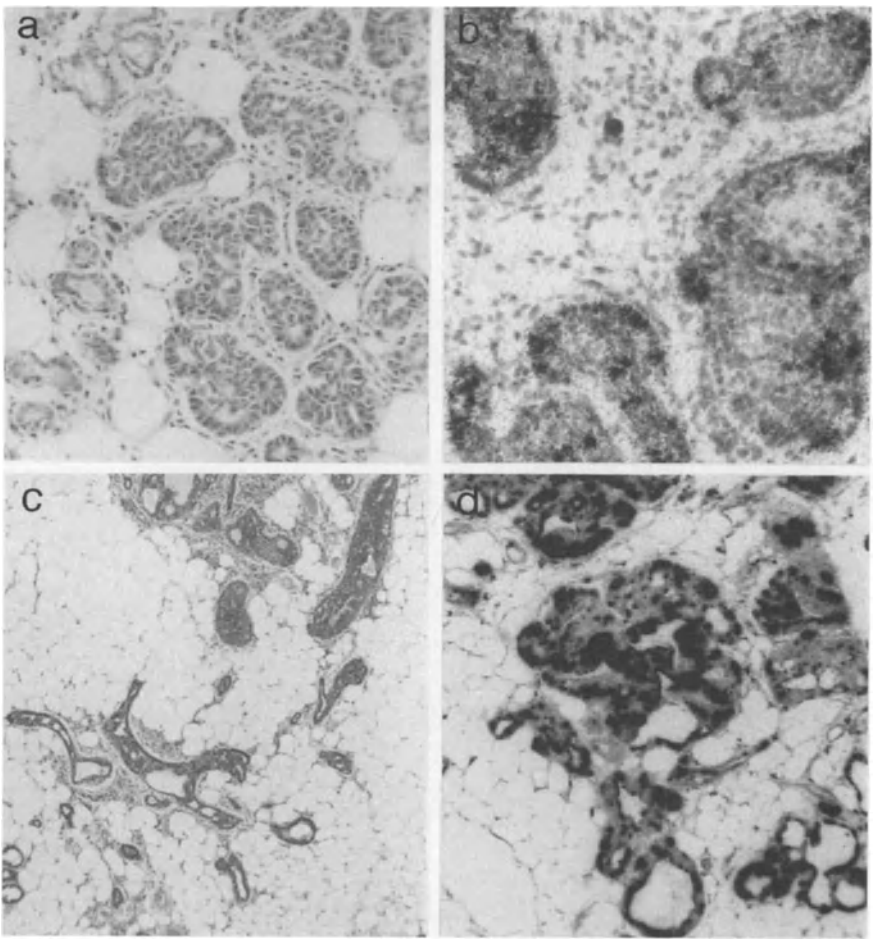

Figure 3. Histological and in situ hybridization analysis of breast tissue from pregnant and virgin mice. (A) An early stage of solid nodular/acinar pattern, where aggregates of undifferentiated cells occupy the glands, enclosing or displacing bilayered tubules. (B) In situ hybridization for Fgf3 mRNA shows labeling concentrated over the peripherally compressed tubules. Note the variability in labeling intensity. (C) A virgin transgenic mouse showing microscopic foci of cellular proliferation in small ducts and tubules. Note the mild stromal hypercellularity in these areas. (D) In situ hybridization localizes Fgf3 mRNA to the cells in the vicinity of the proliferation, but tubules elsewhere in the mammary glands were not labeled.

or undetectable levels in nodular aggregates of cells that appeared less well organized (figures 3B and 3D). Since this patchy transgene expression in mammary tissue has also been reported for MMTV TGF-α, MMTV *neu* and lines transgenic for the MMTV provirus [36,41–43], it may be a characteristic of the viral promoter rather than the transgene or its insertion site. Immuno-

histochemical staining to localize the distribution of the Fgf-3 protein has not been reported, but since Fgf-3 has the characteristic features of a secreted protein it could function as a paracrine growth factor stimulating the growth of nonexpressing cells [22]. However, such a paracrine effect may be restricted in range, since transplantation experiments where transgenic and nontransgenic mammary tissues have been mixed in the same fat pad did not show an enhanced growth of the nontransgenic component [41]. In contrast, virally induced tumors show a less intense but more uniform expression of *Fgf-3* that is not restricted to the more organized cellular structures (figure 4; [59]). This difference in expression pattern may reflect the selective pressure necessary for clonal expansion of an initial cell bearing an activated *Fgf-3* gene. Hence, a cell that continues to produce Fgf-3

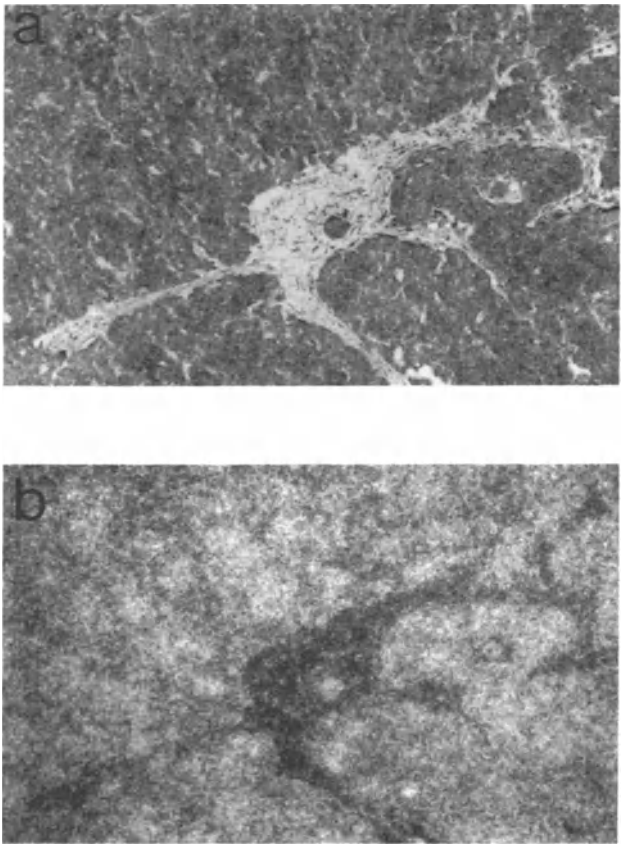

Figure 4. In situ hybridization analysis using ^{35}S-labeled antisense Fgf-3 probe on sections of virally induced mammary tumor. (A) Bright field illumination; (B) dark field illumination. The Fgf-3 sequences localize over the solid aggregates of tumor cells in a relatively uniform pattern, while the stromal elements show background levels of silver grains.

would presumably multiply by autocrine stimulation until larger cell numbers allowed a greater potential contribution from paracrine effects. To further augment the growth properties of such a clone of cells, secondary mutations would need to be established. In MMTV-infected mice, such mutations occur at a high frequency due to further insertions of viral DNA.

Male mice from both transgenic mouse lines expressing *Fgf-3* from a truncated MMTV LTR (see figure 1) have been shown to develop an extensive epithelial cell hyperplasia of the prostate [37]. However, these enlarged prostates did not appear to progress to full malignancy as judged by their lack of transplantability in nude mice. Aberrant growth of the prostate has not been observed in males from other lines where expression of the transgene was from a complete LTR, suggesting that its truncation may change the tissue specificity of the viral promoter [37]. Indeed, male mice from lines expressing *Fgf-3* from an intact promoter region rarely showed overt malignancies of any tissue. However, a few exceptions were found among aged males from line TG.FGF3/1. These mice occasionally presented with distended bladders that resulted from a blockage of the urethra. A detailed histological analysis revealed focal hyperplasia, usually with a papillary configuration, extending into the proximal urethra and causing the obstruction. In situ hybridization showed that such lesions and similar hyperplasias found in the bulbourethral glands were associated with high and much more uniform levels of transgene expression [59]. The prostate from this line, like those from TG.NX and TG.NW-F appeared normal, and *Fgf-3* expression was not detected in this tissue [37,59].

In summary, results of transgenic mouse studies show that aberrant expression of *Fgf-3* in the mammary gland and some male sex accessory glands was responsible for disruption of normal growth control, causing hypertrophy, hyperplasia, and occasionally neoplasia. In the mammary gland, sustained synthesis of Fgf-3 may prolong cell survival, since it appears to interfere with the normal involution process following weaning. Although the mammary glands can become highly enlarged with successive pregnancies, frank neoplasia occurs only at a relatively low frequency, and for the TG.FGF3/1 line the tumors appear to remain pregnancy responsive. This demonstrates that *Fgf-3* expression alone is not sufficient for tumorigenesis, consistent with the notion that tumors develop through a multistep process. These studies also highlight some important differences in tumorigenesis involving aberrant *Fgf-3* expression induced either by MMTV insertion or ectopically as a transgene. With viral infection, the cells of the mammary gland are under prolonged exposure to the insertional mutagen. Therefore, additional mutagenic events may occur at reasonably high frequency. This idea is supported from the finding that two and occasionally three proto-oncogenes are activated in the same tumor cell by proviral integration. For example, in some strains of mice, *Wnt-1* and *Fgf-3* are coactivated in 60%–70% of tumors, suggesting that these genes act in concert to augment tumorigenesis [33,44]. This idea has been tested directly by

crossing *Wnt-1* and *Fgf-3* transgenic mice to create bitransgenic animals expressing both genes in the mammary gland [45]. Previous experiments had shown that both male and female *Wnt-1* transgenic mice develop mammary and salivary gland hyperplasia and that tumors arise stochastically in the mammary gland [35]. The *Wnt-1/Fgf-3* bitransgenic mice showed a much greater propensity to develop mammary tumors, particularly the male mice, demonstrating a co-operative effect of these oncogenes. However, since mammary tumors still occurred stochastically even in these mice, other genetic changes are apparently necessary to induce frank neoplasia.

Concluding remarks

The ability to introduce new DNA into the germline of mice has provided a means to test the potential role of suspected oncogenes directly in a tissue context. The availability of the MMTV promoter, which has been shown to function efficiently in the mammary gland, has led to many suspected oncogenes, identified in a variety of systems, to be tested in this tissue; for example, *c-myc*, SV40 T-antigen, *H-ras*, *neu*, *TGF-α*, *N-ras*, as well as *Wnt-1* and *Fgf-3* [9–11,35–37,45–55]. The transgenic mouse studies concerning the *Fgf-3* and *Wnt-1* genes are of particular relevance because these genes are implicated in mammary tumorigenesis as targets of MMTV proviral insertion. The role of these genes in mammary tumorigenesis and their cooperativity has been strongly supported by these studies, and shows that transgenic animal systems can be especially valuable to study cooperativity between candidate oncogenes. Furthermore, the application of homologous recombination techniques to inactivate selected genes (tumor-suppressor genes) in embryonal stem cells has led to the generation of transgenic mice with a specific gene 'knocked out.' Thus, it will be possible through genetic crossing experiments to investigate the effects of oncogenes on tumor development in the absence of specific suppressor genes. Such studies should provide further insights into the multiple genetic changes that conspire to pervert the growth and differentiation of normal cells in vivo, as well as generating additional animal models to test new forms of cancer therapy.

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Index