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Plenary Lectures

REGULATION OF PROLIFERATION, INVASION AND GROWTH FACTOR SYNTHESIS IN BREAST CANCER BY STEROIDS

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Summary—Endogenous ovarian estrogens and progestins appear to play a critical role in the development and progression of breast cancer. Local productions of growth factors probably also contribute to malignant proliferation, while production and activation of collagenolytic enzymes may be equally critical for local invasive processes. The current review focusses on characterization of growth factor–receptor systems operant in normal and malignant breast epithelium. In addition, the determinants of local invasion are reviewed: attachment, modality, and proteose secretion. Finally, data are discussed concerning the regulation of both proliferation and invasion by hormones and antihormonal agents in hormone-dependent breast cancer. The results suggest new potential pharmacologic targets to explore to suppress onset and progression of breast cancer.

INTRODUCTION: HORMONE-RESPONSIVE BREAST CANCER MODELS

Exposure to the hormonal estrogenic steroids appears to be a critical in the carcinogenesis phase of human breast cancer, and in mitogenesis of the hormone-dependent form of the disease. The initial hormonal requirements are often bypassed with progression of breast cancer to a more advanced disease. Advanced disease also involves basement membrane invasion and distant metastases of the tumor. For experimental studies, established human breast cancer cell lines, generally derived from pleural effusions [1], can be used to study both the estrogen-dependent and -independent forms of the disease. The estrogen receptor positive MCF-7 cell line [2] has been widely studied (along with a few other such lines: T47D, ZR75-1) and provides a useful system to evaluate the molecular basis for action of estrogen and other hormones in breast cancer. This cell line exhibits estrogen sensitivity for progesterone receptor expression [3], proliferation *in vitro* [4–6], basement membrane invasion *in vitro* [7, 8], and expression of a host of specific mRNA species and proteins [9–16]. Of particular note, MCF-7 tumor formation in the nude mouse is dependent upon administration of estrogen [17]. Recent progress indicates that a number of polypeptide growth factors regulate breast cancer cell growth *in vitro*, and may contribute to the proliferative effects of estrogen on MCF-7 cells through autocrine and paracrine actions. In this review, we summarize our current understanding of the regulation of breast cancer cell proliferation and invasiveness by estrogenic hormones, antiestrogenic antagonists and growth factors.

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ANTIESTROGENS *IN VITRO* AND *IN VIVO*

Antiestrogens have been extensively and successfully used in the clinical treatment of breast cancer; and they are also useful agents for *in vitro* study of breast cancer cell lines. The substituted triphenylethylene tamoxifen, and its active 4-hydroxylated metabolite (OHT), display both estrogen agonism and antagonism in various systems [18–32]. They have relative binding affinities (RBAs) for rat uterine estrogen receptor *in vitro* of approximately 2 and 200% of 17β-estradiol, but exhibit nearly equal activity *in vivo* with a minimum effective dose of 0.05–0.1 mg/kg [33]. Partial estrogen agonistic activity of these triphenylethylene compounds

is both species- and tissue-specific. They show pure estrogen antagonism in the chicken oviduct and mouse uterus, but only partial estrogen agonism in the rat uterus, rat liver and some areas of the rat brain [23]. They also stimulate expression of progesterone receptor [26–29], morphological changes [22], and elevation of some estrogen-dependent mRNA species [30–32] in cultured human breast cancer cells. Although long-term treatment of breast cancer cell lines *in vivo* in the nude mouse can lead to tumors that are resistant to [34], and even stimulated by [35] tamoxifen, tamoxifen is generally tumoristatic for MCF-7 cell tumors *in vivo* [36, 37]. Tamoxifen can also suppress the onset of carcinogen-induced rat mammary tumors [38]. Tamoxifen is currently used in the clinical management of advanced human breast cancer where it significantly prolongs disease-free survival, particularly in post-menopausal ER+ patients [39–41].

A second structural class of antiestrogens, the benzothiophenes LY 117018 and LY 156758 (RBAs of 130 and 290% of 17β-estradiol at 30°C respectively [42, 43], exhibit higher antagonism and less agonism in the rat uterotrophic assay [25, 42–44], and less agonism of estrogen effects on MCF-7 cells *in vitro* [8, 18, 45–47]. These antiestrogens, however, are less active than tamoxifen when tested against estrogen-depenent, carcinogen-induced, rat mammary carcinoma [46–49]. This is possibly due to poor pharmacokinetics [38]. These drawbacks, as well as reports of ocular toxicity, have precluded this antiestrogen series from clinical application.

A new steroidal antiestrogen, *N*-butyl-*N*-methyl (10-triene-7-yl) undecanimide (ICI 164,384) appears to be a purely antagonistic agent in a number of experimental model systems. This antiestrogen exhibits no estrogenic effect on uterine growth in immature rats and mice, and it reverses estrogen-induced proliferation and basement membrane invasiveness of MCF-7 cells *in vitro* [50–53]. In contrast to estrogen, it does not appear to induce the DNA-binding conformation of the bound, isolated estrogen receptor [54]. Since partial estrogen agonism may restrict the effectiveness of antiestrogen therapy, the possibility exists that ICI 164,384, or related antiestrogens, may offer clinical advantage over the more agonistic non-steroidal antiestrogens like tamoxifen.

REGULATION OF EXPRESSION OF THE ESTROGEN RECEPTOR

The stimulatory effects of estrogen on MCF-7 cell proliferation are mediated by the estrogen receptor (ER [4, 5]). The intracellular concentration of the estrogen receptor is modulated by cell culture density, growth rate and ligand milieu [55–61]. The mechanism of ER regulation has been examined in response to estrogen and antiestrogen at the levels of ER gene transcription, ER–mRNA stability, ER protein concentration and ligand binding levels. In response to 17β-estradiol (10^{-9} M), ER protein, as measured by immunoassay and estrogen receptor binding assay, decreased by 60% in 6 h, and remained suppressed for 24–48 h. RNase protection experiments showed that steady-state levels of ER–mRNA decreased maximally by 6 h after estrogen treatment, and remained suppressed for 48 h. Transcriptional "run-on" experiments with isolated nuclei demonstrated a transient 90% decrease in ER transcription within 1 h of estrogen treatment, this increased to a level approximately 2-fold higher than controls after 3–6 h, and remained elevated for 48 h. These data suggest that estrogen down-regulates estrogen receptor mRNA by inhibition of estrogen receptor gene transcription at early times, and by a post-transcriptional suppression of receptor mRNA at later times. The level of ER–mRNA in the cellular nucleus declined in a manner similar to that of total ER–RNA, suggesting that post-transcriptional suppression of ER–mRNA is a nuclear event.

ER regulation in MCF-7 cells has been examined in response to treatment with OHT and ICI 164,384 [62]. Treatment with 10^{-7} M OHT had no apparent effect on the steady-state level of ER–mRNA, on ER transcription in transcription "run-on" experiments, nor on the concentration of ER protein as measured by radioreceptor assay. In addition, OHT inhibited estrogen regulation of the receptor at the transcriptional and post-transcriptional levels. While ICI 164,384 also caused no change in steady-state ER–mRNA levels, it induced a 60% decrease in the receptor protein level, suggesting post-translational regulation of ER expression. Taken collectively, these data suggest that ER regulation in MCF-7 cells is a complex process involving multiple levels of regulation by estrogen and antiestrogens: transcriptional, post-transcriptional, translational and post-translational.

HORMONAL REGULATION OF MCF-7 CELL PROLIFERATION

Considerable controversy existed in the 1970s concerning the ability to elicit estrogenic and antiestrogenic effects in hormone-dependent breast cancer cell lines *in vitro*. Estrogenic and antiestrogenic regulation of MCF-7 cell proliferation *in vitro* has recently been enlightened by the finding that Phenol red, the pH indicator dye commonly used in tissue culture media, contains variable quantities of a contaminating, weak estrogen [63, 64]. Growth regulation studies in the absence of Phenol red [18, 53, 65] have demonstrated that, effects of antiestrogens on MCF-7 cell proliferation are considerably blunted in the absence of estrogen, consistent with the hypothesis of estrogen receptor mediation of antiestrogen effects. We have recently shown that both tamoxifen, and the less agonistic LY 117018, stimulate MCF-7 cell proliferation in estrogen-free culture, but that the steroidal pure antiestrogen ICI 164,384 does not [53]. All three antiestrogens, however, are clearly antagonistic in the presence of exogenous estrogen, and are effective against MCF-7 cell tumors in the nude mouse [46, 50].

Estrogen induces a range of enzymes and other proteins involved in nucleic acid synthesis. This is consistent with its effects on MCF-7 cell proliferation. These endpoints include DNA polymerase, *c-myc* protooncogene, thymidine and uridine kinases, thymidylate synthetase, carbamyl phosphate synthetase, aspartate transcarbamylase, dihydroorotase, glucose 6-phosphate dehydrogenase, and dihydrofolate reductase [66–70]. Though increases in global transcription appear to be tightly coupled to estrogen action [71], no study has yet identified the most critically regulated gene(s). The existence of "second message" regulatory systems in this hormonally-regulated proliferation process is also possible but has not yet been proven. Estrogen stimulated MCF-7 cell phosphatidyl inositol turnover occurs after a relatively long lag time of several hours [72]. In contrast, other growth regulators, such as the polypeptide growth factors [73, 74], induce this metabolic effect within minutes. Phosphatidyl inositol turnover could mediate the mitogenic effects of estrogen-induced growth factors. There is evidence that polypeptide growth factors, particularly the transforming growth factors (TGFs) and somatomedins (IGF-I and -II), may be involved in breast cancer cell proliferation via paracrine and autocrine loops, and may contribute, as local mediators, to the mitogenic effects of estrogen on these cells.

TGFa is mitogenic for normal and malignant breast epithelial cells. A TGFa-like activity was initially detected in conditioned media for MCF-7 cells, other breast cancer cell lines, and breast cancer extracts using the colony formaton bioassay with NRK and AKR-2B cells [75–79]. In contrast to the 6 kDa TGFa molecule isolated, cloned and sequenced from transformed fibroblasts, breast cancer cell TGFa has an apparent M_r of 30 kDa. It may be related to the predicted 17–19 kDa TGFa precursor which has been observed in some cell lines [80–82], and further modified by glycosylation or palmitoylation [80], or it may be the product of a novel gene. Estrogen stimulates the production of the 30 kDa TGF-like growth factor some 2–14-fold in the MCF-7 cell line, and other estrogen receptor-positive cell lines T47D and ZR-75-1, depending on culture conditions and cell type [75, 76, 83–85]. TGFa–mRNA induced by estradiol treatment of MCF-7 cells within 6 h, and antibodies directed against TGFa or its receptor (the EGF receptor) slightly and transiently suppress both the anchorage-dependent and -independent growth of these cells [79, 86]. A much stronger effect of anti-EGF receptor antibodies was observed with anchorage-dependent and independent proliferation of the estrogen-independent, EGF receptor amplified MDA-MB-468 breast cancer cell line [87]. It is not yet clear what is the range of cell lines affected *in vitro* by anti-EGF receptor directed reagents, or whether these observations can be extended to *in vivo*. It is probable that the magnitude of response to the EGF receptor-directed antibodies relates to many factors, such as TGFa, EGF receptor levels, and more subtle aspects of malignant transformation.

No correlation has been reported between TGFa expression and ER status in a study of breast tumor tissue. Moreover, TGFa–mRNA was detected in 70% of breast adenocarcinomas. Treatment of breast cancer patients with antiestrogens significantly decreases immunoreactive TGFa [88]. With MCF-7 cells grown as tumors in the nude mouse, estrogen withdrawal decreased TGFa–mRNA levels [29]. TGFa apparently acts through the EGF receptor, overexpression of which is closely associated with poor prognosis breast cancer, and with tumor growth rate in experimental systems *in vivo* [89]. Since TGFa has also been

implicated the proliferation of normal mammary epithelial cells in culture [90], and in normal murine mammary development [91], the effects of TGFa on normal and malignant breast may be distinct at the level of response. For example, in nude mice treated with estrogen, EGF, or breast cancer cell conditioned medium, implanted MCF-7 cells formed small tumors, whereas no effects were seen on the normal mouse breast tissue *in situ* under these conditions [92]. TGFa gene transfection experiments suggests that constitutive expression of physiological levels of TGFa is not sufficient to support hormone-independent tumorigenesis of MCF-7 cells [93]. Indeed, 17β-estradiol and TGFa induce quite distinct profiles of intracellular peptides in MCF-7 cells, as detected by 2-D gel electrophoresis [94]. Other induced products may act in association with estrogen-induced TGFa to constitute autocrine growth regulation. These observations suggest that the TGFa-EGF receptor system may be relevant to a large web of factors modulating breast cancer proliferation.

All human breast cancer cell lines so far examined secrete an IGF-related polypeptide, and IGF-I and IGF-II are mitogenic for some breast cancer cells in culture [95, 96]. Although a complex series of IGF-I cross-reacting mRNAs can be detected in MCF-7 cells by Northern analysis [96], none of these has been identifiable as authentic IGF-I using nuclease protection analysis [97]. Production of an immunoreactive IGF-related protein by MCF-7 cells is induced 3–6-fold by treatment with estrogen, TGFa, EGF or insulin treatment in Phenol red-free culture, and inhibited by treatment with TGF-β, glucocorticoids or growth-inhibitory antiestrogens in the presence of Phenol red in the medium [98]. Recent studies have shown that an antibody which blocks the IGF-I receptor suppresses the clonal proliferation of the estrogen-independent MDA-MB-231 cell line *in vitro*, and its tumor formation *in vivo* [99, 100]. Since IGF-I-related polypeptides can mediate autocrine growth of fibroblasts and smooth muscle cells [101–103], those produced by malignant breast epithelium may also act in a paracrine fashion to mediate stromal cell chemotaxis and proliferation. IGF-II, another somatomedin family member, is also produced by the T47D human breast cancer cell line under estrogenic regulation, and is mitogenic for this line as well as the MCF-7 cell line [104]. IGF-II–mRNA is present in equivalent amounts in paired samples of normal and malignant breast, suggesting that it is also a product of stromal elements [104]. Thus, IGF-II may also function in the autocrine and paracrine regulation of breast cancer. IGF-I acts largely through the IGF-I receptor, but also binds weakly to the insulin receptor, while IGF-II binds to all three related receptors: insulin, IGF-I and IGF-II. All three of these receptors have been detected in human breast cancer [95, 105], as well as lactating bovine breast [106]. Elements with blocking anti-IGF-I-receptor antibodies strongly suggest that the mitogenic effects of IGF-II in many systems are mediated via the IGF-I receptor.

TGF-β inhibits proliferation and induces differentiation responses in a variety of cell types, including normal and malignant breast epithelium (see [107] for review). It is present in human milk [108], it causes cessation of mammary duct development when implanted in the mouse mammary gland [109], and it causes decreased proliferation and increased differentiation (milk fat globule antigen) of normal mammary epithelial cells in culture [110]. Many breast cancer cell lines produce a TGF-β-related activity [75, 107, 111, 112], and both estrogen receptor-positive and -negative cell lines contain TGF-β receptors, and are inhibited by both TGFβ-1 and the closely related TGF-β-2 [107, 113]. TGF-β secretion by MCF-7 cells is inhibited by mitogenic agents like estrogen and insulin, but stimulated by antiproliferative agents like antiestrogens and glucocorticoids [112]. The role of TGF-β in breast cancer progression to the estrogen-indendent state is unknown. Conditioned media from antiestrogen-treated MCF-7 cells strongly inhibits the proliferation of the hormone-independent MDA-MB-231 cell line *in vitro*, and this inhibition is blocked with anti-TGF-β antisera [113]. Since breast cancer is heterogeneous for estrogen receptor expression, antiestrogen stimulated TGF-β secretion from hormone-responsive elements may modulate the proliferation of adjacent, unresponsive elements, thus expanding the effective growth regulatory potential of the antiestrogens.

Many breast cancer cell lines also synthesize PDGF (platelet-derived growth factor) [114–116], a potent mitogen for fibroblasts, smooth muscle cells, and other stromal cells [117]. Since breast cancer cell lines, and epithelial cells in general, lack PDGF receptor, PDGF may play a paracrine role in breast cancer development, possibly at the level of

desmoplasia. Other studies have demonstrated that cultured human breast cancer cells produce a 60 kDa, heparin-binding, FGF-like protein which may also contribute to paracrine and autocrine growth regulation in the tumor environment [118, 119]. Furthermore, the 52 kDa cathespin D-related protease secreted from MCF-7 cells under estrogenic control appears to have mitogenic activity for these cells, and may also participate in an autocrine loop [120], or alternatively may contribute to the activation of other biologically important, secreted polypeptides.

Thus, these data clearly suggest a role for polypeptide growth factors in the control of breast cancer growth, both through autocrine and paracrine mechanims. At least *in vitro*, some of these growth factors are estrogen-regulated in hormone-responsive cell lines, and antiestrogen-inhibited proliferation of the estrogen-dependent T47D cell line *in vitro* can be partially reversed by EGF [121]. Recent reports that antiestrogens can inhibit the mitogenic effects of insulin, EGF, TGFa and IGF-I in the total absence of estrogen suggest a further level of interaction between these factors and estrogen receptor-mediated responses [65, 122]. While these factors appear to be important for the proliferation of estrogen-dependent and -independent breast cancer cell lines *in vitro*, their relationship to estrogenic regulation of malignant breast epithelium *in vivo* is less clear.

TUMOR INVASION OF THE BASEMENT MEMBRANE AND METASTASES

As part of the metastatic process, tumor cells must escape the primary lesion, escape immune surveillance in the circulation, and penetrate and proliferate in normal tissue at distant sites [123, 124]. These are not random events but require distinct cellular and biochemical activities possessed by a limited population of cancer cells which arise during malignant progression [123]. The interaction of tumor cells with basement membranes is a critical step in this process, since the cells usually encounter and pass at least two basement membranes as they disseminate through the body [125]. Several critical determinants in basement membrane invasion have been identified. Tumor cells first attach to the basement membrane glycoprotein laminin. This appears to regulate the production of a protease cascade resulting in activated collagenase IV [126]. This enzyme degrades the type IV collagen structural network of the basement membrane, allowing escape of the tumor cell [127]. A recently described "autocrine motility factor" (AMF) may contribute to this migration [128]. The importance of two of these determinants to tumor cell metastasis has suggested by a significant reduction in lung colonization by metastatic melanoma cells introduced in the presence of peptides which block the attachment of cells to laminin [129] or in the presence of inhibitors of collagenase type IV [130].

We have employed matrigel [131], an extract from the transplantable, basement membrane producing, Engelbreth Holm Swarm (EHS) sarcoma in an *in vitro* basement membrane invasion assay [7, 8, 132–134]. Matrigel is rich in basement membrane components, particularly laminin, collagen type IV and heparan sulfate proteoglycan. It is liquid at 4°C but forms a homogenous gel when heated to 37°C [131]. It has powerful effects on cellular adhesion, proliferation and differentation, particularly with cells of epithelial origin [135–137]. This substratum has proved useful in studies on tumor cell interactions with basement membane [138, 139].

HORMONAL REGULATION OF BASEMENT MEMBRANE INVASIVENESS OF MCF-7 CELLS

Many aspects of malignancy of hormone-dependent breast cancer are regulated by 17β-estradiol. This hormone stimulates MCF-7 cell invasiveness as well as the interaction between these cells and laminin, as measured by attachment to and migration toward laminin, and growth in the presence of basement membrane matrigel [7]. This response was associated with increased expression of laminin receptor binding activity on the cell surface [7]. Further analysis has shown that the effects of estradiol have a rapid onset (less than 9 h) and rapidly reversible if estradiol is withdrawn [8]. Furthermore, 17β-estradiol causes increased secretion of active collagenase-IV in the assay chamber, as measured by a solid phase collagen radiodegradation assay [8]. Thus, estradiol has the potential to coordinately up-regulate a number of parameters which constitute key events in basement membrane invasiveness, and may thus also contribute to the metastatic dissemination as well as proliferation of hormone-dependent breast cancer.

Divergent results have been reported comparing the effects of different antiestrogens on MCF-7 cell invasion [8, 53], apparently due to differential estrogen agonism. At growth inhibitory doses, tamoxifen and OHT markedly stimulated MCF-7 cell invasiveness, whereas the benzothiophene antiestrogens (LY 117018, LY 156758) had no effect. Coordinate effects have been reported on collagenase type IV production in the assay chamber. ICI 164,384 lacks agonistic activity in this assay, and both LY 117018 and ICI 164,384 are capable of competing for the stimulatory effects of either 17β-estradiol or OHT [53]. These assays were performed in serum-free medium containing 0.1% bovine serum albumin, and the effects were independent of the previous culture history of the cells (i.e. complete or estrogen-depleted serum) or the presence or absence of weakly estrogenic Phenol red in the medium.

It also appears that polypeptide growth factors regulate basement membrane invasiveness in breast cancer cell lines, as measured in the *in vitro* chemoinvasion assay [140, 141]. MCF-7 cell invasion was stimulated by TGF-β, and to a lesser degree by EGF. The more aggressive, ER- MDA-MB-231 and Hs578T cell lines were inhibited by TGF-β. As seen for antiestrogen regulation, invasiveness responses to growth factors appear to occur independently of proliferative responses, since both ER+ and ER− cell types are growth inhibited by TGF-β [113].

EXPERIMENTAL MODELS OF PROGRESSION OF BREAST CANCER TO ESTROGEN INDEPENDENCE

The process of malignant progression of breast cancer is poorly understood. Although 70% of primary human breast cancers, and about 50% of metastases contain a significant amount of estrogen receptor, only about two thirds of the estrogen receptor-containing tumors respond to some form of antiestrogen therapy [39–41]. Furthermore, most hormone-responsive tumors eventually become hormone-unresponsive following treatment with antiestrogens, other endocrine therapy, or chemotherapy. Absence or loss of estrogen responsiveness is often, but not always, associated with loss of the estrogen receptor, which is generally associated with the appearance of more malignant or more rapidly growing tumors. Loss of the estrogen receptor is often associated with loss of the receptor for IGF-I [142, 153] and acquisition of elevated levels of the EGF receptor [144]. Poor prognostic indicators which are independent of the estrogen receptor include tumor nuclear grade [145] $erbB_2$/neu amplification [145] possibly TGFa [147] and a putative repressor of metastases, Nm23 [148]. We have shown that estrogen receptor-negative human breast cancer cell lines are significantly more invasive than their estrogen-dependent counterparts, suggesting that metastatic dissemination may contribute to the poor prognosis associated with this group [149]. We have attempted to model this progression by selecting the MCF-7 cell line for antiestrogen resistance *in vitro* (Y-2 subline [150]) and estrogen-independence *in vitro* (MIII subline [151, 152]). These cell lines express functional estrogen receptor, and may thus model some of the subsets of human breast cancer that escape estrogen dependence and/or do not respond to hormonal therapy. In addition, MCF-7 cells selected for permanent chemotherapy resistance coordinately lose expression of the estrogen receptor and acquired increased expression of EGF receptor [153]. One very interesting *in vivo* model also exists for tamoxifen resistance [154]. MCF-7 cells were treated to relapse in the nude mouse tumor model [35]. The resistant line was able to recognize the slight estrogenicity of tamoxifen to stimulate its growth. The pure antiestrogen ICI-164,384 inhibited this growth [154].

The LY-2 subline, selected by stepwise increased antiestrogen for survival and proliferation in medium containing the LY 117018, has a basal growth rate similar to estrogen-stimulated MCF-7 cells, and responds only marginally to added estrogen. This narrow window of estrogen stimulation can be blocked by ICI 164,384, and to a lesser degree by OHT or LY 117018, but these antiestrogens do not reduce the proliferation rate below the relatively high baseline [53, 155]. Thus, while these cells express functional estrogen receptor, they have surpassed their dependence on estrogen for proliferation, and are not growth-inhibited by antiestrogens. This cell line has also lost expression of the progesterone receptor, but not PS2 or 52K proteins; they are regulated normally by estrogen. In contast to these considerations for proliferation, this subline resembles the parent in terms of invasiveness, indicating that these malignancy-related parameters are independently regulated. Basal invasiveness of

the LY-2 subline is similar to that seen for the MCF-7 parent in the absence of estrogen, and is similarly stimulated by estrogen and OHT [53]. In contrast to the parent, however, LY-2 cell invasiveness is stimulated by the less agonistic antiestrogen, LY 117018. These cells also exhibit an increased sensitivity to antiestrogen stimulation of the estrogen-regulated 52K cathespin D-related protease [32], which may be involved in the invasiveness measured in our assay. Conceivably, as with *in vivo* resistance models [35, 154], some aspects of antiestrogen resistance may involve increased recognition of weak agonistic effects of antiestrogens in common clinical use. Two other models of antiestrogen resistance also exist. A random subclone of T47D (T47D-5) has reduced progesterone receptor and resistance to antiestrogens and progestins with slightly elevated estrogen receptor content [155]. A ZR-75-1 variant has been selected against stepwise increased tamoxifen (ZR-75-9al) for 6 months also had an antiestrogen-resistant, progesterone receptor negative phenotype; further selection for 6 more months led to unstable loss of the estrogen receptor as well [156].

The MCF-7 MIII subline was derived from an MCF-7 cell tumor which spontaneously formed in an ovariectomized nude mice. It was further selected *in vitro* through maintenance in estrogen-depleted, Phenol red-free culture medium [151]. It proliferates at an increased basal rate similar to that seen for the LY-2 subline, and although not stimulated by estrogen, the MIII subline is growth inhibited with antiestrogen treatment. The difference in antiestrogen-responsiveness between the LY-2 and M-III cell lines, both of which appear estrogen independent, suggests that estrogen-independence and antiestrogen-resistance can develop independently. The antiestrogen-responsiveness of the M-III cell line in terms of proliferation is consistent with the effects seen on invasiveness, since the M-III subline shows increased constitutive invasiveness [151] which is stimulated only slightly by 17β-estradiol, but markedly stimulated by OHT [152]. Thus, the invasive response to OHT displayed by the parent MCF-7 cells is retained by the M-III subline, while the dependence on and response to estrogen is surpassed. The fact that proliferation of these cells is inhibited by OHT, while their invasiveness is stimulated, further suggests that these malignancy-associated parameters are independently regulated.

FUTURE PROSPECTS

The regulation both of proliferation and invasiveness is clearly important to the emergence and maintenance of the malignant phenotype in human breast cancer. As summarized above, we and others have shown that polypeptide growth factors are constitutively secreted by hormone-independent breast cancer, and are estrogen-regulated in hormone-responsive models. Basement-membrane invasiveness, which relates to the metastatic potential of these cells, is also estrogen-stimulated in responsive models. It is constitutively increased in hormone-independent models, and growth-factor sensitive. While one may speculate that progression to hormone-independence occurs through constitutive production of, or altered responsiveness to these and other estrogen-regulated mediators, this awaits future experimentation. Further analysis of the hormonal regulation of growth factor production and response, and regulation of the invasive phenotype, possibly with the progression models described herein, may serve to clarify this issue. Whatever the outcome, however, polypeptide growth factors and their receptors may suggest new modalities of antihormonal therapy of breast cancer. Furthermore understanding of the differential effects of hormones and growth factors on breast cancer cell invasiveness, in addition to proliferation, should also suggest new modalities for therapy. These modalities could be successfully tested in the near future with the development of more antagonist antiestrogenic and antiprogestational agents.

REFERENCES

1. Engel L. W. and Young N. A.: Human breast carcinoma cells in continuous culture: a review. *Cancer Res.* **38** (1978) 4327–4339.
2. Soule H. D., Vasquez J., Long A., Albert S. and Brennan M. J.: A human cell line from a pleural effusion derived from a breast carcinoma. *J. Natn. Cancer Inst.* **51** (1973) 1409–1416.
3. Horwitz K. B. and McGuire W. L.: Estrogen control of progesterone receptor in human breast cancer. *J. Biol. Chem.* **253** (1978) 2223–2228.
4. Lippman M. E., Bolan G. and Huff K.: The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long term culture. *Cancer Res.* **36,** (1976) 4595–4601.
5. Darbre P., Yates J., Curtis S. and King R. J. B.: Effect of estradiol on human breast cancer cells in culture. *Cancer Res.* **43** (1983) 349–454.
6. Chablos D., Vignon F., Keydar I. and Rochefort H.: Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). *J. Clin. Endocr. Metab.* **55** (1982) 276–283.

7. Albini A., Graf J. G., Kitten G. T., Kleinman H. K., Martin G. R., Veillette A. and Lippman M. E.: 17β-Estradiol regulates and v-Ha-ras transfection constitutively enhances MCF-7 breast cancer cell interactions with basement membrane. *Proc. Natn Acad. Sci. U.S.A.* **83** (1986) 8182–8186.
8. Thompson E. W., Reich R., Shima T. B., Albini A., Graf J., Martin G. R., Dickson R. B. and Lippman M. E.: Differential regulation of growth and invasiveness of MCF-7 breast cancer cells by antiestrogens. *Cancer Res.* **48** (1988) 6764–6768.
9. Ciocca D. R., Adams D. J., Edwards D. P., Bierke R. J. and McGuire W. L.: Distribution of an estrogen induced protein with a molecular weight of 24,000 in normal and malignant human tissues and cells. *Cancer Res.* **43** (1983) 1204–1210.
10. Westley B. and Rochefort H.: A secreted glycoprotein induced by estrogen in human breast cancer cell lines. *Cell* **20** (1980) 353–362.
11. Westley B. R. and May F. E. B.: Oestrogen regulates cathepsin D mRNA levels in oestrogen responsive human breast cancer cells. *Nucleic Acids Res.* **15** (1987) 3773–3780.
12. Bronzert D. A., Silverman S. and Lippman M. E.: Estrogen inhibition of a Mr 39,000 glycoprotein secreted by human breast cancer cells. *Cancer Res.* **47** (1987) 1234–1238.
13. Sheen Y. Y. and Katzenellenbogen B. S.: Antiestrogen stimulation of the production of a 37,000 molecular weight secreted protein and estrogen stimulation of the production of a 32,000 molecular weight secreted protein in MCF-7 human breast cancer cells. *Endocrinology* **120** (1987) 1140–1151.
14. Jakolew S. B., Breathneck R., Jeltsch J. and Chambon P.: Sequence of the pS2 mRNA induced estrogen in the human breast cancer cell line MCF-7. *Nucleic Acids Res.* **12** (1984) 2861–2874.
15. Nunez A. M., Jakolew S., Briand J. P., Gaire M., Krust A., Rio M. C. and Chambon P.: Characterization of the estrogen-induced pS2 protein secreted by the human breast cancer cell line MCF-7. *Endocrinology* **121** (1987) 1759–1765.
16. May F. E. B. and Westley B. R.: Cloning of estrogen-regulated messenger RNA sequences from human breast cancer cells. *Cancer Res.* **46** (1986) 6034–6040.
17. Soule H. D. and McGrath C. M.: Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Lett.* **10** (1980) 177–189.
18. Katzenellenbogen B. S., Kendra K. L., Norman M. J. and Berthois Y.: Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res.* **47** (1987) 4355–4360.
19. Sonnenschein C., Papendorp J. T. and Sato A. M.: Estrogenic effect of tamoxifen and its derivations on the proliferation of MCF-7 human breast tumor cells. *Life Sci.* **37** (1985) 387–394.
20. Reddel R. R. and Sutherland R. L.: Tamoxifen stimulation of human breast cancer cell proliferation *in vitro*: a possible model for tamoxifen tumor flare. *Eur. J. Cancer Clin. Oncol.* **20** (1984) 1419–1424.
21. Darbre P., Yates Y., Curtis S. and King R. J. B.: Effect of estradiol on human breast cancer cells in culture. *Cancer Res.* **43** (1983) 349–354.
22. Sapino A., Pietribiasi F., Rhusolti G. and Marchosio P. C.: Estrogen-and tamoxifen-induced rearrangements of cytoskeletal and adhesion structure in breast cancer MCF-7 cells. *Cancer Res.* **46:** (1986) 2526–2531.
23. Lyman S. D. and Jordan V. C.: Possible mechanism for the agonist actions of tamoxifen and the antagonist actions of MER-25 (ethamoxytriphetol) in the mouse uterus. *Biochem. Pharmac.* **34** (1985) 2795–2806.
24. Jordan V. C., Haldemann B. and Allen K. E.: Geometric isomers of substituted triphenylethylenes and antiestrogen action. *Endocrinology* **108** (1981) 1353–1361.
25. Black L. J. and Goode R. L.: Uterine bioassay of tamoxifen, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. *Life Sci.* **26** (1980) 1453–1458.
26. Dix C. J. and Jordan V. C.: Subcellular effects of monohydroxytamoxifen in the rat uterus: steroid receptors and mitosis. *J. Endocr.* **85** (1980) 393–404.
27. Koseki Y., Zava D. T., Chambers G. C. and McGuire W. L.: Progesterone interaction with estrogen and antiestrogen in the rat uterus-receptor effects. *Steroids* **30** (1977) 169–177.
28. Leavitt W. W., Chen T. J. and Allen T. C.: Regulation of progesterone receptor formation by estrogen action. *Ann. N.Y. Acad. Sci.* **286** (1977) 210–225.
29. Jordan V. C. and Prestwich G.: Effect of non-steroidal antiestrogens on the concentration of rat uterine progesterone receptors. *J. Endocr.* **76** (1978) 363–364.
30. Westley B., May F. E., Brown M. C. *et al.*: Effects of antiestrogens on the estrogen-regulated pS2 RNA and the 52 and 160-kilodalton proteins in MCF-7 cells and two tamoxifen-resistant sublines. *J. Biol. Chem.* **259** (1984) 10,030–10,035.
31. May F. E. B. and Westley B. R.: Effects of tamoxifen and 4-hydroxytamoxifen on the pNR-1 and pNR-2 estrogen-regulated RNA's in human breast cancer cells. *J. Biol. Chem.* **262** (1987) 15,894–15,899.
32. Cavailles V., Augereau P., Garcia M. and Rochefort H.: Estrogens and growth factors induce the mRNA of the 52 K-pro-cathespin-D secreted by breast cancer cells. *Nucleic Acids Res.* **16** (1988) 1903–1919.
33. Wakeling A. E. and Slater S. R.: Estrogen receptor binding and biologic activity of tamoxifen and its metabolites. *Cancer Treat. Rep.* **64** (1980) 741–744.
34. Osborne C. K., Coronado E. B. and Robinson J. R.: Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur. J. Cancer Clin. Oncol.* **23** (1987) 1189–1196.
35. Gottardis M. M. and Jordan V. C.: Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mouse after long-term tamoxifen administration. *Cancer Res.* **48** (1988) 5183–5188.
36. Jordan V. C., Fritz N. F. and Gottardis M. M.: Strategies for breast cancer therapy with antiestrogens. *J. Steroid Biochem.* **27** (1987) 493–498.
37. Gottardis M. M., Robinson S. P. and Jordan V. C.: Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen. *J. Steroid Biochem.* **30** (1988) 311–314.
38. Gottardis M. M. and Jordan V. C.: Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* **47** (1987) 4020–4024.
39. Jackson I. M. and Loweny C.: Clinical uses of antiestrogens. In *Pharmacology and Clinical Uses of Inhibitors of Hormone Secretion and Action* (Edited by B. J. Furr and A. E. Wakeling). Bailliere Tindall, Eastbourne, England (1987).
40. Furr B. J. A. and Jordan V. C.: The pharmacology and clinical uses of tamoxifen. *Pharmac. Ther.* **25** (1984) 127–205.
41. Patterson J. S., Battersley L. A. and Edwards D. G.: Review of the clinical pharmacology and international experience with tamoxifen in advanced breast cancer. *Endocr. Treat. Rev.* **9** (1981) 563–582.
42. Black L. J., Jones C. D. and Goode R. L.: Differential interaction of antiestrogens with cytosolic estrogen receptors. *Molec. Cell Endocr.* **22** (1981) 95–103.

43. Black L. J., Jones C. D. and Falcone J. F.: Antagonism of estrogen action with a new benzothiophene derived antiestrogen. *Life Sci.* **32** (1982) 1031–1036.
44. Black L. J. and Goode R. L.: Evidence for biological action of the antiestrogens LY117018 and tamoxifen by different mechanisms. *Endocrinology* **109** (1981) 987–989.
45. Scholl S. M., Huff K. K. and Lippman M. E.: Antiestrogenic effects of LY117018 in MCF-7 cells. *Endocrinology* **113** (1983) 611–617.
46. Wakeling A. E., Valcaccia B., Newboult E. and Green L. R.: Non-steroidal antiestrogens: receptor binding and biological response in rat uterus, rat mammary carcinoma and human breast cancer cells. *J. Steroid Biochem.* **20** (1984) 111–120.
47. Wakeling A. E. and Valcaccia B.: Antioestrogenic and antitumor activities of a series of non-steroidal antioestrogens. *J. Endocr.* **99** (1983) 455–460.
48. Nicholson R. L., Daniel P., Colin P. and Davis P.: Antitumor and oestrogen receptor binding activities of a series of antioestrogens. *Breast Cancer Res. Treat.* **2** (1982) 277–283.
49. Wakeling A. E.: Chemical structure and pharmacology of anti-estrogens. History, current trends and future prospects. In *Proc. of the Int. Symp. of Hormone Therapy* (Edited by F. Pannait). Excepta Medica, Amsterdam (1985) pp. 43–53.
50. Wakeling A. E. and Bowler J.: Steroidal pure antiestrogens. *J. Endocr.* **112** (1987) R7–R10.
51. Wakeling A. E. and Bowler J.: Biology and mode of action of pure antiestrogens. *J. Steroid Biochem.* **30** (1988) 141–147.
52. Wakeling A. E. and Bowler J.: Novel antioestrogens without partial agonist activity. *J. Steroid Biochem.* **31** (1988) 645–653.
53. Thompson E. W., Katz D., Shima T. B., Wakeling A., Lippman M. E. and Dickson R. B.: ICI 164,384: a pure antiestrogen for basement membrane invasiveness and proliferation of MCF-7 cells. *Cancer Res.* (1990) **49** (1990) 6929–6934.
54. Weatherill P. J., Wilson A. P. M., Nicholson R. I., Davies P. and Wakeling A. E.: Interaction of the antioestrogen ICI 164,384 with the oestrogen receptor. *J. Steroid Biochem.* **30** (1988) 263–266.
55. Jakesz R., Smith C. A., Aitken S., Huff K. K., Schuette W., Shakney S. and Lippman M. E.: Influence of cell proliferation and cell cycle phase on expression of estrogen receptor in MCF-7 breast cancer cells. *Cancer Res.* **44** (1984) 619–625.
56. Horwitz K. B. and McGuire W. L.: Actinomycin D prevents nuclear processing of estrogen receptor. *J. Biol. Chem.* **253** (1978) 6319–6322.
57. Horwitz K. B. and McGuire W. L.: Nuclear estrogen receptors. *J. Biol. Chem.* **255** (1980) 9699–9705.
58. Horwitz K. B. and McGuire W. L.: Nuclear mechanisms of estrogen action. *J. Biol. Chem.* **253** (1978) 8185–8191.
59. Kasid A., Strobl J. S., Huff K. Greene G. L. and Lippman M. E.: A novel nuclear form of estradiol receptor in MCF-7 human breast cancer cells. *Science* **225** (1984) 1162–1165.
60. Saceda M., Lippman M. E., Lindsey R. K., Puente M. and Martin M. B.: Regulation of the estrogen receptor in MCF-7 cells by estradiol. *Molec. Endocr.* **2** (1988) 1157–1162.
61. Saceda M., Lippman M. E., Chambon P., Lindsey R. K., Puente M. and Martin M. B.: Role of an estrogen receptor-dependent mechanism in the regulation of estrogen receptor mRNA in MCF-7 cells. *Molec. Endocr.* **3** (1989) 1782–1787.
62. Martin M. B., Lindsey R., Saceda M. and Puente M.: Anti-estrogen regulation of estrogen receptor expression in MCF-7 cells. *7th Annual Meeting of The Endocrine Society* (1988) p. 932.
63. Berthois Y., Katzenellenbogen J. A. and Katzenellenbogen B. S.: Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 2496–2500.
64. Nelson J. N., Clarke R., McFerran N. V. and Murphy R. F.: Morpho-functional effects of phenol red on oestrogen-sensitive breast cancer cells. *Biochem. Soc. Trans.* **15** (1987) 244.
65. Wakeling A. E. (ICI Pharmaceuticals, Manchester, England): Personal communication. (1990).
66. Edwards D. P., Murphy S. R. and McGuire W. L.: Effect of estrogen and antiestrogen on DNA polymerase in human breast cancer. *Cancer Res.* **40** (1980) 1722–1726.
67. Dubik D., Dembriniki T. C. and Shiu R. P.: Stimulation of c-myc oncogene expression associated with estrogen-induced proliferation of human breast cancer cells. *Cancer Res.* **47** (1987) 6517–6521.
68. Aitken S. C. and Lippman M. E.: Hormonal regulation of *de novo* pyrimidine synthesis and utilization in human breast cancer cells in tissue culture. *Cancer Res.* **43** (1983) 4681–4690.
69. Aitken S. C. and Lippman M. E.: Effect of estrogens and antiestrogens on growth-regulatory enzymes in human breast cancer cells in tissue culture. *Cancer Res.* **45** (1985) 1611–1620.
70. Dickson R. B., Aitken S. and Lipman M. E.: Assay of mitogen-induced effects on cellular thymidine incorporation. In *Methods in Enzymology 46, Hormone Action*, Part II—*Peptide Growth Factors* (Edited by D. Barnes and D. A. Sirbasku). Academic Press, New York (1987) pp. 327–340.
71. Aitken S. C., Lippman M. E., Kasid A. and Schoenberg D. R.: Relationship between the expression of estrogen regulated genes and estrogens-stimulated proliferation of MCF-7 mammary tumor cells. *Cancer Res.* **45** (1985) 2608–2615.
72. Freter C. E., Lippman M. E., Cheville A. and Gelmann E. P.: Alterations in phosphoinositide metabolism associated with 17β-estradiol and growth factor treatment of MCF-7 breast cancer cells. *Molec. Endocr.* **2** (1988) 159–166.
73. Carney D. H., Scott D. L., Gordon E. A. and LaBelle E. F.: Phosphoinositides in mitogenesis: neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. *Cell* **42** (1985) 479–488.
74. Nishizuka Y.: Protein kinases in a signal transduction. *Trends Biochem. Sci.* **9** (1984) 163–171.
75. Bates S. E., McManaway M. E., Lippman M. E. and Dickson R. B.: Characterization of estrogen responsive transforming activity in human breast cancer cell lines. *Cancer Res.* **46** (1986) 1707–1713.
76. Dickson R. B., Huff K. K., Spencer E. M. and Lippman M. E.: Induction of epidermal growth factor related polypeptides by 17-beta estradiol in MCF-7 human breast cancer cell lines. *Endocrinology* **118** (1986) 138–142.
77. Nickell K. A., Halper J. and Moses H. L.: Transforming growth factors in solid human malignant neoplasms. *Cancer Res.* **43** (1983) 1966–1971.
78. Salomon D. S., Zweibel J. A., Bano M., Losonczy I., Felnel P. and Kidwell W. R.: Presence of transforming growth factors in human breast cancer cells. *Cancer Res.* **44** (1984) 4069–4077.
79. Bates S. E., Davidson N. E., Valverius E., Dickson R. B., Kudlow J. E., Freter C., Tam J. P., Lippman M. E. and Salamon D. S.: Expression of TGFa and its mRNA in human breast cancer: its regulation and its

possible functional significance. *Molec. Endocr.* **2** (1988) 543–555.
80. Bringman T. S., Lindquist P. B. and Derynck R.: Different transforming growth factor a species are derived from a glycosylated and palmitolated transmembrane precursor. *Cell* **48** (1987) 429–440.
81. Gentry L. E., Twardzik D. R., Lim G. J., Ranchalis J. E. and Lee D. C.: Expression and characterization of transforming growth factor precursor protein in transfected mammalian cells. *Molec. Cell. Biol.* **7** (1987) 1585–1591.
82. Ignotz R. A., Kelly B., Davis R. J. and Massague J.: Biologically active precursor for transforming growth factor type a released by retrovirally transformed cells. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 6307–6311.
83. Dickson R. B., Kasid A., Huff K. K., Davidson N. and Dickson R. B.: Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17-beta estradiol or v-ras^H oncogene. *Proc. Natn Acad. Sci. U.S.A.* **84** (1987) 837–841.
84. Perroteau E., Salomon D., Debortali M., Kidwell W. R., Huzavika P., Pardue R., Dedman J. and Tam J.: Immunologic detection and quantitation of alpha transforming growth factors in human breast carcinoma cells. *Breast Cancer Res. Treat.* **7** (1986) 201–210.
85. Dickson R. B. and Lippman M. E.: Estrogenic regulation of growth and polypeptide growth factor secretin in human breast carcinoma. *Endocr. Rev.* **8** (1987) 29–43.
86. Arteaga C. L., Coronado E. and Osborne C. K.: Blockade of the epidermal growth factor receptor inhibits transforming growth factor a -induced but not estrogen-induced growth of hormone-dependent breast cancer. *Molec. Endocr.* **2** (1988) 1064–1069.
87. Ennis B. W., Valverius E. M., Lippman M. E., Bellot F., Kris R., Schlessinger J., Masui H., Goldberg A., Mendelsohn J. and Dickson R. B.: Monoclonal anti-EGF antibodies inhibit the growth of malignant and nonmalignant human mammary epithelial cells. *Molec. Endocr.* (1989) **3** (1989) 1830–1838.
88. Gregory H., Thomas C. E., Willshire I. R., Young J. A., Anderson H., Baildman A. and Howell A.: Epidermal and transforming growth factor a in patients with breast tumors. *Br. J. Cancer* **59** (1989) 605–609.
89. Harris A. L. and Nicholson S.: Epidermal growth factor receptors in human breast cancer. In *Breast Cancer: Cellular and Molecular Biology* (Edited by M. E. Lippmann and R. B. Dickson). Kluwer, Boston (1988) pp. 93–118.
90. Stampfer M. and Bartley J. C.: Development of human mammary epithelial cell culture systems for studies of carcinogenesis and differentiation. In *In Vitro Models for Cancer Research* (Edited by L. Sekley and M. Webber). CRC Press, Florida. Vol. 3 (1986) pp. 11–29.
91. Tonelli Q. J. and Sorof S.: Epidermal growth factor requirement for development of cultured mammary gland. *Nature* **285** (1980) 250–252.
92. Dickson R. B., McManaway M. and Lippman M. E.: Estrogen induced factors of breast cancer cells partially replace estrogen to promote tumor growth. *Science* **232** (1986) 1540–1543.
93. Clarke R., Brunner N., Katz D., Glanz P., Dickson R. B., Lippman M. E. and Kern F.: The effects of a constitutive expression of TGFa on growth of the MCF-7 human breast cancer cells *in vitro* and *in vivo*. *Molec. Endocr.* **3** (1989) 372–380.
94. Worland P. J. (Medicine Branch, NCI, NIH, Bethesda, Md): Personal communication (1990).
95. Furlanetto R. W. and DiCarlo J. N.: Somatomedin C receptors and growth effects in human breast cells maintained in long-term culture. *Cancer Res.* **44** (1984) 2122–2128.
96. Huff K. K., Kaufman D., Gabbay K. H., Spencer E. M., Lippman M. E. and Dickson R. B.: Human breast cancer cells secrete an insulin-like growth factor I-related polypeptide. *Cancer Res.* **46** (1986) 4613–4619.
97. Jansen M., Van Schaik F. M. A., Ricker A. T., Bullock B., Woods P. E., Gabbay K. H., Nissbaum A. L., Sussenbach J. S. and Van der Branch J. R.: Sequence of cDNA encoding human insulin-like growth factor I precursor. *Nature (Lond.)* **306** (1983) 609–611.
98. Huff K. K., Knabbe C., Kaufman D., Lindsay R., Lippman M. E. and Dickson R. B.: Multihormonal regulation of insulin-like growth factor-I-related protein in MCF-7 human breast cancer cells. *Molec. Endocr.* **2** (1988) 200–208.
99. Rohlik Q. T., Adams D., Kull F. C. and Jacobs S.: An antibody to the receptor for insulin-like growth factor I inhibits the growth of MCF-7 cells in tissue culture. *Biochem. Biophys. Res. Commun.* **149** (1988) 276–281.
100. Arteaga C. L., Kitten L., Coronado E., Jacobs C., Kull F. and Osborne C. K.: Blockade of the type I somatomedin receptor inhibits growth of estrogen receptor negative human breast cancer cells in athymic nude mice. *Proceedings of the Annual Meeting of the Endocrine Society* (1988) Abstr. 683.
101. Clemmons D. R. and Van Wyk J. J.: Evidence for a functional role of endogenously produced somatomedin-like peptides in the regulation of DNA synthesis in cultured human fibroblasts and porcine smooth muscle cells. *J. Clin. Invest.* **75** (1986) 1914–1918.
102. Clemmons D. R. and Shaw D. S.: Variables controlling somatomedin production by cultured human fibroblasts. *J. Cell. Physiol.* **115** (1983) 137–143.
103. Clemmons D. R. and van Wyk J. J.: Evidence for a functional role of endogenously produced somatomedin-like peptides in the regulation of DNA synthesis in cultured human fibroblasts and porcine smooth muscle cells. *J. Clin. Invest.* **75** (1986) 1914–1918.
104. Yee D., Cullen K. J., Paik S., Purdue J. F., Hampton B., Schwartz A., Lippman M. E. and Rosen N.: Insulin-like growth factor II mRNA expression in human breast cancer. *Cancer Res.* **48** (1988) 6691–6696.
105. De Leon D. D., Bakker B., Wilson D. M., Hintz R. L. and Rosenfeld R. G.: Demonstration of insulin-like growth factor (IGF-I and -II) receptors and binding protein in human breast cancer cell lines. *Biochem. Biophys. Res. Commun.* **152** (1988) 398–405.
106. Dehoff M. H., Elgin R. G., Collier R. J. and Clemmons D. R.: Both type I and II insulin-like growth factor receptor binding increase during lactogenesis in bovine mammary tissue. *Endocrinology* **122** (1988) 2412–2417.
107. Roberts A. B., Anzano M. A., Wakefield L. M., Roche N. S., Stern D. F. and Sporn M. B.: Type β transforming growth factor: a bifunctional regulator of cellular growth. *Proc. Natn Acad. Sci. U.S.A.* **82** (1985) 119–123.
108. Noda K., Umeda M. and Ono T.: Transforming growth factor activity in human colostrum. *Gann* **75** (1984) 109–112.
109. Silberstein G. B. and Daniel C. W.: Reversible inhibition of mammary gland growth by transforming growth factor β. *Science* **237** (1987) 291–295.
110. Walker-Jones D., Valverius E. M., Stampfer M. S., Lippman M. E. and Dickson R. B.: Transforming growth factor beta (TGFβ) stimulates expression of epithelial membrane antigen in normal and oncogene transformed human mammary epithelial cells. *Cancer Res.* **49** (1989) 6407–6411.

111. Derynck R., Jarrett J. A., Chen E. Y., Eaton D. H., Bell J. R., Assoian R., Roberts A. B., Sporn M. B. and Goeddel D. U.: Human transforming growth factor β: Complementary DNA sequence and expression in normal and transformed cells. *Nature* **316** (1985) 701–705.
112. Knabbe C., Lippman M. E., Wakefield L., Flanders K., Kasid A., Derynck R. and Dickson R. B.: Evidence that TGFβ is a hormonally regulated negative growth factor in human breast cancer. *Cell* **48** (1987) 417–428.
113. Zugmaier G., Knabbe C., Deschauer B., Lippman M. E. and Dickson R. B.: Inhibition of anchorage independent growth of estrogen receptor positive and estrogen receptor negative human breast cancer cell lines by TGFβ and TGFβ2. *J. Cell. Physiol.* **141** (1989) 353–361.
114. Bronzert D. A., Pantazis P., Antoniades H. N., Kasid A., Davidson N., Dickson R. B. and Lippman M. E.: Synthesis and secretion of PDGF-like growth factor by human breast cancer cell lines. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 5763–5767.
115. Rozengurt E., Sinnett-Smith J. and Taylor-Papadimitriou J.: Production of PDGF-like growth factor by breast cancer lines. *Int. J. Cancer* **36** (1985) 247–252.
116. Betsholtz C., Hohnsson A., Heldin C. H., Westermark B., Lind P., Ureda M. S., Eddy R., Shows T. B., Philpott K., Mellor A. L., Knoff T. J. and Scott J.: cDNA sequence and chromosomal localization of human platelet derived growth factor A chain and its expression in tumor cell lines. *Nature* **320** (1986) 695–700.
117. Ross R., Raines E. W. and Bowen-Pope D. F.: The biology of platelet-derived growth factor. *Cell* **46** (1986) 155–169.
118. Halper J. and Moses H. L.: Purification and characterization of a novel transforming growth factor. *Cancer Res.* **47** (1987) 4552–4559.
119. Swain S., Dickson R. B. and Lippman M. E.: Anchorage independent epithelial colony stimulating activity in human breast cancer cell lines. *Procs American Association for Cancer Research Annual Meeting*, Los Angeles, Calif. (1986) Abstr. 844.
120. Vignon F., Capony F., Chambon M., Freiss G., Garcia M. and Rochefort H.: Autocrine growth stimulation of the MCF-7 breast cancer cells by the estrogen regulated 52Kd protein. *Endocrinology* **118** (1986) 1537–1545.
121. Koga M. and Sutherland R. L.: Epidermal growth factor partially reverses the inhibitory effects of antiestrogens on T47D human breast cancer cell growth. *Biochem. Biophys. Res. Commun.* **146** (1987) 739–745.
122. Vignon F., Bouton M.-M. and Rochefort H.: Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. *Biochem. Biophys. Res. Commun.* **146** (1987) 1502–1508.
123. Fidler I. J.: Tumor heterogeneity and the biology of cancer invasion and metastasis. *Cancer Res.* **38** (1978) 2651–2660.
124. Liotta L. A.: Tumor invasion and metastases: role of the basement membrane. *Am. J. Path.* **117** (1984) 339–348.
125. Terranova V. P., Hujanen E. S. and Martin G. R.: Basement membrane and the invasive activity of metastatic tumor cells. *J. Natn. Cancer Inst.* **77** (1986) 311–316.
126. Terranova V. P., Liotta L. A., Russo R. G. and Martin G. R.: Role of laminin in the attachment and metastasis of murine tumor cells. *Cancer Res.* **42** (1982) 2265–2269.
127. Liotta L. A., Thorgeirsson U. P., Garbisa S.: Role of collagenases in tumor cell invasion. *Cancer Metab. Rev.* **1** (1982) 277–288.
128. Liotta L. A., Mandler R., Murano G., Katz D. A., Gordon R. K., Chiang P. K. and Schiffman E.: Tumor cell autocrine motility factor. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 3302–3306.
129. Iwamoto Y., Robey F. A., Graf J., Sasaki M., Kleinmann H. K., Yamada Y. and Martin G. R.: YIGSR, a synthetic laminin pentapeptide, inhibits experimental metastasis formation. *Science* **238** (1987) 1132–1134.
130. Reich R., Thompson E. W., Iwamoto Y., Martin G. R., Deason J., Fuller G. C. and Miskin R.: Inhibitors of plasminogen activator, serine proteases and collagenase IV prevent the invasion of basement membranes by metastatic cells. *Cancer Res.* **48** (1988) 3307–3312.
131. Kleinman H. K., McGarvey M. L., Hassell J. R., Star V. L., Cannon F. B., Laurie G. W. and Martin G. R.: Basement membrane complexes with biological activity. *Biochemistry* **25** (1986) 312–318.
132. Albini A., Iwamoto Y., Kleinman H. K., Martin G. R., Aaronson S. A., Kozlowski J. M. and McEwan R. N.: A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res.* **47** (1987) 3239–3245.
133. Iwamoto Y., Albini A., Reich R., Romish K., Thompson E. W., Graf J., Yamada Y. and Martin G. R.: Use of a reconstituted basement membrane to study the invasiveness of tumor cells. *UCLA Symp. on Tumor Progression and Metastasis* Keystone, Colorado (1987) Abstr.
134. Albini A., Auckerman S. L., Melchiori A., Thompson E. W., Reich R., Shima T. B., Martin G. R. and Iwamoto Y.: Basement membranes: Reconstituted to assess invasiveness of tumor cells. *Proceedings of the UCLA Symposium on Tumor Progression and Metastasis*. Liss, New York (1988).
135. Kleinman H. K., Luckenbill-Edds L., Cannon F. W. and Sephel G. C.: Use of extracellular matrix components for cell culture. *Analyt. Biochem.* **166** (1987) 1–13.
136. Kleinman H. K., Graf J., Iwamoto Y., Kitten G. T., Ogle R. C., Sasaki M., Yamada Y., Martin G. R. and Luckenbill-Edds L.: Role of basement membranes in cell differentiation. *Ann. N.Y. Acad. Sci.* **513** (1988) 134–145.
137. Kleinman H. K., McGarvey M. L., Hassell J. R., Martin G. R., Baron van Evercoorern A. and Dubois-Dalc M.: The role of laminin in basement membranes and in the growth, adhesion and differentiation of cells. In *The Role of Extracellular Matrix in Development*. Liss, New York (1984).
138. Kramer R. H., Bensch K. G. and Wong J.: Invasion of reconstituted basement membrane matrix by metastatic human tumor cells. *Cancer Res.* **46** (1986) 1980–1986.
139. Hendrix M. J. C., Seftor E. A., Seftor R. E. B. and Fidler I. J.: A simple quantitative assay for studying the invasive potential of high and low human metastatic variants. *Cancer Lett.* **38** (1987) 137–147.
140. Thompson E. W., Shima T. B., Martin G. R., Valvertus E. M., Lippman M. E. and Dickson R. B.: Effects of TGFb and EGF on basement membrane invasiveness of human breast cancer cell lines. *Inter. Symp. on Critical Determinants in Cancer Progression and Metastasis*, Houston, Texas (March 1989).
141. Thompson E. W., Shima T. B., Martin G. R., Zugmaier G., Lippman M. E. and Dickson R. B.: TGFb independently regulates invasion, chemotaxis, and proliferation of human breast cancer cells. *Ann. N.Y. Acad. Sci.* (1989).
142. Foekens J. A., Portengen H., Janson M. and Klijn J. G. M.: Insulin-like growth factor—1 receptors and insulin-like growth factor-1-like activity in human primary breast cancer. *Cancer* **63** (1989) 2139–2147.

143. Peyrat J. P., Bonneterre J., Beuscart R., Djiane J. and Demaille A.: Insulin-like growth factor-1 receptors in human breast cancer and their relation to estradiol and progesterone receptors. *Cancer Res.* **48** (1988) 6429–6433.
144. Nicholson S., Halcrow P., Farndon J. R., Sainsbury J. R. C., Chambers P. and Harris A. L.: Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. *The Lancet* **January 28** (1989) 182–185.
145. Fisher B., Redmond C., Fisher E. R. and Caplan R.: Relative worth of estrogen or progesterone receptor and pathologic charactersitics of differentiation as indicators of prognosis in node negative breast cancer patients: findings from National Surgical Adjuvant Breast and Bowel Project Protocol B-06. *J. Clin. Oncol.* **6** (1988) 1076–1087.
146. Slamon D. J., Godolphin W., Jones L. A., Holt J. A., Wong S. G., Keith D. E., Levin W. J., Stuart S. G., Udove J., Ullrich A. and Press M. F.: Studies of the HER-2/neu protooncogene in human breast and ovarian cancer. *Science* **244** (1989) 707–710.
147. Arteaga C. L., Hanauske A. R., Clark G. M., Osborne C. K., Hazarika P., Pardue R. C., Tio F. and Von Hoff D. D.: Immunoreactive a transforming growth factor activity in effusions from cancer patients as a marker of tumor burden and patient prognosis. *Cancer Res.* **48** (1988) 5023–5028.
148. Steeg P. S., Bevilacqua G., Kopper K., Thorgeirsson U. P., Talmadge J. E., Liotta L. A. and Sobel M. E.: Evidence for a novel gene associated with low tumor metastatic potential. *J. Natn. Cancer Inst.* **80** (1988) 200–204.
149. Thompson E. W., Shima T. B., Reich R., Martin G. R., Paik S., Zugmaien G., Dickson R. B. and Lippman M. E.: Estrogen receptor negative human breast cancer cell lines are more invasive than estrogen receptor positive lines. *American Association for Cancer Research* (Abstr.), New Orleans (May, 1988).
150. Bronzert D. A., Greene G. L. and Lippman M. E.: Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY 117018. *Endocrinology* **117** (1985) 1409–1417.
151. Clarke R., Brunner N., Thompson E. W., Katzenellenbogen B. S., Norman M. J., Koppi C., Paik S., Lippman M. E. and Dickson R. B.: Progression of human breast cancer cells from hormone dependent to hormone independent growth both *in vitro* and *in vivo*. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 3649–3653.
152. Clarke R., Glanz P., Brunner N., Thompson E. W., Katz D., Dickson R. B. and Lippman M. E.: The inter-relationships between ovarian-independent growth, tumourigenicity, invasiveness and anti-oestrogen-resistance in the malignant progression of human breast cancer. *J. Endocr.* **122** (1989) 331–340.
153. Vickers P. J., Dickson R. B., Shoemaker R. and Cowan K. H.: Multidrug resistant human breast cancer cells exhibit cross-resistance to antiestrogens and hormone-independent growth *in vivo*. *Molec. Endocr.* **2** (1988) 886–892.
154. Gottardis M. M., Jiang S. Y., Jeng M. H. and Jordan V. C.: Inhibition of tamoxifen-stimulated growth of a MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res.* **49** (1989) 4090–4093.
155. Reddel R. R., Alexander I. E., Koga M., Shine J. and Sutherland R. L.: Genetic instability and the development of steroid hormone insensitivity in cultured T47D human breast cancer cells. *Cancer Res.* **48** (1988) 4340–4347.
156. Van den Berg H. W., Lynch M., Martin J., Nelson J., Dickson G. R. and Cockard A. D.: Characterization of a tamoxifen-resistant variant of the ZR-75-1 human breast cancer cell line (ZR-75-9a1) and stability of the resistant phenotype. *Br. J. Cancer* **59** (1989) 522–526.