MECHANISMS OF HORMONE RESISTANCE IN BREAST CANCER

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MECHANISMEN VAN HORMOONRESISTENTIE BIJ BORSTKANKER

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2. Induction of estrogen independence of ZR-75-1 human breast cancer cells by epigenetic alterations. Ton van Agthoven, Thecla L. A. van Agthoven, Anita Dekker, John A. Foekens, and Lambert C. J. Dorssers. Molecular Endocrinology 8, 1474-1483, 1994. 59

3. Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry. Ton van Agthoven, Mieke Timmermans, John A. Foekens, Lambert C. J. Dorssers, and Sonja C. Henzen-Logmans. American Journal of Pathology 144, 1238-1246, 1994. 69

4. Expression of estrogen, progesterone, and epidermal growth factor receptors in primary and metastatic breast cancer. Ton van Agthoven, Mieke Timmermans, Lambert C. J. Dorssers, and Sonja C. Henzen-Logmans. International Journal of Cancer 63, 790-793, 1995. 79

5. Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: Identification of bcar-1, a common integration site. Lambert C. J. Dorssers, Ton van Agthoven, Anita Dekker, Thecla L. A. van Agthoven, and Elisabeth M. Kok. Molecular Endocrinology 7, 870-878, 1993. 83

6. Identification of BCAR3 by a random search for genes involved in antiestrogen resistance of human breast cancer cells. Ton van Agthoven, Thecla L. A. van Agthoven, Anita Dekker, Peter J. van der Spek, Lilian Vreede and Lambert C.J. Dorssers. The EMBO Journal 17, 2799-2808, 1998. 93

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Abbreviations

AZA	5-azacytidine
BCAR	breast cancer antiestrogen resistance
CAS	Crk-associated substrate
cDNA	complementary DNA
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ER	estrogen receptor
ERE	estrogen response elements
ICI	Imperial Chemical Industries
IGF	insulin-like growth factor
kb	kilo base
kD	kilo dalton
LTR	long terminal repeat
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
MDR	multidrug resistance
PCR	polymerase chain reaction
PR	progesterone receptor
RNA	ribonucleic acid
SH2	Src homology 2
SSCP	single strand conformation polymorphism
TGF	transforming growth factor

CHAPTER 1

Introduction

Antiestrogenic drugs such as tamoxifen are important in the management of hormone-dependent breast cancer. Treatment with tamoxifen prolongs disease-free survival and reduces mortality. Tamoxifen has few harmful side effects, it has low cost, is easy to administer, and has potential benefits on lipid and bone metabolism. However, the treatment is palliative and, hormone resistance of the tumor cells is almost inevitable, resulting in disease recurrence. The mechanisms underlying the progression of breast cancer, either spontaneously or therapeutically induced, to a form unresponsive to endocrine treatment, are largely unknown. Insight in these mechanisms may result in the development of new treatment strategies. The work described in this thesis focuses on possible mechanisms and genes involved in hormone resistance in breast cancer.

1.1 Development of breast cancer

The biological function of the mammary gland is to produce and secrete milk. This unique feature of mammals is vital for the feeding of the newborn and thus for survival of the species. The normal glandular epithelium of the adult female breast consists of ducts and lobular secretory alveoli. The stroma of the breast consists of fat, connective tissue, blood vessels and lymphatics. Two different cell types line both ducts and alveoli: the luminal epithelial cells and the myoepithelial cells, which have clearly distinguishable phenotypes. Luminal cells are characterized by the expression of keratins 7, 8, 18, and 19, whereas keratins 5 and 14, alpha smooth muscle actin, vimentin, collagen IV and laminin have been demonstrated in myoepithelial cells [1-4]. Also the expression pattern of hormone receptors and epidermal growth factor (EGF) receptor is different. Luminal cells express classical estrogen receptor α (ER) and progesterone receptor (PR, an estrogen-responsive gene product) and EGF receptor. Myoepithelial cells show strong expression of EGF receptor and are negative for ER and PR [5].

Over 95% of primary breast malignancies originate from the luminal epithelial cells of the glandular epithelium [6]. Adenocarcinomas of the breast are classified in either ductal or lobular lesions, and can further be divided in non-invasive or invasive tumors [7]. Invasive tumor cells can disseminate by infiltrating through tissue spaces, and can invade both lymphatics and blood vessels. Metastases primarily develop in lymph nodes, and later at distant sites as lungs, bones and liver. At presentation, approximately 40-50% of patients has lymph node metastases and <10% already has clinically manifested distant metastases. When there is no evidence of metastasis at presentation of the disease, therapy is usually restricted to local treatment of the tumor. Since a substantial percentage of women with apparently localized breast cancer will have occult (or micro) metastases by the time of diagnosis, success of local treatment will often be limited. Patients with no evidence of progressive disease may therefore benefit from adjuvant chemo- or endocrine therapy [8]. Patients with metastatic disease will require some form of systemic treatment such as endocrine- or chemotherapy.

In the Western world today, breast cancer is the most common cancer in women (approximately one third of all types of cancer), affecting one out of ten women. The most clearly identified risk factors for breast cancer are female sex and age, with occurrence predominantly in postmenopausal women. However, more than 70% of the patients over the age of 50 have no apparent additional risk factors. This implies that the etiology of breast cancer is probably multifactorial, including inherited, hormonal and environmental factors [9-14]. Hormones and growth factors play an important role in the development and the physiology of the adult breast. Presumably estrogen, one of the major regulatory hormones for the normal breast, plays a crucial role in promoting breast cancer [15-17]. Several lines of evidence support this hypothesis. In the normal ductal and lobular epithelial cells, from which most breast tumors arise, ER is expressed in varying amounts, depending on the developmental stage [5,18]. Approximately 70% of primary breast tumors contain ER, and these presumably depend on the proliferative effect of estrogens [19,20]. The risk of breast cancer is correlated with the number of menstrual cycles and thus with the time of activation of the ovaries, which are the primary producers of estrogens, and the time of menopause, the inactivation of the ovaries. Ovariectomy, when performed early in life, reduces the risk of breast cancer to that of men, approximately 1% of the risk in women [21]. Finally, ovariectomy can inhibit growth of breast cancer, which can be explained by the elimination of estrogens, which are required to activate the ER and to stimulate proliferation of the tumor cells [18,22]. These observations strongly suggest that the development of the majority of breast cancers is estrogen-dependent.

1.2 Antiestrogen therapy

A large proportion of breast tumors expresses ER and appears dependent on estrogens for proliferation. Consequently, the ER is an important target for pharmacotherapy. The rationale for many endocrine therapies for breast cancer is the inhibition of the proliferative effects of estrogens. The first compounds developed to interfere with the ER were highly toxic [23]. In 1969, tamoxifen developed as a possible drug for oral contraception by Imperial Chemical Industries (ICI) Pharmaceuticals (now Zeneca) was shown to be an effective inhibitor of breast cancer growth (reviewed by [24]). Most *in vitro* studies suggest that tamoxifen, like other antiestrogens, competes with estradiol for binding to the ligand-binding domain of the ER [25-27]. The antiestrogen complexes with the ER, and the complex may still bind to the DNA. However, this complex will not result in modulation of expression of the ER-target genes [28]. Antiestrogens may thus block hormone signaling and induce growth arrest of the tumor cells [25,29-32]. Tamoxifen has been the first choice endocrine therapy in the adjuvant setting after surgery for breast cancer, and in patients with advanced breast cancer for >20 years now. Presently, it is evaluated as a preventive agent in women at high-risk for developing breast cancer [33-35].

Nonsteroidal antiestrogens such as tamoxifen display also some estrogenic characteristics [36]. In some tissues and cell types, they can activate gene expression [37] and are therefore classified as partial agonists. An important estrogenic activity of tamoxifen is the increase of osteoblastic activity resulting in the preservation of bone density in postmenopausal women [38,39]. Tamoxifen also appears to have an effect on

lipid metabolism, resulting in lower serum cholesterol levels, which may decrease the incidence of myocardial infarction [40,41]. Increased risk of endometrial cancer due to tamoxifen treatment was reported. This association is insignificant compared to the beneficial effects of tamoxifen, and until now no causal association has been established (reviewed by [42]). Tamoxifen has other cellular effects not mediated through the ER, among others enzyme inhibition (protein kinase C and calmodulin) and growth factor modulation [43-46]. Treatment with tamoxifen induces synthesis of transforming growth factor β (TGFβ), a negative growth regulator of breast cancer cells [47-49], and decreases insulin-like growth factor I (IGFI) levels, a growth stimulator of breast (cancer) cells [50,51]. New and more effective antiestrogens have been developed [52-54]. Different antiestrogens may differ in their effects on various cell types and in their interactions with ER [55-57]. Some of these compounds, including ICI 164,384 and ICI 182,780, lack agonistic activity [53,58-60]. These so-called pure antiestrogens are currently evaluated in clinical trials for the treatment of patients with primary breast cancer and advanced breast cancer resistant to tamoxifen [61,62]. Pure antiestrogens will not have the beneficial effects on bone and lipid metabolism of tamoxifen. New compounds have to be designed, combining the antagonistic effects on breast cancer cells and agonistic effects on bone and lipid metabolism.

Tamoxifen has been used for more than 20 years. In the adjuvant setting after surgery for early breast cancer it reduces the recurrence rate by 25%, mortality by 16%, and the incidence of contralateral breast cancer by 40% (reduction in annual odds) [8]. Although these reductions are small, the impact is significant due to the high incidence of breast cancer. In patients with metastatic disease who originally had ER-positive primary tumors, treatment with tamoxifen causes remissions in approximately 50% of the patients [63-66]. Although treatment with antiestrogens can delay recurrences or induce remissions of the disease, metastases refractory to treatment will develop in almost all cases. Several factors may be responsible for tamoxifen resistance (reviewed by [17,26,67-75]), including decreased levels of ER, mutation or splice variants of ER, agonistic effects of tamoxifen, altered drug metabolism, *de novo* production of estrogens, overexpression of several genes, interactions with the environment and increase in antiestrogen binding sites. Nevertheless, for the majority of breast cancers the mechanisms responsible for development of resistance to hormonal therapy are still not known. Better understanding of tamoxifen resistance may lead to new treatment strategies or the development of new therapeutic agents. Knowing the mechanism of antiestrogen resistance is also important for patients non-responsive to second-line hormone therapy. Finally, understanding of tamoxifen action and associated resistance may be of particular importance for preventing breast cancer in women who are at high-risk of developing the disease [76].

1.3 Estrogen receptor alpha

The major effects of estrogens and antiestrogens are mediated through the ER. Changes in structure and function of ER have been implicated in antiestrogen resistance. Therefore, a concise review will be given of the known structure and function of the ER. A schematic representation of the ER is presented in Figure 1. The ER belongs to the superfamily of nuclear hormone receptors. These receptors function as transcription factors when they are activated by their ligand. Other members of this family include receptors for other steroid hormones, vitamin D, thyroid hormone, ecdysone and retinoic acid [77-83].

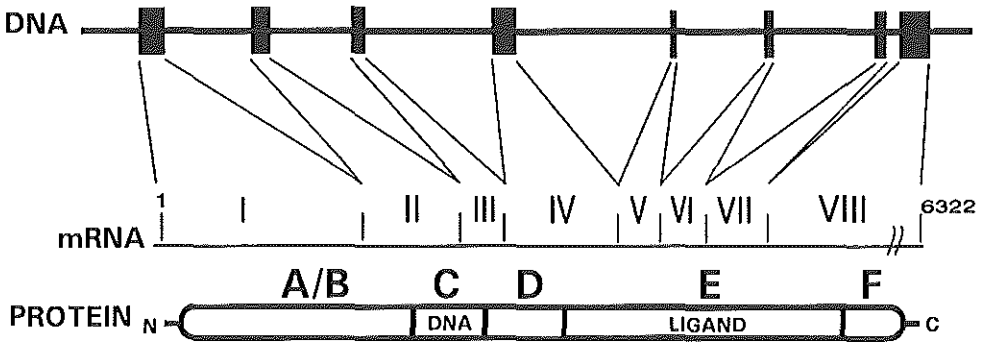


Figure 1.
Schematic representation of the estrogen receptor. The top portion of the figure shows the genomic organisation. The eight exons are indicated by black boxes. Below the genomic DNA the configuration of the spliced mRNA is presented. The bottom part of the figure shows the functional domains of the estrogen receptor protein.

Estrogens diffuse across the cell membrane and bind to the ER, to form homodimers that bind with high affinity to specific DNA enhancer elements termed estrogen response elements (ERE) and characterized by the consensus sequence GGATCnnnGATCC [84]. Consequently, most estrogen-responsive genes are controlled by EREs located either upstream or downstream of the transcription initiation site. The structure and number of EREs, may vary noticeably between different genes. The estrogen-ER complex bound to the DNA interacts with other transcription factors and with components of the transcriptional complex to modulate gene transcription [85]. The exact mechanism of estrogen-induced transcription activation is still largely unknown. Recent studies have led to the identification of various coactivator proteins that appear to modulate the functional activity of the ER [86-88].

The ER gene has eight exons spanning more than 140 kb, and is located on human chromosome 6q 25.1 [89]. It encodes a nuclear protein of 593 amino acid residues with a molecular mass of 66 kilodalton (kDa). Comparison of the sequences of the ER complementary DNA (cDNA) with that of different members of the hormone superfamily revealed six regions with varying homology, labeled A to F starting from the N-terminus [90]. The many reported functional activities to these domains are indicated in Figure 1. ER can activate transcription through two distinct activation regions. The NH2-terminal region A/B contains the ligand-independent activation function-1 (AF-1) domain. The AF-2 domain is located in the COOH-terminal E-region, which can only activate transcription in the presence of ligand [77,79,82]. The individual activities of the two activation domains depend on the target promoter and cell type [91]. Region C, contains two zinc finger motifs which are responsible for the specific DNA binding activity of the protein based on the recognition of the ERE of target genes. Region D functions as a hinge between the hormone- and the DNA-binding domains and contains the sequences required for nuclear localization of the receptor [90]. Region E, the hormone-binding domain, is responsible for ligand binding, receptor dimerization and activation of transcription of estrogen-responsive genes. Region E also contains a site for binding of heat-shock proteins. It is suggested that in the absence of estrogen, the DNA-binding domain is masked by the hormone-binding domain. Hormone binding results in relieving this masking effect. Region F has a modulatory effect on the transcriptional activity of the ER [92].

Recently, an ERβ subtype was described, which may explain some of the cell and promoter-specific effects [93-95]. The gene encodes a protein of 485 amino acid residues that shares respectively 96% and 58% identity with the DNA- and ligand binding domains of ERα. In contrast to the A/B domain, the hinge region and the F-domain are not conserved [93,96]. Expression of ERβ was observed in many tissues including thymus, spleen, ovary, testis, and in both ERα positive- and negative tumors [93,97]. The formation of heterodimers of ERα and ERβ was demonstrated in a mammalian two-hybrid system and in transfection experiments [98,99]. This suggests the existence of three pathways of estrogen signaling: via ERα or ERβ homodimers, and via the formation of heterodimers in cells expressing both receptor subtypes. The identification of this second estrogen receptor will lead to a re-evaluation of estrogen signaling and physiology.

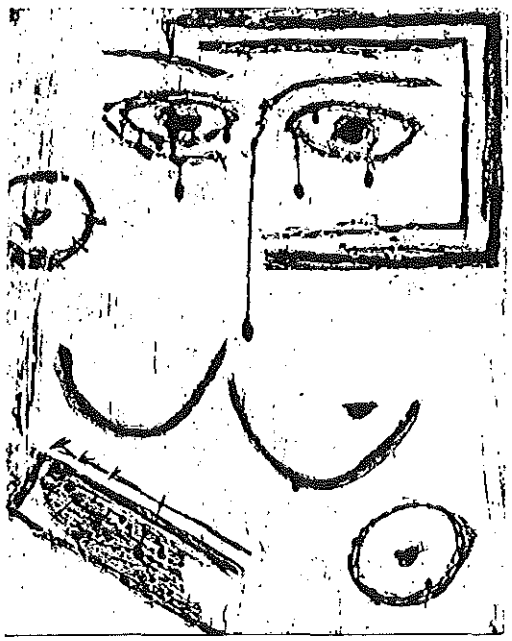
1.4 Hormone resistance in breast cancer

During development and progression of a tumor, the cells may undergo diverse changes [100-102]. Breast tumors are composed of malignant cells and non-malignant cells, including stromal, adipose, and vasculature cells, macrophages and lymphocyte infiltrations. During tumor progression complex cell-cell interactions take place involving malignant and non-malignant cells and the extracellular matrix, eventually resulting in the lethal ability of tumor cells to invade tissues and to metastasize. Hormone-resistant breast tumors can be classified in three groups. First, intrinsic resistant tumors; the ER-negative group of tumors. Most of the actions of estrogens leading to cell proliferation appear to be mediated by the ER. Consequently, progression to ER-negative disease would result in tumors unresponsive to

antiestrogen therapy. Second, intrinsic resistant ER-positive tumors, which are refractory to initial tamoxifen therapy. Third, tumors with acquired resistance; the remainder of the ER-positive tumors, which initially responds to antiestrogens, but becomes resistant under therapy. The mechanisms underlying the resistance of these latter two categories may be similar and only reflect a difference in the state of tumor progression at the moment of starting with therapy. Progression from a hormone-dependent state to hormone insensitivity may be the result of a mutational event, followed by clonal selection [103,104]. Other observations have implied epigenetic mechanisms [105,106], which are heritable alterations in gene function that are mediated by factors other than changes in primary DNA sequence, for example 5-methylcytosine DNA methylation. Epigenetic changes as opposed to mutations may result in the same phenotypic change simultaneously in a large proportion of the tumor cells.

Many possible mechanisms for antiestrogen resistance have been postulated and studied, including: alteration of metabolism or availability of tamoxifen, changes in ER and function, and overexpression of different genes. Data supporting multiple possible mechanisms and the model systems used in these studies are reviewed in the next chapter.

CHAPTER 2



Mechanisms of antiestrogen resistance

2.1 Pharmacology of tamoxifen and its resistance

Alteration in the pharmacology of the drug was postulated as a possible mechanism for tamoxifen resistance by experimental and clinical observations [70]. Tamoxifen is metabolized in the liver to several compounds with different biological properties. N-desmethyl tamoxifen is the major metabolite, which has a comparable biological activity as tamoxifen. Another major metabolite is 4-hydroxy-tamoxifen, which has a higher affinity for the ER and a stronger biological activity. An increased metabolism of tamoxifen to agonistic compounds could explain the existence of tumors with a tamoxifen-stimulated phenotype. However, tamoxifen analogues with fixed ring structures, resistant to metabolic conversion into estrogenic metabolites, were also able stimulate cell proliferation [72,107-109]. Furthermore, tamoxifen metabolites acting as agonists are only found in low amounts in tumors so far [110,111]. However, accumulation of tamoxifen in the tumor does not always lead to the same level as the circulating concentration [112-114]. Tamoxifen may be bound to other binding proteins than the ER, designated antiestrogen binding sites [115,116]. Metabolism of tamoxifen may be responsible for some of the resistant tumor phenotypes, but there is no clear evidence that it can explain the majority of the cases with observed clinical tamoxifen resistance.

2.2 Alterations in ER expression, structure and function

Most of the actions of estrogens and antiestrogens are exerted via the ER. However, expression of ER in the (primary) tumor does not guarantee successful antiestrogen therapy. Half of the patients with ER-positive tumors do not respond to the drug despite the presence of a functional ER. Furthermore, over 50% of the tumors with acquired tamoxifen resistance continue to express ER and often PR, an indicator of a functional ER. Therefore, resistance cannot be explained simply by loss of ER expression [117-119]. ER-negative metastases may be the result of tumor heterogeneity and clonal selection of ER-negative tumor cells. ER may also be lost as a result of progression to a more malignant and metastatic tumor phenotype. Expression of ER is very heterogeneous in ER-positive primary tumors. Gross differences in ER expression levels and percentages of ER-positive cells are observed. Immunohistochemical analysis seldom shows biopsies containing 100% ER-positive tumor cells. Consequently this variation in expression may lead to the outgrowth of ER-negative tumor populations. Similar differences in expression levels were observed in ER-positive breast cancer cell lines. When these cell lines are subcloned, all resulting cell clones are ER-positive, again with heterogeneous expression, suggesting regulatory mechanism for the ER level [5,120].

Several groups of investigators have studied the ER gene at different cellular levels, i.e., at the genomic, the mRNA, and the protein level. Loss of heterozygosity at the ER locus has previously been reported [121] but does not correlate with ER status of the tumor [122]. Neither can the majority of the ER-negative tumors be explained by loss

of chromosome 6q [123]. Southern blot analysis of ER-negative breast tumors did not show major rearrangements in the ER locus, indicating that the loss of ER expression is not associated with large chromosomal rearrangements or deletions [124,125]. These results were confirmed by a more detailed study, using polymerase chain reaction (PCR) analysis of the ER gene. Analysis of all eight exons of ER-negative breast tumors did not show abnormalities in the size of the PCR product compared to ER-positive tumors [126]. Other investigators performed a detailed and sensitive analysis of the eight exons by single-strand conformation polymorphism (SSCP) analysis. This technique can detect point mutations and very small deletions or rearrangements, which may have been missed by the former studies. In a series of 188 breast tumors, including 70 ER-negative tumors, only two missense mutations were identified in the same ER-negative tumor. In another study, SSCP analysis of the ER cDNA of 20 tamoxifen-sensitive and 20 tamoxifen-resistant tumors, all ER-positive, revealed two mutations in the tamoxifen-resistant tumors, leading to a truncated ER with a defective hormone binding domain [127]. These studies show that mutations in the ER gene do occur, but at low frequency. They cannot account for the majority of ER-negative or tamoxifen-resistant breast tumors [128-130].

The DNA methylation status of CpG residues is an important factor regulating eukaryotic gene expression and differentiation. Although exceptions have been reported, for most genes there is an inverse relationship between the degree of DNA methylation and the level of expression (reviewed by [131-133]). Constitutively expressed housekeeping genes are generally present in a demethylated state. Genes with tissue-specific expression patterns are usually hypomethylated in the cells expressing the gene and hypermethylated in the cells lacking expression. Aberrations in methylation patterns and levels have been described in tumors and do play a role in the development of neoplasia [134]. Changes in the methylation pattern of the ER could result in altered gene expression, and this could influence the phenotype of the tumor. Methylation status was analyzed of both regulatory and coding sequences of the ER gene [125,135]. It was shown that DNA methylation of the ER gene is different in estrogen target and non-target tissues. In primary breast tumors, upstream sequences of the ER tend to be hypomethylated in ER-positive tumors and more methylated in ER-negative tumors. No association was observed between ER status of a tumor and different levels of methylation in the coding sequences of the gene. Another study [136] showed that in cell lines the NotI restriction enzyme site present in the 5' CpG island of the ER gene is methylated in ER-negative cell lines but not in ER-positive cell lines. Furthermore, a higher level of DNA methyltransferase was observed in ER-negative cell lines, suggesting an increased ability to methylate DNA, which may explain the loss of ER expression in cell lines. Recently, these *in vitro* observations were confirmed in biopsies of primary breast tumors. A significant correlation between aberrant methylation of the ER and the PR genes and lack of gene expression was shown [137]. This mechanism of gene silencing could account for loss of ER and PR gene expression in a significant proportion of breast tumors, resulting in antiestrogen resistance. If methylation changes are involved, demethylating agents possibly can be used to reinduce ER expression. From the therapeutic point of view, this approach is only meaningful when the tumor cells starting to reexpress the ER also loose their estrogen-independent phenotype.

In the last few years, much attention has been given to expression of variant ER

mRNAs. Several variant ER mRNAs have been detected in breast cancer cell lines, breast cancers, and in normal breast tissues [138-141]. The presence of ER variants derived from alternatively spliced mRNAs functioning as dominant positive, estrogen-independent transcription factors may induce hormone independent tumor growth. Expression of variant ER appears to be a process that occurs in every cell that expresses the primary transcript. Until now, it is not clear whether these alternatively spliced RNAs result in variant proteins, and the clinical significance remains to be established [142,143].

2.3 Overexpression of various genes

Overexpression of growth factors, growth factor receptors, or other components of signaling cascades leading to proliferation, may provide selective growth advantage to cells progressing along the malignant pathway. A possible role for suspected genes in the development of resistance might be established by overexpressing the gene of interest in hormone-dependent breast cancer cells. To this end, a number of candidate genes have been studied in transfection experiments, including the genes encoding Ras, EGF receptor, HER2, FGF4, RAF1, IGFII, cyclin D1 and MDR1.

Ras

One of the first candidate genes for hormone resistance examined by transfection experiments was v-HRas [144], the oncogene of the Harvey murine sarcoma virus and the homologue of human c-HRas. Isolation of members of the Ras oncogene family (HRas, KRas and NRas), as the transforming gene from genomic DNA of different human carcinomas, implicated an important role of this gene family in the development of neoplasia. In human neoplasms, Ras genes can be activated by single amino acid changes at codons 12, 13 or 61, or by 5- to 50- fold amplification of the wild type gene [145]. Ras plays a major role as a molecular switch in many signal transduction pathways, which lead to cell proliferation and differentiation. Ras is a GTPase that cycles between an inactive GDP-bound and an active GTP-bound form. Many ligands that stimulate cell surface receptors including EGF receptor, HER2 and FGF receptor, transduce their signals via Ras. Overexpression of Ras has been described in breast tumors. Also point mutations at amino acid codon 12 (glycine to valine) were detected in HRas. Mutations or overexpression may therefore play a role in tumorigenesis and progression of some breast tumors, although Ras mutations occur infrequently in breast cancer (less than 5%).

A possible role for activated Ras genes in hormone independence was studied by transfection of v-HRas into MCF-7 cells, which resulted in an ER-positive, estrogen-independent phenotype [144]. The v-HRas gene product differs from the c-HRas protein, p21, at two amino acid residues: glycine is replaced by arginine at position 12 and alanine is replaced by threonine at position 59. Overexpression or single mutation of c-HRas had no effect on hormone dependence [146]. Over the last few years many components of the Ras signal cascade have been identified [147,148]. It is possible that deregulation of the Ras signal transduction pathway via other mechanisms than mutation of Ras itself may play a role in the progression of breast cancer [149].

EGF receptor

In comparing hormone-dependent and hormone-independent breast cancer cell lines and primary tumors, the absence of ER and expression of a high level of EGF receptor in the latter group is often noted, as reviewed by [150]). The EGF receptor/ ErbB-1 gene is the cellular homologue of the v-ErbB oncogene, originally identified in avian erythroblastosis viruses [151,152]. The EGF receptor is a 170 kDa transmembrane glycoprotein with tyrosine kinase activity encoded by a gene on human chromosome 7p12 [153]. Overexpression of EGF receptor has been observed in several types of cancer and cancer-derived cell lines, such as squamous cell carcinomas of the lung, glioblastomas, gastric carcinomas, bladder carcinomas, phyllodes tumors, and ovary- and breast carcinomas. Overexpression of the recombinant EGF receptor cDNA into NIH-3T3 mouse fibroblasts lacking endogenous EGF receptor expression resulted in ligand-dependent transformation *in vitro* and *in vivo* [154,155].

EGF receptor protein has been detected in normal breast, breast cancers and breast cancer cell lines. Six different ligands that bind to the EGF receptor have been identified. These include EGF, transforming growth factor α (TGFα), amphiregulin, heparin-binding EGF, beta-cellulin and epiregulin [156]. Binding of a ligand to the EGF receptor results in receptor dimerization and activation of tyrosine kinase activity, leading to receptor autophosphorylation on multiple tyrosine residues [157]. Signaling proteins with Src homology 2 (SH2) domains bind to these tyrosine-phosphorylated residues, initiating multiple signaling cascades leading to stimulation of proliferation or differentiation [158]. EGF receptor can form heterodimers with the other members of the ErbB family. In combination with the different ligands, this may result in signal diversification, explaining the various functions of the ErbB family [159-161]. In approximately half of the human breast cancers, EGF receptor expression is detected (range 14-91%, dependent on the assay used) [150]. Expression of EGF receptor is inversely related to expression of ER in breast cancer cell lines and in primary breast tumors both on the mRNA and the protein level [5,150,162]. The presence of EGF receptor indicates a poor prognosis in primary breast cancer and correlates with failure of response to endocrine therapy in recurrent breast cancer [163,164].

Two reports have been published involving ectopic expression of EGF receptor in human ZR-75-1 breast cancer cells. The first study failed to demonstrate a relation between EGF receptor expression and hormone dependence [165]. However, our study showed that expression of EGF receptor could bypass hormone dependence [166]. Estrogen-dependent ZR-75-1 cells were infected with a retrovirus carrying the EGF receptor cDNA. The parental ZR-75-1 cells are fully estrogen-dependent for proliferation, and growth is inhibited in the presence of antiestrogens. The transduced cells, designated ZR/HERc, were ER-positive and EGF receptor-positive and acquired a proliferative response to EGF. Treatment with antiestrogens in the presence of EGF resulted in bypassing of estrogen-dependent growth. Furthermore, in contrast to the parental cells, prolonged antiestrogen treatment of ZR/HERc cells resulted in progression to estrogen independence and down-regulation of ER. Interference of ER and EGF receptor signal transduction pathways in ZR/HERc cells was observed when simultaneously activated [166]. Influences of ER on expression of EGF receptor were demonstrated by antisense ER experiments in MCF-7 cells.

Decrease in the level of ER resulted in up-regulation of EGF receptor mRNA [167]. These results may explain the observed inverse relationship of ER and EGF receptor in primary breast tumors and provide a potential link between loss of estrogen sensitivity and the acquisition of autonomous growth.

HER2

Another member of the ErbB family is HER2 (also called ErbB-2, neu or p185). This proto-oncogene encodes a 185 kDa transmembrane tyrosine kinase receptor with close similarity to the EGF receptor [168]. Amplification of the HER2 proto-oncogene, localized on human chromosome 17q21-22, leading to overexpression of the receptor protein is reported in approximately 20% of primary breast cancers. Overexpression can also occur from a single-copy gene (reviewed by [169]). HER2 can enhance and stabilize dimerization of other ErbB members, but a ligand has not been clearly identified. Overexpression of HER2 is thought to lead to dimerization of the receptor, resulting in its autophosphorylation and activation of the protein tyrosine kinase domain. Overexpression of HER2 is sufficient to transform mouse NIH 3T3 cells [170]. Overexpression of HER2 is inversely related to expression of ER and PR in breast cancer and is associated with high tumor grade. Furthermore, HER2 overexpression or gene amplification in breast tumors is associated with poor response to tamoxifen treatment in both ER-positive and ER-negative tumors [171-175].

To determine a possible causal role of HER2 in estrogen independence, MCF-7 cells were transfected with a HER2 cDNA construct. Cell clones showing over-expression of the recombinant gene, in comparison with expression levels observed in the parental cells, were no longer sensitive to tamoxifen. However, tumor take in nude mice of these transfectants was still estrogen-dependent, but tamoxifen could not inhibit further outgrowth of the tumors [176,177].

FGF4

Fibroblast growth factors (FGFs) are a family of proteins that are important in development and angiogenesis. FGFs are transforming in NIH-3T3 cells and have been implicated in tumorigenesis and metastasis of breast tumors [178,179]. Transfection of MCF-7 cells with FGF4 resulted in cell lines that are able of forming progressively growing, metastatic tumors in ovariectomized and tamoxifen-treated nude mice [180,181]. MCF-7/FGF4 cells are stimulated by tamoxifen and inhibited by estrogens. Whether this reflects some of the clinical observations remains to be established. Interestingly, FGF4 transfected cell lines could support growth and metastasis of the parental cell line, when both cell lines were coinjected in nude mice into the same site. This supports a role for paracrine factors in mixed populations of tumor cells of different states of malignant progression [182]. FGF expression may be important in the progression of breast tumors from estrogen dependence to estrogen independence, and in the development of a metastatic phenotype.

RAFI

One of the major pathways activated by the EGF receptor ligands is the mitogen-activated protein kinase (MAPK) phosphorylation cascade [158]. The activated receptor interacts with an activated Ras, resulting in subsequent activation of the RAFI proto-oncogene. RAFI is a serine threonine kinase that can directly phosphorylate nuclear

factors like p53, and indirectly transcription factors such as Myc, Elk and Rsh. MCF-7 cells were transfected with a RAFI expression construct containing an amino-truncated protein with constitutive kinase activity. Constitutive RAFI activity in MCF-7 cells is incompatible with growth in the presence of estrogen. However, under estrogen depleted conditions, RAF1 activity can stimulate growth [183].

IGFII

Insulin and insulin-like growth factors type I and II can stimulate growth of a number of breast cancer cell lines. These effects are mediated via the IGFI receptor which synergizes with estrogen in stimulating the growth of breast cancer cells *in vitro*. IGFI and IGFII are expressed by fibroblasts in benign and malignant breast tissue [184]. IGFII was overexpressed in estrogen-responsive MCF-7 cells to determine whether this would result in an autocrine growth mechanism. Increased expression, following transfection, of IGFII resulted in loss of estrogen dependence for proliferation and enhanced anchorage independent growth [105,185].

Cyclin D1

Cell cycle progression is controlled at a series of checkpoints to ensure orderly progress of cell division. The mechanism for these control points involves sequential transcriptional activation of cyclin genes, and consequent transient accumulation of different cyclins with cyclin-dependent kinases [186,187]. Deregulated expression of components of this strictly regulated system may lead to loss of normal growth control. Overexpression of cyclin D1 with or without amplification of the chromosomal locus 11q13 has been described for many tumors including breast cancer [188-192]. Treatment with antiestrogens of breast cancer cells *in vitro* results in rapid down-regulation of cyclin D1 mRNA expression, and G1 cell cycle arrest [31]. Therefore, overexpression of cyclin D1 could result in antiestrogen resistance. This hypothesis is supported by transfection experiments of cyclin D1 in breast cancer cells [193]. T-47D cells were chosen because they are ER-positive and there is no evidence for cyclin D1 overexpression or amplification. Overexpression of cyclin D1 was sufficient for growth factor-independent acceleration of cells into S-phase. Similar experiments were performed in MCF-7 cells with an inducible cyclin D1 expression construct [194]. Overexpression of cyclin D1 resulted in a continued proliferation potential under low-serum conditions, in contrast to control cells, which ceased to grow. This phenomenon is caused by a reduced cell cycle exit from G_1 to G_0 phase in the cells with overexpression of cyclin D1 [193]. Similar experiments performed with MCF-7 cells showed that growth of cyclin D1-overexpressing cells is still efficiently inhibited by antiestrogens [195]. Recently, it was suggested that cyclin D1 binds to the ER and activates its transcriptional activity independent of estrogen [196,197]. The role for overexpression of cyclin D1 in clinical breast cancer is contradictory with the experiments in T-47D cells. High levels of the protein are detected in well differentiated, ER-positive tumors, which respond well to tamoxifen treatment for metastatic disease [198,199].

MDR1/ p-glycoprotein

Multidrug resistance (MDR) is a major problem in the chemotherapeutic treatment of cancer [200]. Almost all breast cancers treated with cytotoxic chemotherapy develop multidrug resistance. Often this resistance is associated with high expression of the MDR1 gene, an energy-dependent efflux pump that reduces intracellular drug levels. If tamoxifen was a substrate of MDR1 this pump could be involved in antiestrogen resistance. MCF-7 cells transduced with the MDR1 gene exhibit a multidrug resistant phenotype. Nevertheless, overexpression of MDR1 did not result in cross-resistance to antiestrogens or loss of ER expression [201]. Although this study showed that transfection of MDR1 did not result in tamoxifen resistance in MCF-7 cells, some evidence exists for involvement of MDR1 in antiestrogen resistance. In the first study, a correlation was observed between p-glycoprotein level in tumors post treatment and response to tamoxifen [202]. In the second study, MDR1 mRNA expression in the primary tumor was found to correlate with resistance to hormone therapy [203].

CHAPTER 3

Concluding remarks

In Chapter 2, a variety of possible mechanisms causing antiestrogen resistance have been reviewed. For most of the described genes and mechanisms direct evidence for involvement in acquired tamoxifen resistance in clinical breast cancer is still required. Expression of EGF receptor and amplification of HER2 in primary tumors will predict a failure to respond to tamoxifen therapy [173,204]. The transfection experiments reviewed above have revealed important facts leading to a better understanding of tumor progression. The major drawback of this approach, however, is its limitation to known and candidate genes. Unknown genes with yet unknown functions are not addressed. Review of these studies strongly suggests that many different mechanisms may account for antiestrogen resistance. The molecular mechanisms responsible for clinical tamoxifen resistance in the majority of ER-negative and ER-positive breasts cancers still have to be elucidated. Further search for different gene products conferring antiestrogen resistance is needed.

Random search for genes involved in hormone independence may be accomplished by transfection of total cDNA libraries into hormone-dependent breast cancer cells. High quality libraries containing full-length cDNAs are required for this approach. Transfer of cDNAs causing tamoxifen resistance would result in estrogen-independent cell clones. Subsequently, the putative resistance gene may be identified and isolated using the expression vector sequences of the integrated cDNA.

Thus far, one study was published applying this strategy [205]. Mixtures of several cDNA libraries were transfected into tamoxifen sensitive MCF-7 cells. Tamoxifen selection of the transfected MCF-7 cells resulted in isolation of several resistant clones. One of these clones was further characterized, and had retained ER expression which is the phenotype of typical clinical tamoxifen resistance. Although this cell clone was isolated after cDNA transfection, involvement of the integrated cDNA has still to be established, and a spontaneous event, leading to antiestrogen resistance, has to be excluded. In Chapter 4 we discuss the use of retroviral insertional mutagenesis, to identify genes involved in antiestrogen resistance.

CHAPTER 4

Experimental work and discussion

The aim of the experimental work described in this thesis was to gain insight into the mechanisms involved in antiestrogen resistance in human breast cancer, and to develop tools for the identification of involved genes. To accomplish this, a random search for involved genes was initiated. An approach was chosen in which estrogen-dependent breast cancer cells were altered by retroviral insertion mutagenesis. Retroviruses integrate into the DNA of the host cell and introduce random mutations, which may result in altered gene expression. Selection of the infected cells in the presence of antiestrogens (4-hydroxy-tamoxifen) should allow cells, which have acquired a favorable mutation to proliferate. The integrated retrovirus serves as a tag, which facilitates the isolation of the involved gene near or at the integration site. To assess the feasibility of this approach different experiments were performed, described in Chapter 5.

Breast cancer cell lines for the study of hormone resistance

It is widely accepted that human breast cancers are initially dependent upon estrogens for development and progression [69,206]. During tumor progression eventually resulting in dissemination of the disease, estrogen-dependence is lost, and finally ER-negative tumor cell clones may emerge. The cellular and molecular mechanisms underlying these changes are poorly understood. Primary cultures of breast cancer specimens would provide ideal model systems for studying the progression of breast cancer. However, solid tumors are difficult to grow *in vitro*, and large-scale experiments are therefore at present not feasible [207]. Hence, cell lines derived from breast tumors are widely used instead. With some limitations they provide valuable models in the study of hormone resistance. Most breast cancer cell lines are ER-negative and are used for the study of hormone resistant disease. MCF-7, ZR-75-1, and T-47D are ER-positive cell lines, used in the study of the progression of hormone dependent breast cancer [105,208,209]. Both MCF-7 and ZR-75-1 cells express functional ER and are estrogen dependent for growth both *in vitro* and *in vivo*. The T-47D cell line is less suitable for our studies because of its genetic instability [210,211], which could result in a high rate of spontaneous development of resistant cell clones. The MCF-7 cell line is the most widely used model and many variant cell lines have been established [17,208,209]. Our initial experiments indicated that in comparison with MCF-7 cells, ZR-75-1 cells exhibited a more stable estrogen-dependent phenotype during prolonged large-scale culture experiments. For this reason ZR-75-1 cells were chosen for most of the experiments described in this thesis.

The ZR-75-1 human breast cancer cell line was first described by Engel et al. [212]. It was established from a patient treated for infiltrating ductal carcinoma by mastectomy and subsequent chemo- and antiestrogen therapy. Cells from a malignant ascites effusion were brought successfully into culture. This cell line is estrogen-dependent for growth, although the patient did not respond to treatment with tamoxifen. The ZR-75-1 cell line has been used for the study of hormone dependence in human breast cancer in many laboratories.

ZR-75-1 cells maintained in our laboratory are fully estrogen-dependent for proliferation *in vitro* and *in vivo* [166]. Depletion of estrogens or addition of an excess of

antiestrogens to the culture medium results in long-term growth inhibition of the cells. The rate of spontaneous progression of ZR-75-1 cells to hormone independence in the presence of 1 μM 4-hydroxy-tamoxifen (a more active metabolite of tamoxifen) was estimated to be less than 10^{-8} within a period of 5 weeks of selection [213]. This stable estrogen-dependent phenotype is a valuable characteristic of the ZR-75-1 cells for the study of mechanisms involved in the progression to hormone independence.

Ectopic expression of EGF receptor in ZR-75-1 cells.

In Chapter 5.1, we show that transduction of the EGF receptor to estrogen-dependent breast cancer cells is sufficient to bypass hormone dependence. This implicates that activation of a single gene is sufficient to induce hormone-resistance. This notion is important, because it is a prerequisite for successful retroviral insertional mutagenesis. Coordinate alteration of more than one gene in a single cell is technically not feasible in a restricted number of cells.

Epigenetic mechanisms in antiestrogen resistance.

In Chapter 5.2, we describe the effects of epigenetic changes in gene expression as a possible mechanism of development of hormone independence. Estrogen-dependent ZR-75-1 cells were treated with 5-azacytidine to cause DNA demethylation. This may result in activation of genes controlled by CpG containing promoters. High numbers of cell clones developed upon treatment with 5-azacytidine in the absence of estradiol or in the presence of antiestrogens. Expression of ER, PR and pS2 (two estrogen-responsive genes) was down-regulated and expression of EGF receptor and HER2 was up-regulated in these ZR/AZA cells. Proliferation of ZR/AZA cells could be inhibited with an EGF receptor-blocking antibody, suggesting an autocrine growth mechanism. Our data show that hormone resistance may be caused by epigenetic mechanisms, affecting a large proportion of the cells simultaneously.

Expression of ER, PR, and EGF receptor in breast tumors.

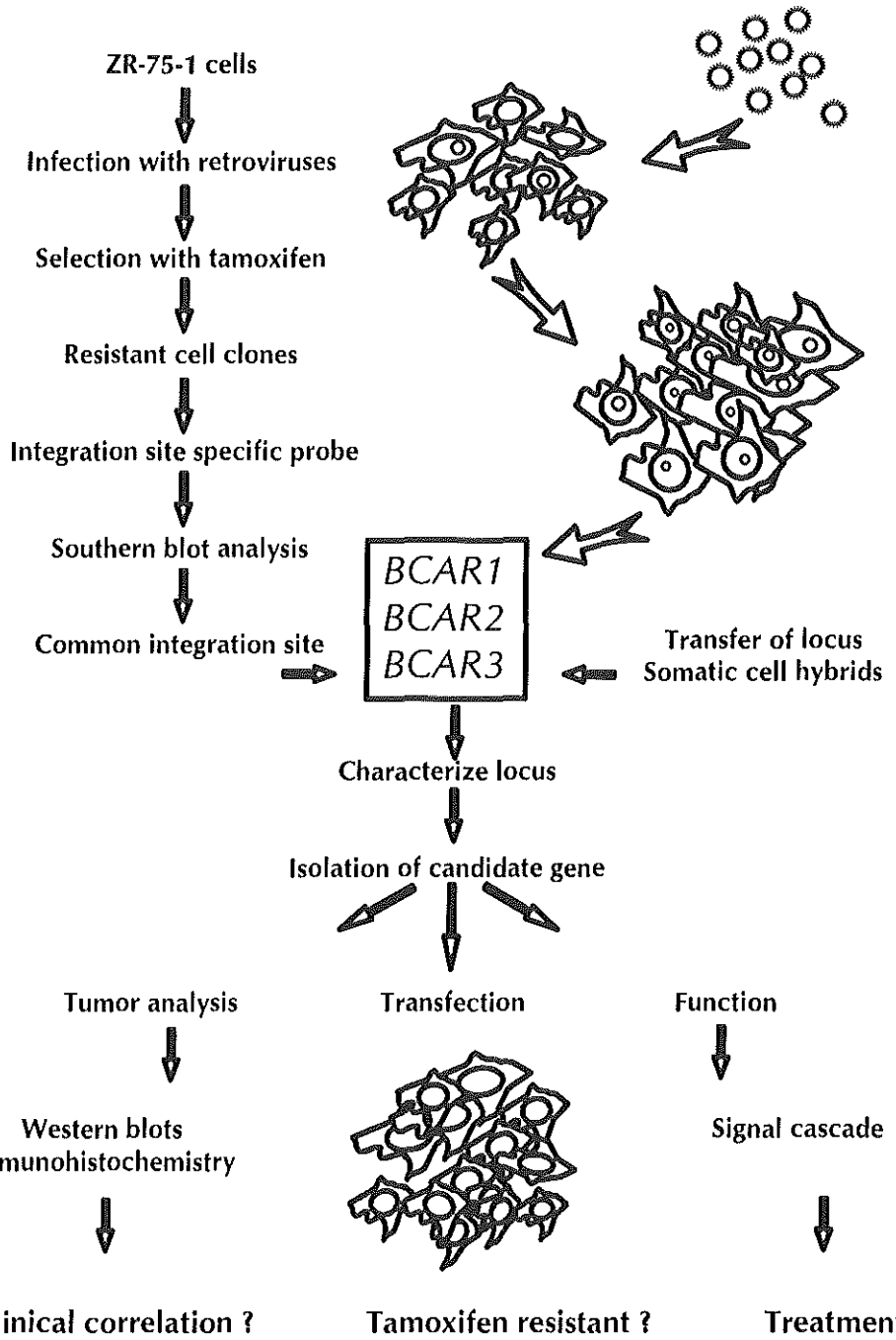
Chapters 5.3 and 5.4: It has been shown that EGF receptor is expressed in tumors unresponsive to endocrine therapy. As described in Chapter 5.1, interference of ER and EGF receptor signal transduction pathways was observed when both receptors were activated simultaneously. Furthermore, ER and EGF receptor double positive cells rapidly progressed to a hormone-resistant phenotype. EGF receptor expression is inversely related to expression of ER in primary breast tumors. However, approximately 50% of ER-positive tumors express EGF receptor. It was not clear whether these two different receptors were simultaneously expressed in individual cells. In Chapters 5.3 and 5.4 we describe the expression patterns of ER, PR, and EGF receptor at the single cell level in a series of primary breast tumors and non-malignant breast tissue biopsies. The observations were extended to a comparison of expression patterns in primary tumors and their metastases. It was shown that coexpression of ER and EGF receptor, and of PR and EGF receptor in tumor cells is a rare phenomenon, but does occur in non-malignant epithelial breast cells. Expression of ER and PR was determined at the single cell level in both primary tumors and their corresponding lymph node metastases, or in synchronously or metachronously

occurring bilateral breast cancer. Surprisingly, little differences were observed between steroid receptor expression of the primary tumor and the corresponding metastasis, or in bilateral breast cancers [5,214]. This indicates that loss of ER is not a prerequisite for tumor spread to the lymph nodes, and may represent a late event in tumor progression and development of hormone-resistance.

Retroviral insertional mutagenesis.

In Chapter 5.5, our approach to unravel the mechanisms of antiestrogen resistance by retroviral insertional mutagenesis and the first common integration site linked with antiestrogen resistance are described. A random search for involved genes by retroviral insertional mutagenesis was performed. Retroviruses may act as naturally occurring mutagens, because integration of proviruses into the DNA of a host cell is part of their life cycle [215]. Insertion of the provirus results in rearrangement of the chromosomal DNA of the host cell, which may lead to alteration of gene expression. Genes in the vicinity of or at the integration site may be activated, inactivated or truncated by the retrovirus. Consequently, retroviruses may mimic (epi-)genetic changes leading to tumor progression. Development of convenient retroviral vectors [216] and safe packaging cell lines [217] have made retroviral tagging an attractive tool for *in vitro* mutagenesis. Retroviruses show little selectivity for specific DNA sequences, which is a prerequisite for random mutagenesis [218]. Another favorable property of the retroviruses for gene targeting is the reported selection for transcriptionally active regions in the genome [219]. Furthermore, infection is a highly efficient process, compared with frequencies obtainable by stable transfection of plasmids. In mouse models, many proto-oncogenes have been identified as key genes involved in tumorigenesis by insertional mutagenesis (reviewed by [220]). Three major mechanisms for up-regulation of gene expression have been identified; promoter insertion, readthrough activation and enhancer insertion (reviewed by [220,221]). Inactivation of gene expression by retroviruses is a less likely mechanism due to the presence of the second allele, or even additional chromosomes in the situation of polyploidy, which is often observed in established cell lines. Comparison of the genomic DNA of a number of related tumors or cell lines may show the existence of common integration sites. This term refers to a particular locus that is occupied by proviruses in different independent tumors or cell lines. The presence of a retroviral genome integrated in a specific locus of several independently arisen tumors or cell lines strongly suggests that a specific gene function was altered by the viral integration, which resulted in the development of the tumor or the outgrowth of a cell clone. Our efforts to infect as many as possible cells in a particular experiment has the disadvantage that a single cell clone may have been infected by a number of retroviruses not all contributing to the altered phenotype. Identification of a common integration site facilitates the identification of the responsible locus involved in a specific process. The retrovirus with its unique DNA sequence in the host cell serves as a tag to isolate the locus and the involved gene.

Identification of genes involved in tamoxifen resistance



BCAR1 locus

Retroviral insertional mutagenesis and selection in the presence of tamoxifen resulted in eighty tamoxifen resistant cell clones. Search for common integration sites resulted in the identification of BCAR1 (Chapter 5.5). Further evidence for the involvement of this locus in tamoxifen resistance was obtained by somatic cell hybrid experiments. Cell hybrids of parental ZR-75-1 cells and one of the BCAR1 clones showed that the resistant phenotype co-segregated with the BCAR1 locus. Molecular cloning of the gene adjacent to the integrations and subsequent expression analysis showed that it was up-regulated in the BCAR1 clones compared to the ZR-75-1 cells. Sequence comparison analysis demonstrated that BCAR1 is the human homologue of the rat p130 Crk-associated substrate (CAS) gene (A. Brinkman et al.; manuscript in preparation). $p130^{CAS}$ has the structural characteristics of an adapter gene [222-224]. It was localized on human chromosome 16q22-23, by fluorescent in situ hybridization (T. van Agthoven, unpublished results). Distinct signal transduction pathways have been identified that led to tyrosine phosphorylation of $p130^{CAS}$ suggesting that it may play a role in integrin- [225,226] and mitogen-mediated signal transduction [227]. The exact role of BCAR1 in development of hormone resistance has to be determined.

BCAR2 locus

Further experiments involving somatic cell fusion identified a second resistance locus BCAR2 affected in two cell lines of the panel [228]. Recently a possible candidate gene in this locus was isolated using cDNA selection, screening of cDNA libraries, and RACE strategies. Northern analysis with these cDNA probes identified transcripts of 5 and 8 kb in BCAR2 cell lines and in other human tissues. Transfection experiments to demonstrate the role of this candidate gene are progress (J. Veldscholte, unpublished results).

BCAR3 locus

In Chapter 5.6 we report on the cloning and partial characterization of the BCAR3 gene, involved in tamoxifen resistance [229]. The BCAR3 locus was identified as a common integration site in six independently derived cell clones with a proviral integration in a 5 kb region, strongly suggesting that this locus carries the gene involved in tamoxifen resistance. Furthermore, cell fusion experiments showed that transfer of this locus confers tamoxifen resistance to the tamoxifen-sensitive recipient cells. Screening of Northern blots with the common integration site-specific DNA probe identifies a transcript in estrogen-independent MDA-MB-231 breast cancer cells and in different normal human tissues. This 3,4 kb mRNA is up-regulated in the antiestrogen-resistant cell lines in comparison with the expression level observed in the parental ZR-75-1 cells. The cDNA was isolated from a testis library. Transfection of a BCAR3 cDNA construct induces antiestrogen resistance in ZR-75-1 and MCF-7 cells. Sequence comparisons of the BCAR3 cDNA clone show that this transcript represents a novel gene containing an SH2 domain, suggesting a role in signal transduction. Another domain shows some homology with a part of the yeast CDC48 gene which has a role in cell division and apoptosis.

Cumulative data from *in vitro* studies and analyses of tumor biopsies strongly suggest that many different mechanisms may account for antiestrogen resistance. The molecular mechanisms that are operative in clinical tamoxifen resistance still have to be explained for the majority of ER-negative and ER-positive breast cancers. Presently, three new genes that play a role in tamoxifen resistance *in vitro* have been identified in our laboratory. We have shown that retroviral insertional mutagenesis is a useful approach to identify (novel) genes involved in antiestrogen resistance. The identification of the BCAR1/p130^{CAS} and BCAR2 and BCAR3 genes may provide important new insights in the biology of breast cancer. Further search for different gene products conferring antiestrogen resistance is needed. They may be identified from our panel of cell lines. It is essential to establish the role of these gene products in clinical breast cancer. Furthermore, it will be of great importance to establish which signaling cascades, leading to proliferation, are active in hormone-resistant breast cancer cells. Therapeutic strategies may ultimately be developed intervening with these escape routes of the tumor cells.

References

1. Moll R, Franke WF, Schiller DL, tumors and cultured cells. The catalog of human cytokeratins: patterns of expression in normal epithelia. *Cell* 1982; 31:11-24.
2. Ramaekers F, Huysmans A, Schaart G, Moesker O, Vooijs P. Tissue distribution of keratin 7 as monitored by a monoclonal antibody. *Exp Cell Res* 1987; 170:235-249.
3. Trask DK, Band V, Zajchowski DA, Yaswen P, Suh T, Sager R. Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. *Proc Natl Acad Sci USA* 1990; 87:2319-2323.
4. Bocker W, Bier W, Freytag G, Brommelkamp B, Jarasch ED, Edel G, Dockhorn-Dworniczak B, Schmid KW. An immunohistochemical study of the breast using antibodies to basal and luminal keratins, alpha-smooth muscle actin, vimentin, collagen IV and laminin. Part I: Normal breast and benign proliferative lesions. *Virchows Arch A Pathol Anat Histopathol* 1992; 421:315-322.
5. Van Agthoven T, Timmermans M, Foekens JA, Dorssers LCJ, Henzen-Logmans SC. Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry. *Am J Pathol* 1994; 144:1238-1246.
6. Perez-Mesa CM. Gross and Microscopic Pathology. In: Donegan WL, Spratt JS., eds. Cancer of the Breast. Philadelphia: W.B. Saunders Company, 1995; 240-278.
7. Page DL, Anderson TJ. Diagnostic histopathology of the breast. New York: Churchill Livingstone, 1987.
8. Early Breast Cancer Trial Collab Group . Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 Randomised trials involving 31 000 recurrences and 24 000 deaths among 75 000 women. *Lancet* 1992; 339:1-15-71-85.
9. Malkin D. Germline *p53* mutations and heritable cancer. *Annu Rev Genet* 1994; 28:443-465.
10. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, Bell R, Rosenthal J, Hussey C, Tran T, McClure M, Frye C, Hattier T, Phelps R, Haugen-Strano A, Katcher H, Yakumo K, Gholami Z, Shaffer D, Stone S. A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science* 1994; 266:66-71.
11. Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G, Barfoot R, Hamoudi R, Patel S, Rice C, Biggs P, Hashim Y, Smith A, Connor F, Arason A, Gudmundsson J, Ficenec D, Kelsell D, Ford D, Tonin P. Identification of the breast cancer susceptibility gene BRCA2. *Nature* 1995; 378:789-792.
12. Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Clines GA, Sartiel A,

Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NGJ, Shiloh Y. A single ataxia telangiectasia gene with a product similar to PI- 3 kinase. *Science* 1995; 268:1749-1753.
13. McPherson K, Steel CM, Dixon JM. ABC of Breast Diseases: Breast cancer–Epidemiology, risk factors, and genetics. *BMJ* 1994; 309:1003-1006.
14. Spratt JS, Donegan WL, Sigdestad CP. Epidemiology and Etiology. In: Donegan WL, Spratt JS., eds. Cancer of the Breast. Philadelphia: W.B. Saunders Company, 1995; 116-141.
15. Henderson BE, Ross R, Bernstein L. Estrogens as a cause of human cancer: The Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res* 1988; 48:246-253.
16. King RJB. Estrogen and progestin effects in human breast carcinogenesis. *Breast Cancer Res Treat* 1993; 27:3-15.
17. Clarke R, Skaar T, Baumann K, Leonessa F, James M, Lippman J, Thompson EW, Freter C, Brunner N. Hormonal carcinogenesis in breast cancer: Cellular and molecular studies of malignant progression. *Breast Cancer Res Treat* 1994; 31:237-248.
18. Boyd M, Hildebrandt RH, Bartow SA. Expression of the estrogen receptor gene in developing and adult breast. *Breast cancer Res Treat* 1996; 37:243-251.
19. Foekens JA, Van Putten WLJ, Portengen H, De Koning HYWCM, Thirion B, Alexieva-Figusch J, Klijn JGM. Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: Multivariate analysis. *J Clin Oncol* 1993; 11:899-908.
20. Spyratos F, Andrieu C, Hacène K, Chambon P, Rio MC. pS2 and response to adjuvant hormone therapy in primary breast cancer. *Br J Cancer* 1994; 69:394-397.
21. Dickson RB, Lippman ME. Estrogenic regulation of growth and polypeptide growth factor excretion in human breast carcinoma. *Endocr Rev* 1987; 8:29-43.
22. Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma. Suggestions for a new method of treatment with illustrative cases. *Lancet* 1896; 2:104-107.
23. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eight Cain memorial award lecture. *Cancer Res* 1990; 50:4177-4189.
24. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI146474. *Br J Cancer* 1971; 25:270-275.
25. Jordan VC, Murphy CS. Endocrine pharmacology of antiestrogens as antitumor agents. *Endocr Rev* 1990; 11:578-610.
26. Jordan VC. Molecular mechanisms of antiestrogen action in breast cancer. *Breast Cancer Res Treat* 1994; 31:41-52.
27. Wolf DM, Fuqua SAW. Laboratory-Clinic Interface: Mechanisms of action of antiestrogens. *Cancer Treatm Rev* 1996; 21:247-271.
28. Reese JC, Katzenellenbogen BS. Examination of the DNA-binding ability of estrogen receptor in whole cells: Implications for hormone-independent transactivation and the actions of antiestrogens. *Mol Cell Biol* 1992; 12:4531-4538.
29. Sutherland RL, Lee CSL, Feldman RS, Musgrove EA. Regulation of breast cancer cell cycle progression by growth factors, steroids and steroid antagonists. *J Steroid Biochem Mol Biol* 1992; 41:315-321.
30. Musgrove EA, Hamilton JA, Lee CSL, Sweeney KJE, Watts CKW, Sutherland RL. Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. *Mol Cell Biol* 1993; 13:3577-3587.
31. Watts CKW, Sweeney KJE, Warlters A, Musgrove EA, Sutherland RL. Antiestrogen regulation of cell cycle progression and cyclin D1 gene expression in MCF-7 human breast cancer cells. *Breast Cancer Res Treat* 1994; 31:95-105.
32. Watts CKW, Brady A, Sarcevic B, DeFazio A, Musgrove EA, Sutherland RL. Antiestrogen inhibition of cell cycle progression in breast cancer cells is associated with inhibition of cyclin-dependent kinase activity and decreased retinoblastoma protein phosphorylation. *Mol Endocrinol* 1995; 9:1804-1813.
33. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. *Science* 1997; 278:1073-1077.
34. McKeon VA. The Breast Cancer Prevention Trial: evaluating tamoxifen's efficacy in preventing breast cancer. *J Obstet Gynecol Neonatal Nurs* 1997; 26:79-90.
35. Bruzzi P. Tamoxifen for the prevention of breast cancer - Important questions remain unanswered, and existing trials should continue. *Br Med J* 1998; 316:1181-1182.

36. Jordan VC. Long-term adjuvant tamoxifen therapy for breast cancer. *Breast Cancer Res Treat* 1990; 15:125-136.
37. Ramkumar T, Adler S. Differential positive and negative transcriptional regulation by Tamoxifen. *Endocrinology* 1995; 136:536-542.
38. Wright CDP, Mansell RE, Gazet JC, Compston JE. Effect of long term tamoxifen treatment on bone turnover in women with breast cancer. *BMJ* 1993; 306:429-430.
39. Kristensen B, Ejlertsen B, Dalgaard P, Larsen L, Holmegaard SN, Transbol I, Mouridsen HT. Tamoxifen and bone metabolism in postmenopausal low-risk breast cancer patients: A randomized study. *J Clin Oncol* 1994; 12:992-997.
40. Wiseman H. Taking tamoxifen to heart. *Nature Med* 1995; 1:1226.
41. Costantino JP, Kuller LH, Ives DG, Fisher B, Dignam J. Coronary heart disease mortality and adjuvant tamoxifen therapy. *J Natl Cancer Inst* 1997; 89:776-782.
42. Morgan RW. Risk of endometrial cancer after tamoxifen treatment. *Oncology* 1997; 11:25-33.
43. Colletta AA, Benson JR, Baum M. Alternative mechanisms of action of anti-oestrogens. *Breast Cancer Res Treat* 1994; 31:5-9.
44. Kiss Z. Tamoxifen stimulates phospholipase D activity by an estrogen receptor-independent mechanism. *FEBS Lett* 1994; 355:173-177.
45. Zhang JJ, Jacob TJC, Valverde MA, Hardy SP, Mintenig GM, Sepúlveda FV, Gill DR, Hyde SC, Trezise AEO, Higgins CF. Tamoxifen blocks chloride channels. A possible mechanism for cataract formation. *J Clin Invest* 1994; 94:1690-1697.
46. Hoelting T, Duh QY, Clark OH, Herfarth C. Tamoxifen antagonizes proliferation and invasion of estrogen receptor-negative metastatic follicular thyroid cancer cells via protein kinase C. *Cancer Lett* 1996; 100:89-93.
47. Thompson AM, Kerr DJ, Steel CM. Transforming growth factor Beta1 is implicated in the failure of tamoxifen therapy in human breast cancer. *Br J Cancer* 1991; 63:609-614.
48. Knabbe C, Zugmaier G, Schmahl M, Dietel M, Lippman ME, Dickson RB. Induction of transforming growth factor beta by the antiestrogens droloxifene, tamoxifen, and toremifene in MCF-7 cells. *Am J Clin Oncol* 1997; 14 Suppl 2:15-20.
49. Butta A, MacLennan K, Flanders KC, Sacks NPM, Smith I, McKinna A, Dowsett M, Wakefield LM, Sporn MB, Baum M, Colletta AA. Induction of transforming growth factor Beta1 in human breast cancer in vivo following tamoxifen treatment. *Cancer Res* 1992; 52:4261-4264.
50. Ruan W, Newman CB, Kleinberg DL. Intact and amino-terminally shortened forms of insulin-like growth factor I induce mammary gland differentiation and development. *Proc Natl Acad Sci USA* 1992; 89:10872-10876.
51. Paik S. Expression of IGF-I and IGF-II mRNA in breast tissue. *Breast Cancer Res Treat* 1992; 22:31-38.
52. Chander SK, McCague R, Luqmani Y, Newton C, Dowsett M, Jarman M, Coombes RC. Pyrrolidino-4-iodotamoxifen and 4-iodotamoxifen, new analogues of the antiestrogen tamoxifen for the treatment of breast cancer. *Cancer Res* 1991; 51:5851-5858.
53. Wakeling AE, Bowler J. ICI 182,780, a new antioestrogen with clinical potential. *J Steroid Biochem Mol Biol* 1992; 43:173-177.
54. Rauschning W, Pritchard KI. Droloxifene, a new antiestrogen: Its role in metastatic breast cancer. *Breast Cancer Res Treat* 1994; 31:83-94.
55. Dauvois S, Danielian PS, White R, Parker MG. Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. *Proc Natl Acad Sci USA* 1992; 89:4037-4041.
56. Fujimoto N, Katzenellenbogen BS. Alteration in the agonist/antagonist balance of antiestrogens by activation of protein kinase A signaling pathways in breast cancer cells: Antiestrogen selectivity and promoter dependence. *Mol Endocrinol* 1994; 8:296-304.
57. McDonnell DP, Clemm DL, Hermann T, Goldman ME, Pike JW. Analysis of estrogen receptor function *in vitro* reveals three distinct classes of antiestrogens. *Mol Endocrinol* 1995; 9:659-669.
58. Thompson EW, Katz D, Shima TB, Wakeling AE, Lippman ME, Dickson RB. ICI 164,384, a pure antagonist of estorgen-stimulated MCF-7 cell proliferation and invasiveness. *Cancer Res* 1989; 49:6929-6934.
59. DeFriend DJ, Anderson E, Bell J, Wilks DP, West CML, Mansel RE, Howell A. Effects of 4-hydroxytamoxifen and a novel pure antioestrogen (ICI 182780) on the clonogenic growth of human breast cancer cells *in vitro*. *Br J Cancer* 1994; 70:204-211.

60. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, McCue BL, Wakeling AE, McClelland RA, Manning DL, Nicholson RI. Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. *JNCI* 1995; 87:746-750.
61. DeFriend DJ, Howell A, Nicholson RI, Anderson E, Dowsett M, Mansel RE, Blamey RW, Bundred NJ, Robertson JF, Saunders C, Baum M, Walton P, Sutcliffe F, Wakeling AE. Investigation of a new pure antiestrogen (ICI 182780) in women with primary breast cancer. *Cancer Res* 1994; 54:408-414.
62. Howell A, Robertson J. Response to a specific antioestrogen (ICI 182780) in tamoxifen- resistant breast cancer. *Lancet* 1995; 345:989-990.
63. DeSombre ER, Thorpe SM, Rose C, Blough RR, Andersen KW, Rasmussen BB, King WJ. Prognostic usefulness of estrogen receptor immunohistochemical assays for human breast cancer. *Cancer Res* 1986; 46:4256S-4264S.
64. McClelland RA, Berger U, Miller LS, Powles TJ, Jensen EV, Coombes RC. Immunocytochemical assay for estrogen receptor: relationship to outcome of therapy in patients with advanced breast cancer. *Cancer Res* 1986; 46:4241S-4243S.
65. Henderson BE, Ross RK, Pike MC. Toward the primary prevention of cancer. *Science* 1991; 254:1131-1138.
66. Ravdin PM, Green S, Dorr TM, McGuire WL, Fabian C, Pugh RP, Carter RD, Rivkin SE, Borst JR, Belt RJ, Metch B, Osborne CK. Prognostic significance of progesterone receptor levels in estrogen receptor-positive patients with metastatic breast cancer treated with tamoxifen: Results of a prospective Southwest Oncology Group study. *J Clin Oncol* 1992; 10:1284-1291.
67. King RJB. Receptors, growth factors and steroid insensitivity of tumours. *J Endocrinol* 1990; 124:179-181.
68. Johnston SRD, Dowsett M, Smith IE. Towards a molecular basis for tamoxifen resistance in breast cancer. *Ann Oncol* 1992; 3:503-511.
69. Horwitz KB. Mechanisms of hormone resistance in breast cancer. *Breast Cancer Res Treat* 1993; 26:119-130.
70. Wolf DM, Jordan VC. Drug resistance to tamoxifen during breast cancer therapy. *Breast Cancer Res Treat* 1993; 27:27-40.
71. Klijn JGM, Berns EMJJ, Dorssers LCJ, Foekens JA. Molecular markers of resistance to endocrine treatment of breast cancer. In: Dickson RB, Lippman ME., eds. Drug and hormonal resistance in breast cancer,part II: cellular and molecular mechanisms. Chichester: Ellis Horwood Ltd, 1994; 133-168.
72. Osborne CK, Fuqua SAW. Mechanisms of tamoxifen resistance. *Breast Cancer Res Treat* 1994; 32:49-55.
73. Clarke R, Brünner N. Cross-resistance and molecular mechanisms in antiestrogen resistance. *Endocrine-Related Cancer* 1995; 2:59-72.
74. Dowsett M. Endocrine resistance in advanced breast cancer. *Acta Oncol* 1996; 35:91-95.
75. Katzenellenbogen BS, Montano MM, Ekena K, Herman ME, McInerney EM. Antiestrogens: mechanisms of action and resistance in breast cancer. *Breast cancer Res Treat* 1997; 44:23-38.
76. Henderson BE, Ross RK, Pike MC. Hormonal chemoprevention of cancer in women. *Science* 1993; 259:633-638.
77. Evans RM. The steroid and thyroid hormone receptor superfamily. *Science* 1988; 240:889-895.
78. Beato R. Gene regulation by steroid hormones. *Cell* 1989; 56:335-344.
79. Gronemeyer H. Transcription activation by estrogen and progesterone receptors. *Annu Rev Genet* 1991; 25:89-123.
80. Parker MG, Arbuckle N, Dauvois S, Danielian P, White R. Structure and function of the estrogen receptor. *Ann NY Acad Sci* 1993; 684:119-126.
81. Truss M, Beato M. Steroid hormone receptors: Interaction with deoxyribonucleic acid and transcription factors. *Endocr Rev* 1993; 14:459-479.
82. Beato M, Herrlich P, Schütz G. Steroid hormone receptors: Many actors in search of a plot. *Cell* 1995; 83:851-857.
83. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: The second decade. *Cell* 1995; 83:835-839.
84. Kumar V, Chambon P. The estrogen receptor binds tightly to its responsive element as a

ligand-induced homodimer. *Cell* 1988; 55:145-156.
85. Meyer ME, Gronemeyer H, Turcotte B, Bocquel MT, Tasset D, Chambon P. Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell* 1989; 57:433-442.
86. Cavaillès V, Cauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ, Parker MG. Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J* 1995; 14:3741-3751.
87. Hanstein B, Eckner R, DiRenzo J, Halachmi S, Liu H, Searcy B, Kurokawa R, Brown M. p300 is a component of an estrogen receptor coactivator complex. *Proc Natl Acad Sci U S A* 1996; 93:11540-11545.
88. McInerney EM, Tsai MJ, O'Malley BW, Katzenellenbogen BS. Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. *Proc Natl Acad Sci USA* 1996; 93:10069-10073.
89. Menasce LP, White GR, Harrison CJ, Boyle JM. Localisation of the estrogen receptor locus (ESR) to chromosome 6q25.1 by FISH and a simple post-FISH banding technique. *Genomics* 1993; 17:263-265.
90. Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P. Functional domains of the human estrogen receptor. *Cell* 1987; 51:941-951.
91. Katzenellenbogen JA, O'Malley BW, Katzenellenbogen BS. Tripartite steroid hormone receptor pharmacology: Interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. *Mol Endocrinol* 1996; 10:119-131.
92. Montano MM, Müller V, Trobaugh A, Katzenellenbogen BS. The carboxy-terminal F domain of the human estrogen receptor: Role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol Endocrinol* 1995; 9:814-825.
93. Mosselman S, Polman J, Dijkema R. ERβ: Identification and characterization of a novel human estrogen receptor. *FEBS Lett* 1996; 392:49-53.
94. Kuiper GGJM, Gustafsson J. The novel estrogen receptor-β subtype: Potential role in the cell- and promoter-specific actions of estrogens and anti- estrogens. *FEBS Lett* 1997; 410:87-90.
95. Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. *Science* 1997; 277:1508-1510.
96. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguère V. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. *Mol Endocrinol* 1997; 11:353-365.
97. Dotzlaw H, Leygue E, Watson PH, Murphy LC. Expression of estrogen receptor-*beta* in human breast tumors. *J Clin Endocrinol Metab* 1997; 82:2371-2374.
98. Pettersson K, Grandien K, Kuiper GGJM, Gustafsson J. Mouse estrogen receptor β forms estrogen response element- binding heterodimers with estrogen receptor α. *Mol Endocrinol* 1997; 11:1486-1496.
99. Cowley SM, Hoare S, Mosselman S, Parker MG. Estrogen receptors α and β form heterodimers on DNA. *J Biol Chem* 1997; 272:19858-19862.
100. Sato T, Akiyama F, Sakamoto G, Kasumi F, Nakamura Y. Accumulation of genetic alterations and progression of primary breast cancer. *Cancer Res* 1991; 51:5794-5799.
101. Remvikos Y, Gerbault-Seureau M, Magdelenat H, Prieur M, Dutrillaux B. Proliferative activity of breast cancers increases in the course of genetic evolution as defined by cytogenetic analysis. *Breast Cancer Res Treat* 1992; 23:43-49.
102. O'Connell P, Pekkel V, Fuqua S, Osborne CK, Allred DC. Molecular genetic studies of early breast cancer evolution. *Breast Cancer Res Treat* 1994; 32:5-12.
103. Dorssers LCJ, Van Agthoven T, Sieuwerts AM. Genetic mechanisms involved in progression to hormone independence of human breast cancer. In: Berns PMJJ, Romijn JC, Schroeder FH., eds. Mechanisms of progression to hormone-independent growth of breast and prostatic cancer. Carnforth, UK: Parthenon Publishing Group Ltd, 1991; 169-182.
104. Clarke R, Thompson EW, Leonessa F, Lippman J, McGarvey M, Frandsen TL, Brünner N. Hormone resistance, invasiveness, and metastatic potential in breast cancer. *Breast Cancer Res Treat* 1993; 24:227-239.
105. Darbre PD, Daly RJ. Transition of human breast cancer cells from an oestrogen responsive state. *J Steroid Biochem Mol Biol* 1990; 37:753-763.
106. Van Agthoven T, Van Agthoven TLA, Dekker A, Foekens JA, Dorssers LCJ. Induction of estrogen

independence of ZR-75-1 human breast cancer cells by epigenetic alterations. *Mol Endocrinol* 1994; 8:1474-1483.
107. Osborne CK. Mechanisms for tamoxifen resistance in breast cancer: Possible role of tamoxifen metabolism. *J Steroid Biochem Mol Biol* 1993; 47:83-89.
108. Wolf DM, Langan-Fahey SM, Parker CJ, McCague R, Jordan VC. Investigation of the mechanism of tamoxifen-stimulated breast tumor growth with nonisomerizable analogues of tamoxifen and metabolites. *JNCI* 1993; 85:806-812.
109. Wolf DM, Jordan VC. Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. *Breast Cancer Res Treat* 1994; 31:117-127.
110. Wiebe VJ, Osborne CK, McGuire WL, DeGregorio MW. Identification of estrogenic tamoxifen metabolite(s) in tamoxifen-resistant human breast tumors. *J Clin Oncol* 1992; 10:990-994.
111. Osborne CK. Tamoxifen metabolism as a mechanism for resistance. *Endocrine-Related Cancer* 1995; 2:53-58.
112. Osborne CK, Wiebe VJ, McGuire WL, Ciocca DR, DeGregorio MW. Tamoxifen and the isomers of 4-hydroxytamoxifen in tamoxifen- resistant tumors from breast cancer patients. *J Clin Oncol* 1992; 10:304-310.
113. Johnston SRD, Haynes BP, Sacks NPM, McKinna JA, Griggs LJ, Jarman M, Baum M, Smith IE, Dowsett M. Effect of oestrogen receptor status and time on the intra- tumoural accumulation of tamoxifen and N-desmethyltamoxifen following short-term therapy in human primary breast cancer. *Breast Cancer Res Treat* 1993; 28:241-250.
114. Johnston SRD, Haynes BP, Smith IE, Jarman M, Sacks NPM, Ebbs SR, Dowsett M. Acquired tamoxifen resistance in human breast cancer and reduced intra-tumoral drug concentration. *Lancet* 1993; 342:1521-1522.
115. Pavlik EJ, Nelson K, Srinivasan S, Powell DE, Kenady DE, DePriest PD, Gallion HH, Van Nagell JR,Jr.. Resistance to tamoxifen with persisting sensitivity to estrogen: Possible mediation by excessive antiestrogen binding site activity. *Cancer Res* 1992; 52:4106-4112.
116. Parisot JP, Hu XF, Sutherland RL, Wakeling A, Zalcberg JR, DeLuise M. The pure antiestrogen ICI 182,780 binds to a high-affinity site distinct from the estrogen receptor. *Int J Cancer* 1995; 62:480-484.
117. Taylor RE, Powles TJ, Humphreys J, Bettelheim R, Dowsett M, Casey AJ, Neville AM, Coombes RC. Effects of endocrine therapy on steroid-receptor content of breast cancer. *Br J Cancer* 1982; 45:80-85.
118. Johnston SRD, Saccani-Jotti G, Smith IE, Salter J, Newby J, Coppen M, Ebbs SR, Dowsett M. Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. *Cancer Res* 1995; 55:3331-3338.
119. Robertson JFR. Oestrogen receptor: A stable phenotype in breast cancer. *Br J Cancer* 1996; 73:5-12.
120. Palmari J, Dussert C, Berthois Y, Penel C, Martin PM. Distribution of estrogen receptor heterogeneity in growing MCF-7 cells measured by quantitative microscopy. *Cytometry* 1997; 27:26-35.
121. Devilee P, Van Vliet M, Van Sloun P, Kuipers Dijkshoorn N, Hermans J, Pearson PL, Cornelisse CJ. Allelotype of human breast carcinoma: A second major site for loss of heterozygosity is on chromosome 6q. *Oncogene* 1991; 6:1705-1711.
122. Iwase H, Greenman JM, Barnes DM, Bobrow L, Hodgson S, Mathew CG. Loss of heterozygosity of the oestrogen receptor gene in breast cancer. *Br J Cancer* 1995; 71:448-450.
123. Chappell SA, Walsh T, Walker RA, Shaw JA. Loss of heterozygosity at chromome 6q in preinvasive and early invasive breast carcinomas. *Br J Cancer* 1997; 75: 1324-1329.
124. Hill SM, Fuqua SAW, Chamness GC, Greene GL, McGuire WL. Estrogen receptor expression in human breast cancer associated with an estrogen receptor gene restriction lenght polymorphism. *Cancer Res* 1989; 49:145-148.
125. Piva R, Rimondi AP, Hanau S, Maestri I, Alvisi A, Kumar VL, Del Senno L. Different methylation of oestrogen receptor DNA in human breast carcinomas with and without oestrogen receptor. *Br J Cancer* 1990; 61:270-275.
126. Yaich L, Dupont WD, Cavener DR, Parl FF. Analysis of the PvuII restriction fragment-length polymorphism and exon structure of the estrogen receptor gene in breast cancer and peripheral blood. *Cancer Res* 1992; 52:77-83.
127. Karnik PS, Kulkarni S, Liu XP, Budd GT, Bukowski RM. Estrogen receptor mutations in

tamoxifen-resistant breast cancer. *Cancer Res* 1994; 54:349-353.
128. Catherino WH, Wolf DM, Jordan VC. A naturally occurring estrogen receptor mutation results in increased estrogenicity of a tamoxifen analog. *Mol Endocrinol* 1995; 9:1053-1063.
129. Ince BA, Schodin DJ, Shapiro DJ, Katzenellenbogen BS. Repression of endogenous estrogen receptor activity in MCF- 7 human breast cancer cells by dominant negative estrogen receptors. *Endocrinology* 1995; 136:3194-3199.
130. Roodi N, Bailey LR, Kao W-Y, Verrier CS, Yee CJ, Dupont WD, Parl FF. Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer. *JNCI* 1995; 87:446-451.
131. Cedar H. DNA methylation and gene activity. *Cell* 1988; 53:3-4.
132. Bird A. The essentials of DNA methylation. *Cell* 1992; 70:5-8.
133. Siegfried Z, Cedar H. DNA methylation: a molecular lock. *Curr Biol* 1997; 7:305-307.
134. Jones PA, Gonzalgo ML. Altered DNA methylation and genome instability: A new pathway to cancer. *Proc Natl Acad Sci USA* 1997; 94:2103-2105.
135. Falette NS, Fuqua SA, Chamness GC, Cheah MS, Greene GL, McGuire WL. Estrogen receptor gene methylation in human breast tumors. *Cancer Res* 1990; 50:3974-3978.
136. Ottaviano YL, Issa J-P, Parl FF, Smith HS, Baylin SB, Davidson NE. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res* 1994; 54:2552-2555.
137. Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, Weitzman SA, Baylin SB, Issa J-P,J., Davidson NE. Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. *Clin Cancer Res* 1996; 2:805-810.
138. Sluyser M. Steroid/thyroid receptor-like proteins with oncogenic potential: a review. *Cancer Res* 1990; 50:451-458.
139. Castles CG, Klotz DM, Fuqua SAW, Hill SM. Coexpression of wild-type and variant oestrogen receptor mRNAs in a panel of human breast cancer cell lines. *Br J Cancer* 1995; 71:974-980.
140. Daffada AAI, Dowsett M. Tissue-dependent expression of a novel splice variant of the human oestrogen receptor. *J Steroid Biochem Mol Biol* 1995; 55:413-421.
141. Pfeffer U, Fecarotta E, Vidali G. Coexpression of multiple estrogen receptor variant messenger RNAs in normal and neoplastic breast tissues and in MCF- 7 cells. *Cancer Res* 1995; 55:2158-2165.
142. Daffada AAI, Johnston SRD, Smith IE, Detre S, King N, Dowsett M. Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. *Cancer Res* 1995; 55:288-293.
143. Murphy LC, Hilsenbeck SG, Dotzlaw H, Fuqua SAW. Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. *Clin Cancer Res* 1995; 1:155-159.
144. Kasid A, Lippman ME, Papageorge AE, Lowy DR, Gelman EP. Transfection of v-RasH DNA into MCF-7 human breast cancer cells bypasses dependence on estrogen for tumorgenicity. *Science* 1985; 228:725-728.
145. Bos JL. The ras gene family and human carcinogenesis. *Mutation Research* 1988; 195:255-271.
146. Sommers CL, Papageorge A, Wilding G, Gelmann EP. Growth properties and tumorigenesis of MCF-7 cells transfected with isogenic mutants of RASH. *Cancer Res* 1990; 50:67-71.
147. Qiu R-G, Chen J, Kirn D, McCormick F, Symons M. An essential role for Rac in Ras transformation. *Nature* 1995; 374:457-459.
148. Peeper DS, Upton TM, Ladha MH, Neuman E, Zalvide J, Bernards R, DeCaprio JA, Ewen ME. Ras signaling linked to the cell-cycle machinery by the retinoblastoma protein. *Nature* 1997; 386:177-181.
149. Clark GJ, Der CJ. Aberrant function of the Ras signal transduction pathway in human breast cancer. *Breast Cancer Res Treat* 1995; 35:133-144.
150. Klijn JGM, Berns PMJJ, Schmitz PIM, Foekens JA. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: A review on 5232 patients. *Endocr Rev* 1992; 13:3-17.
151. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature* 1984; 307:521-527.

152. Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 1984; 309:418-425.
153. Wang Y, Minoshima S, Shimizu N. Precise mapping of the EGF receptor gene on the human chromosome 7p12 using an improved fish technique. *Jpn J Hum Genet* 1993; 38:399-406.
154. Di Fiore PP, Pierce JH, Fleming TP, Hazan R, Ullrich A, King CR, Schlessinger L, Aaronson SA. Overexpression of the human EGF receptor confers an EGF- dependent transformed phenotype to NIH 3T3 cells. *Cell* 1987; 51:1063-1070.
155. Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I, Lowy DR. Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene. *Science* 1987; 238:1408-1410.
156. Kannan S, De Santis M, Lohmeyer M, Riese DJ, Smith GH, Hynes N, Seno M, Brandt R, Bianco C, Persico G, Kenney N, Normanno N, Martinez-Lacaci I, Ciardiello F, Stern DF, Gullic WJ, Salomon DS. Cripto enhances the tyrosine phosphorylation of Shc and activates mitogen-activated protein kinase (MAPK) in mammary epithelial cells. *J Biol Chem* 1997; 272:3330-3335.
157. Lemmon MA, Bu ZM, Ladbury JE, Zhou M, Pinchasi D, Lax I, Engelman DM, Schlessinger J. Two EGF molecules contribute additively to stabilization of the EGFR dimer. *EMBO J* 1997; 16:281-294.
158. Hill CS, Treisman R. Transcriptional regulation by extracellular signals: Mechanisms and specificity. *Cell* 1995; 80:199-211.
159. Earp HS, Dawson TL, Li X, Yu H. Heterodimerization and functional interaction between EGF receptor family members: A new signaling paradigm with implications for breast cancer research. *Breast Cancer Res Treat* 1995; 35:115-132.
160. Karunagaran D, Tzahar E, Beerli RR, Chen XM, Graus-Porta D, Ratzkin BJ, Seger R, Hynes NE, Yarden Y. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: Implications for breast cancer. *EMBO J* 1996; 15:254-264.
161. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J* 1997; 16:1647-1655.
162. Sharma AK, Horgan K, Douglas-Jones A, McClelland R, Gee J, Nicholson R. Dual immunocytochemical analysis of oestrogen and epidermal growth factor receptors in human breast cancer. *Br J Cancer* 1994; 69:1032-1037.
163. Sainsbury JRC, Farndon JR, Needham GK, Malcolm AJ, Harris AL. Epidermal growth factor receptor as predictor of early recurrence of and death from breast cancer. *Lancet* 1987; 1398-1402.
164. Nicholson S, Halcrow P, Sainsbury JRC, Angus B, Chambers P, Farndon JR, Harris AL. Epidermal growth factor receptor (EGFr) status associated with failure of primairy endocrine therapy in elderly postmenopausal patients with breast cancer. *Br J Cancer* 1988; 58:810-814.
165. Valverius EM, Velu T, Shankar V, Ciardiello F, Kim N, Salomon DS. Over-expression of the epidermal growth factor receptor in human breast cancer cells fails to induce an estrogen-independent phenoptype. *Int J Cancer* 1990; 46:712-718.
166. Van Agthoven T, Van Agthoven TLA, Portengen H, Foekens JA, Dorssers LCJ. Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res* 1992; 52:5082-5088.
167. DeFazio A, Chiew YE, McEvoy M, Watts CKW, Sutherland RL. Antisense estrogen receptor RNA expression increases epidermal growth factor receptor gene expression in breast cancer cells. *Cell Growth Differ* 1997; 8:903-911.
168. Coussens L, Yang-Feng TL, Liao Y, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, Levinson A, Ullrich A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* 1985; 230:1132-1138.
169. Klijn JGM, Berns PMJJ, Bontenbal M, Alexieva-Figusch J, Foekens JA. Clinical breast cancer, new developments in selection and endocrine treatment of patients. *J Steroid Biochem Mol Biol* 1992; 43:211-221.
170. Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science* 1987; 237:178-182.
171. Wright C, Nicholson S, Angus B, Sainsbury JRC, Farndon J, Cairns J, Harris AL, Horne CHW. Relationship between c-erbB-2 protein product expression and response to endocrine therapy in

advanced breast cancer. *Br J Cancer* 1992; 65:118-121.
172. Borg A, Baldetorp B, Ferno M, Killander D, Olsson H, Ryden S, Sigurdsson H. ERBB2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. *Cancer Lett* 1994; 81:137-144.
173. Berns EMJJ, Foekens JA, Van Staveren IL, Van Putten WLJ, De Koning HYWCM, Portengen H, Klijn JGM. Oncogene amplification and prognosis in breast cancer: relationship with systemic treatment. *Gene* 1995; 159:11-18.
174. Thomas JH. Genomic imprinting proposed as a surveillance mechanism for chromosome loss. *Proc Natl Acad Sci USA* 1995; 92:480-482.
175. Carlomagno C, Perrone F, Gallo C, De Laurentiis M, Lauria R, Morabito A, Pettinato G, Panico L, D'Antonio A, Bianco AR, De Placido S. c-*erb*B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. *J Clin Oncol* 1996; 14:2702-2708.
176. Liu Y, El-Ashry D, Chen D, Ding IYF, Kern FG. MCF-7 breast cancer cells overexpressing transfected *c- erb*B-2 have an *in vitro* growth advantage in estrogen- depleted conditions and reduced estrogen-dependence and tamoxifen- sensitivity *in vivo*. *Breast Cancer Res Treat* 1995; 34:97-117.
177. Pietras RJ, Arboleda J, Reese DM, Wongvipat N, Pegram MD, Ramos L, Gorman CM, Parker MG, Sliwkowski MX, Slamon DJ. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene* 1995; 10:2435-2446.
178. Birnbaum D, DeLapeyriere O, Adnane J, Dionne C, Crumley G, Jaye M, Schlessinger J, Xerri L, Escot C, Gaudray P, Theillet C. Role of FGFs and FGF receptors in human carcinogenesis. *Ann NY Acad Sci* 1991; 638:409-411.
179. Lehtola L, Partanen J, Sistonen L, Korhonen J, Waerri A, Haerkoenen P, Clarke R, Alitalo K. Analysis of tyrosine kinase mRNAs including four FGF receptor mRNAs expressed in MCF-7 breast-cancer cells. *Int J Cancer* 1992; 50:598-603.
180. Kurebayashi J, McLeskey SW, Johnson MD, Lippman ME, Dickson RB, Kern FG. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and LacZ. *Cancer Res* 1993; 53:2178-2187.
181. McLeskey SW, Kurebayashi J, Honig SW, Zwiebel J, Lippman ME, Dickson RB, Kern FG. Fibroblast growth factor 4 transfection of MCF-7 cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen- treated athymic nude mice. *Cancer Res* 1993; 53:2168-2177.
182. Clark GM, Mathieu MC, Owens MA, Dressler LG, Eudey L, Tormey DC, Osborne CK, Gilchrist KW, Mansour EG, Abeloff MD, McGuire WL. Prognostic significance of S-phase fraction in good-risk, node- negative breast cancer patients. *J Clin Oncol* 1992; 10:428-432.
183. El-Ashry D, Miller DL, Kharbanda S, Lippman ME, Kern FG. Constitutive Raf-1 kinase activity in breast cancer cells induces both estrogen-independent growth and apoptosis. *Oncogene* 1997; 15:423-435.
184. Singer C, Rasmussen A, Smith HS, Lippman ME, Lynch HT, Cullen KJ. Malignant breast epithelium selects for insulin-like growth factor II expression in breast stroma: Evidence for paracrine function. *Cancer Res* 1995; 55:2448-2454.
185. Cullen KJ, Lippman ME, Chow D, Hill S, Rosen N, Zwiebel JA. Insulin-like growth factor-II overexpression in MCF-7 cells induces phenotypic changes associated with malignant progression. *Mol Endocrinol* 1992; 6:91-100.
186. Peter M, Herskowitz I. Joining the complex: Cyclin-dependent kinase inhibitory proteins and the cell cycle. *Cell* 1994; 79:181-184.
187. Dynlacht BD. Regulation of transcription by proteins that control the cell cycle. *Nature* 1997; 389:149-152.
188. Berns EMJJ, Klijn JGM, Van Staveren IL, Portengen H, Noordegraaf E, Foekens JA. Prevalence of amplification of the oncogenes c-myc, HER2/neu, and int-2 in one thousand human breast tumours: Correlation with steroid receptors. *Eur J Cancer* 1992; 28:697-700.
189. Callender T, El-Naggar AK, Lee MS, Frankenthaler R, Luna MA, Batsakis JG. PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. *Cancer* 1994; 74:152-158.
190. Dickson C, Fantl V, Gillett C, Brookes S, Bartek J, Smith R, Fisher C, Barnes D, Peters G.

Amplification of chromosome band 11q13 and a role for cyclin D1 in human breast cancer. *Cancer Lett* 1995; 90:43-50.
191. Michalides R, Hageman P, Van Tinteren H, Houben L, Wientjens E, Klompmaker R, Peterse J. A clinicopathological study on overexpression of cyclin D1 and of p53 in a series of 248 patients with operable breast cancer. *Br J Cancer* 1996; 73:728-734.
192. Worsley SD, Jennings BA, Khalil KH, Mole M, Girling AC. Cyclin D1 amplification and expression in human breast carcinoma: Correlation with histological prognostic markers and oestrogen receptor expression. *J Clin Pathol Clin Mol Pathol* 1996; 49:M46-M50.
193. Musgrove EA, Lee CSL, Buckley MF, Sutherland RL. Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle. *Proc Natl Acad Sci USA* 1994; 91:8022-8026.
194. Zwijsen RML, Klompmaker R, Wientjens EBHGM, Kristel PMP, Van der Burg B, Michalides RJAM. Cyclin D1 triggers autonomous growth of breast cancer cells by governing cellcycle exit. *Mol Cell Biol* 1996; 16:2554-2560.
195. Pacilio C, Germano D, Addeo R, Altucci L, Petrizzi VB, Cancemi M, Cicatiello L, Salzano S, Lallemand F, Michalides RJAM, Bresciani F, Weisz A. Constitutive overexpression of cyclin D1 does not prevent inhibition of hormone-responsive human breast cancer cell growth by antiestrogens. *Cancer Res* 1998; 58:871-876.
196. Neuman E, Ladha MN, Lin N, Upton TM, Miller SJ, DiRenzo J, Pestell RG, Hinds PW, Dowdy SF, Brown M, Ewen ME. Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. *Molecular Cell Biology* 1997; 17:5338-5347.
197. Zwijsen RML, Wientjens E, Klompmaker R, Van der Sman J, Bernards R, Michalides RJAM. CDK-independent activation of estrogen receptor by cyclin D1. *Cell* 1997; 88:405-415.
198. Gillet C, Smith P, Gregory W, Richards M, Millis R, Peters G, Barnes D. Cyclin D1 and prognosis in human breast cancer. *Int J Cancer* 1996; 69:92-99.
199. Van Diest PJ, Michalides RJAM, Jannink I, Van der Valk P, Peterse HL, De Jong JS, Meijer CJLM, Baak JPA. Cyclin D1 expression in invasive breast cancer - Correlations and prognostic value. *Am J Pathol* 1997; 150:705-711.
200. Nooter K, Stoter G. Molecular mechanisms of multidrug resistance in cancer chemotherapy. *Pathol Res Pract* 1996; 192:768-780.
201. Clarke R, Currier S, Kaplan O, Lovelace E, Boulay V, Gottesman MM, Dickson RB. Effect of P-glycoprotein expression on sensitivity to hormones in MCF-7 human breast cancer cells. *J Natl Cancer Inst* 1992; 84:1506-1512.
202. Keen JC, Miller EP, Bellamy C, Dixon JM, Miller WR. P-glycoprotein and resistance to tamoxifen. *Lancet* 1994; 343:1047-1048.
203. Wang CS, LaRue H, Fortin A, Gariepy G, Tetu B. MDR1 mRNA expression by RT-PCR in patients with primary breast cancer submitted to neoadjuvant therapy. *Breast Cancer Research and Treatment* 1997; 45:63-74.
204. Nicholson S, Halcrow P, Farndon JR, Sainsbury JRC, Chambers P, Harris AL. Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. *The Lancet* 1989; i:182-185.
205. Toi M, Harris AL, Bicknell R. cDNA transfection followed by the isolation of a MCF-7 breast cell line resistant to tamoxifen in vitro and in vivo. *Br J Cancer* 1993; 68:1088-1096.
206. Jensen EV. Hormone dependency of breast cancer. *Cancer (Phila)* 1981; 47:2319-2326.
207. Taylor-Papadimitriou J, Berdichevsky F, D'Souza B, Burchell J. Human models of breast cancer. *Cancer Surv* 1993; 16:59-78.
208. Brünner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SAW, Clarke R. MCF7/LCC9: An antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182, 780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. *Cancer Res* 1997; 57:3486-3493.
209. Levenson AS, Jordan VC. MCF-7: The first hormone-responsive breast cancer cell line. *Cancer Res* 1997; 57:3071-3078.
210. Reddel RR, Alexander IE, Koga M, Shine J, Sutherland RL. Genetic instability and the development of steroid hormone insensitivity in cultured T 47D human breast cancer cells. *Cancer Res* 1988; 48:4340-4347.
211. Graham ML, Dalquist KE, Horwitz KB. Simultaneous measurement of progesterone receptors

and DNA indices by flow cytometry: analysis of breast cancer cell mixtures and genetic instability of the T47D line. *Cancer Res* 1989; 49:3943-3949.
212. Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ, Joyce MJ. Establishment and characterization of three continuous cell lines derived from human breast carcinomas. *Cancer Res* 1978; 38:3352-3364.
213. Dorssers LCJ, Van Agthoven T, Dekker A, Van Agthoven TLA, Kok EM. Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: Identification of bcar-1, a common integration site. *Mol Endocrinol* 1993; 7:870-878.
214. Van Agthoven T, Timmermans M, Dorssers LCJ, Henzen-Logmans SC. Expression of estrogen, progesterone, and epidermal growth factor receptors in primary and metastatic breast cancer. *Int J Cancer* 1995; 63:790-793.
215. Varmus H. Retroviruses. *Science* 1988; 240:1427-1435.
216. Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. *BioTechniques* 1989; 7:980-990.
217. Danos O, Mulligan RC. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci USA* 1988; 85:6460-6464.
218. Kulkosky J, Skalka AM. Molecular mechanism of retroviral DNA integration. *Pharmacol Ther* 1994; 61:185-203.
219. Kitamura Y, Lee YMH, Coffin JM. Nonrandom integration of retroviral DNA in vitro: Effect of CpG methylation. *Proc Natl Acad Sci USA* 1992; 89:5532-5536.
220. Kung HJ, Boerkoel C, Carter TH. Retroviral mutagenesis of cellular oncogenes: A review with insights into the mechanisms of insertional activation. *Curr Top Microbiol Immunol* 1991; 171:1-25.
221. Jonkers J, Berns A. Retroviral insertional mutagenesis as a strategy to identify cancer genes. *Biochim Biophys Acta Rev Cancer* 1996; 1287:29-57.
222. Ogawa S, Toyoshima H, Kozutsumi H, Hagiwara K, Sakai R, Tanaka T, Hirano N, Mano H, Yazaki Y, Hirai H. The C-terminal SH3 domain of the mouse c-Crk protein negatively regulates tyrosine-phosphorylation of Crk associated p130 in rat 3Y1 cells. *Oncogene* 1994; 9:1669-1678.
223. Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Nishida J, Yazaki Y, Hirai H. Characterization, partial purification, and peptide sequencing of p130, the main phosphoprotein associated with v-Crk oncoprotein. *J Biol Chem* 1994; 269:32740-32746.
224. Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Mano H, Yazaki Y, Hirai H. A novel signaling molecule, p130, forms stable complexes *in vivo* with v-Crk and v-Src in a tyrosine phosphorylation- dependent manner. *EMBO J* 1994; 13:3748-3756.
225. Petch LA, Bockholt SM, Bouton A, Parsons JT, Burridge K. Adhesion-induced tyrosine phosphorylation of the p130 SRC substrate. *J Cell Sci* 1995; 108:1371-1379.
226. Harte MT, Hildebrand JD, Burnham MR, Bouton AH, Parsons JT. $p130^{Cas}$, a substrate associated with v-Src and v-Crk, localizes tofocal adhesions and binds to focal adhesion kinase. *J Biol Chem* 1996; 271:13649-13655.
227. Casamassima A, Rozengurt E. Tyrosine phosphorylation of $p130^{cas}$ by bombesin, lysophosphatidic acid, phorbol esters, and platelet-derived growth factor - Signaling pathways and formation of A $p130^{cas}$- Crk complex. *J Biol Chem* 1997; 272:9363-9370.
228. Dorssers LCJ, Veldscholte J. Identification of a novel breast-cancer-anti-estrogen-resistance (BCAR2) locus by cell-fusion-mediated gene transfer in human breast-cancer cells. *Int J Cancer* 1997; 72:700-705.
229. Van Agthoven T, Van Agthoven TLA, Dekker A, Van der Spek PJ, Vreede L, Dorssers LCJ. Identification of BCAR3 by a random search for genes involved in antiestrogen resistance of human breast cancer cells. *EMBO J* 1998; 17:2799-2808.

CHAPTER 5

[CANCER RESEARCH 52, 5087–5088, September 15, 1992]

Ectopic Expression of Epidermal Growth Factor Receptors Induces Hormone Independence in ZR-75-1 Human Breast Cancer Cells[1]

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ABSTRACT

Epidermal growth factor (EGF) receptor is inversely related to expression of estrogen receptor (ER) and progesterone receptor in primary breast tumors and is a negative predictor for response to endocrine therapy. To investigate a possible causal role of EGF receptor expression in breast cancer progression to hormone independence, we have created an experimental cell system. Epidermal growth factor receptor complementary DNA was introduced in estrogen-dependent ZR-75-1 breast cancer cells, and the resulting ZR/HERc cells exhibited a mitogenic response to epidermal growth factor, thus bypassing estrogen dependence. This EGF-induced proliferation could not be inhibited by antiestrogens. In addition, we noted changes in cell morphology and keratin expression of EGF-stimulated ZR/HERc cells, suggestive of an altered differentiation state. Furthermore, intolerance of functional ER and EGF receptor signal transduction pathways in ZR/HERc cells was observed during simultaneous activation, which possibly explains the inverse relationship of ER and EGF receptor expression in primary tumors. In contrast to the parental cells, ZR/HERc cells rapidly progressed to a stable ER-negative phenotype when cultured in the presence of the antiestrogen hydroxy-tamoxifen. These results suggest a possible role for EGF receptor in progression of breast cancer to hormone independence.

INTRODUCTION

The development of the normal mammary gland is regulated by steroid hormones and polypeptide growth factors. Insights into the mechanisms by which these factors induce cell proliferation and differentiation and how these factors are involved in breast tumor proliferation have remained limited (1–3). Antagonists of steroid hormones are clinically important in the management of breast cancer, especially the antiestrogen, tamoxifen (4, 5). Antiestrogens compete with estrogen for binding to the ER[3] and can antagonize the action of estrogens on gene expression via different mechanisms (6–8). About one-third of patients with advanced breast cancer show an objective response to endocrine treatment. In case of response, progression from a hormone-sensitive to a hormone-insensitive state occurs almost inevitably during prolonged therapy. The mechanisms underlying this progression are complex and, as yet, unclear (9–13).

Numerous studies have established that, in human primary breast cancer, expression of EGF receptor is inversely correlated with ER expression (14). In addition, expression of EGF receptor and ER in breast cancer cell lines is also inversely related (15). Generally, patients with ER-negative primary tumors have a worse prognosis than those with ER-positive tumors (16). Whether patients with EGF receptor-positive tumors have an unfavorable prognosis is at present a matter of debate (for a review, see Ref. 17). Nevertheless, it has been shown that tumors expressing EGF receptors fail to respond to hormonal therapy (18, 19). The relation between EGF receptor expression and a possible causal role in the development of hormone-independent tumors is unknown. A feasible approach to address this question is to investigate *in vitro* the biological consequences of ectopic expression of EGF receptors in hormone-dependent cells.

For the present study, we created a new cell system to study *in vitro* the role of EGF receptor gene expression in progression of human breast tumor cells to hormone independence. Since growth of the ZR-75-1 human breast cancer cell line is fully estrogen dependent, this cell line provided an attractive *in vitro* model. We transduced the human EGF receptor cDNA in estrogen-dependent ZR-75-1 cells and studied the effects of EGF receptor expression on hormone- and EGF-mediated cell proliferation, differentiation, and progression to hormone independence.

MATERIALS AND METHODS

Cells. ZR-75-1 and ZR/HERc cells are maintained in RPMI-1640 medium (Gibco BRL, Life Technologies Ltd, Paisley, United Kingdom) supplemented with 10% heat-inactivated BCS (HyClone Laboratories Inc, Logan, UT) and 1 nM 17β-estradiol. The karyotypic characteristics of the ZR-75-1 human breast cancer cell line maintained in our laboratory and the derived ZR/HERc cells are similar to the original published data for ZR-75-1 (20). Cytogenetic analyses were carried out using R-banding. PA317 cells (21) were maintained in Ham's F-12/Dulbecco's modified Eagle's medium, 1:1 (Sigma, St. Louis, MO), with 10% BCS. Cell cultures were routinely checked for *Mycoplasma* contamination with a DNA-staining reaction using the bisbenzimidazole fluorochrome, Hoechst 33258.

Hormones. 17β-Estradiol (Merck, Darmstadt, Germany), 4-OH-tamoxifen, and ICI 164.384 (ICI, Mecclesfield, United Kingdom) stock solutions were made in absolute ethanol and stored at 4°C. EGF, from mouse submaxillary glands, was obtained from Boehringer Mannheim Gmbh, Mannheim, Germany.

Retroviral Gene Transfer. Clonal amphotropic NTK-HERc virus-producing cell lines were established by infection of PA317 cells with cell-free (0.45 μm filtrated) supernatant of NTK/HERc virus-producing (22) psi-2 cells for 17 h in the presence of 8 μM Polybrene (Sigma). Clones were selected in medium with 1 mg/ml of G418 (Gibco BRL). Using the same procedure, we infected the ZR-75-1 cells with the amphotropic NTK/HERc receptor virus, and cell clones (ZR/HERc) were isolated using G418 selection and expanded in estradiol-containing medium.

Cell Proliferation Experiments. Cells from subconfluent cultures were harvested by treatment with trypsin-EDTA (trypsin, 0.05%; EDTA, 0.5 mM). Single cells were plated into 25-cm² plastic culture flasks (Costar Europe Ltd., Cambridge, MA) in medium with 10% BCS, without estradiol. After 24 h, to allow for cell attachment, the culture medium was replaced with experimental medium. RPMI-1640 medium without phenol red, supplemented with 10% heat-inactivated FCS and stripped by DCC, was used. The mathematical technique of Leibovitz *et al.* (23) was used to obtain the generation time of each cell

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[3] The abbreviations used are: ER, estrogen receptor; EGF, epidermal growth factor; cDNA, complementary DNA; BCS, bovine calf serum; SFM, serum-free medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BSA, bovine serum albumin; PR, progesterone receptor; PBS, phosphate-buffered saline; DCC, dextran-coated charcoal; FCS, fetal calf serum.

clone. Experiments to evaluate the effects of the additives in SFM were performed using the MTT assay (24). Cells were distributed in 96-well plates in RPMI-1640 plus 10% BCS to allow for cell attachment. After 24 h, the culture medium was replaced with a chemically defined SFM, consisting of Ham's F-12/Dulbecco's modified Eagle's medium (1:1) lacking phenol red and supplemented with 0.2% BSA (purified; Behringwerke AG, Marburg, Germany), 2 mM glutamine, 10^{-8} M transferrin, 10 μg/ml porcine insulin (Organon, Oss, the Netherlands), and 50 ng/ml sodium selenite. MTT reduction was assayed after 6 days of culture by measuring the absorbance at 510 nm with an automatic microplate reader (Titertec, Flow Laboratories Ltd., Irvine, Scotland).

Quantitation of Hormone and EGF Receptors. Cells were cultured in 225-cm² flasks in medium containing 10% BCS and 1 nM estradiol. Approximately 5–7 × 10^7 cells were preincubated for 17 h in SFM, prior to harvesting with 0.5 mM EDTA. This preincubation was performed, since the presence of growth factors in serum and/or endogenously produced by the cells interfered with the estimation of receptor numbers. Binding of ^{125}I-EGF to ZR/HERc cells was masked up to 80% when preculture in SFM was omitted. Cell pellets were stored at −80°C until use. Cell pellets were pulverized and homogenized with a microdismembrator at −196°C, as described elsewhere (25). The homogenate was centrifuged for 20 min at 100,000 × *g* at 4°C, and the supernatant fraction (cytosolic extract) was used for ER and PR determinations, using commercially available enzyme immunoassay kits (ER-EIA, PgR-EIA; Abbott Laboratories, Chicago, IL). The membrane fraction for EGF receptor determination was resuspended by homogenization of the 100,000 × *g* pellet in 2.5 ml PBS solution containing Bacitracin (Serva, Heidelberg, Germany) (70 μg/ml), with three 5-s bursts of an Omni-1000 tissue homogenizer (Omni International, Waterbury, CT), and subsequently clarified by centrifugation for 10 min at 1000 × *g*. Multiple-point Scatchard analyses of ^{125}I-EGF binding to membrane preparations were performed as described previously with minor modifications (26, 27). Briefly, 100-μl membrane preparations were incubated in a final volume of 140 μl for 18 h at 20°C with increasing concentrations (0.15–3.5 nM) of ^{125}I-EGF (specific activity, 500 Ci/mmol, mouse EGF, receptor grade, Sigma; radiolabeled with Protag 125, Baker Fine Chemicals, Philipsburg, NJ) in the absence and presence of a 200-fold excess of nonradioactive EGF. Separation of bound and free EGF was performed by hydroxylapatite adsorption. Hydroxylapatite-bound radioactivity was counted.

Immunofluorescence. Cells were harvested with 0.5 mM EDTA, washed with PBS plus 0.5% BSA, and cytocentrifuged on slides. The slides were fixed for 5 min in methanol and 1 min in acetone at −20°C. Slides were kept frozen until use. Indirect immunofluorescence staining was performed at ambient temperature. Slides were rinsed in PBS plus 0.5% BSA prior to specific antibody binding. Incubation of primary antibodies was performed in a wet chamber for 60 min at ambient temperature. After washing, the slides were incubated for 30 min with 15 μl goat anti-mouse immunoglobulin-fluorescein isothiocyanate (Dacopatts, Glostrup, Denmark). After a final washing with PBS containing 0.5% BSA for 15 min, the slides were mounted in glycerol/PBS (4:1) with an antibleach agent, 1,4-Diazabicyclo-octane (Sigma). The antibodies used in this study were RCK 105 to keratin 7, M 20 to keratin 8, RCK 106 to keratin 18, LP2K to keratin 19, and RV 202 to vimentin and HMFG-2. Anti-desmin was a kind gift from Sanbio BV, Uden, The Netherlands.

RESULTS

Expression of the EGF Receptor cDNA in ZR-75-1 Cells. The human EGF receptor cDNA (28) was efficiently introduced into ZR-75-1 cells with the retroviral vector NTK-HERc (22), which carries the human EGF receptor cDNA under control of the herpes simplex thymidine kinase promotor. The combination with the neomycin resistance gene expressed from the 5' LTR provides a selectable marker. After the ZR-75-1 cells were infected with the retroviral vector NTK/HERc, six G418-resistant clones were selected with one integrated copy of the EGF receptor cDNA and a different integration pattern, indicating their independent origin (determined by Southern analysis using a Neo probe, data not shown). ZR/HERc clones were studied for their EGF receptor expression by Scatchard analysis using ^{125}I-EGF. ZR/HERc clones contained high-affinity ^{125}I-EGF binding sites (K_d = 0.9–1.5 nM) with different EGF receptor levels (range, 43–251 fmol/mg membrane protein) (Table 1 and Fig. 1). In contrast, the parental cell line ZR-75-1 contained no detectable EGF receptors, as determined in 8 independently performed Scatchard analyses. EGF receptor mRNA was found only in ZR/HERc clones and not in the ZR-75-1 parental cells (Northern analysis not shown). All generated clones retained ER and PR receptor expression, similar to the parental ZR-75-1 cells (Table 1).

Proliferation-inducing Effects of EGF on ZR-75-1 and ZR/HERc Cells. To test the functionality of the ectopically expressed EGF receptors, ZR/HERc cells were examined for a proliferative response to EGF. The number of generations during a 12-day culture period of six independently derived EGF receptor-expressing clones is presented in Fig. 2. Virtually all examined clones responded to EGF with cell proliferation, and all clones exhibited a clear increase in cell size and change of cell morphology (see Fig. 7 and below). EGF induced a significant increase in ZR/HERc cell population-doubling time compared to stimulation with estradiol. The parental cell line ZR-75-1, expressing no detectable EGF-binding sites, did not show any response to EGF in >10 independent experiments. Subsequently a short-term culture assay was performed to determine the effect of EGF and estradiol on MTT reduction in SFM. Although estradiol is a poor inducer of ZR-75-1 cell proliferation in our serum-free system as determined by cell counting (not shown), MTT reduction in both ZR-75-1 cells and ZR/HERc cell clones was significantly increased after 6 days of culture in the presence of estradiol (Fig. 3). EGF stimulation of ZR-75-1 cells in this serum-free assay did not affect MTT reduction. In contrast, ZR/HERc clones exhibited a clear enlargement of the cell size, a change in morphology, and a strong increase in MTT reduction (Fig. 3), indicating that EGF induced an elevation of the mitochondrial activity in these cells independently of proliferation.

EGF Can Bypass Inhibition of OH-Tamoxifen. ZR-75-1 cells maintained in our laboratory are strictly estrogen dependent for proliferation (Fig. 4, see also Figs. 2 and 3). Estrogen-induced stimulation could be completely inhibited by the simultaneous addition of the antiestrogen OH-tamoxifen (1 μM). Similarly to ZR-75-1 cells, the estrogen-induced stimulation of ZR/HERc cells was completely suppressed by the antiestrogen OH-tamoxifen. In contrast, the EGF-induced 9-fold growth stimulation of the ZR/HERc cell culture could not be inhibited by OH-tamoxifen (Fig. 4). These results demonstrate that the

Table 1 *Expression of hormone receptors and EGF receptors on ZR-75-1 and ZR/HERc cells*

Cells were grown in medium containing 10% BCS and estradiol (1 nM). Cells (5–7 × 10^7) were harvested with 0.5 mM EDTA, after overnight incubation in SFM, for receptor determinations. Values are in fmol/mg protein.

Cell line	EGF receptor	ER	PR
ZR-75-1	[a]	34	>500
ZR/HERc 1A	187	34	>500
ZR/HERc 1D	47	62	>500
ZR/HERc 1E	43	33	>500
ZR/HERc 1F	251	48	>500
ZR/HERc 1G	81	92	>500
ZR/HERc 2B	43	53	>500

[a] No specific ^{125}I-EGF binding.

estrogen-dependent proliferation pathway can be bypassed by exogenous EGF in cells carrying ectopically expressed EGF receptors.

Estrogen Responsiveness after Preculture with EGF. To establish whether preculture of ZR/HERc (or ZR-75-1) cells with EGF would affect subsequent estrogen-dependent proliferation, cells were cultured for 12 days in the absence and presence of EGF (Fig. 5*A*) and subsequently replated in estradiol-containing medium. Fig. 5*B* shows that preculture of ZR/HERc cells with EGF reduced secondary colony formation to 15% when compared to preculture in the absence of EGF. The ability to form colonies by the parental ZR-75-1 cells was not affected by preculture with EGF. The differences observed were not a result of variation in plating efficiencies. To investigate whether down-regulation of ER in EGF-treated ZR/HERc cells had occurred and thus could explain the loss of estrogen response, the number of steroid receptors was determined on the same batch of cells (Fig. 5*A*). Following additional culture for 2 days in estradiol-containing medium, to allow also measurement of

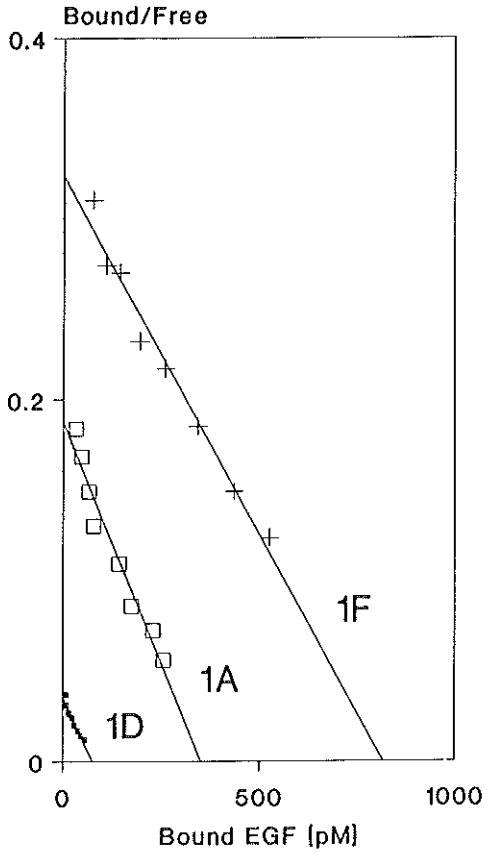

Fig. 1. Scatchard analysis of EGF binding. ^{125}I-EGF-binding studies were performed as described in "Materials and Methods." Specific binding of three representative clones (HERc-1A, -1D, and -1F) is plotted.

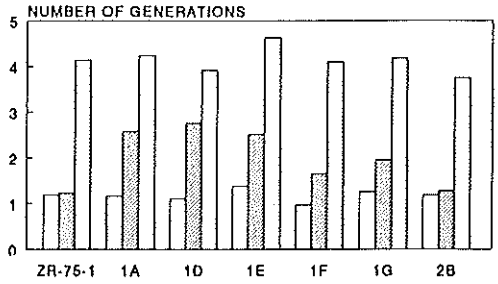

Fig. 2. Proliferation-inducing effects of EGF. ZR-75-1 and ZR/HERc cells (2×10^5) were plated in RPMI/BCS medium in 25-cm^2 culture flasks. After 24 h, the medium was replaced with RPMI/DCC-FCS, without EGF (□), with EGF (□), or with estradiol (■). Cells were harvested on day 12 and counted, and the number of generations was calculated.

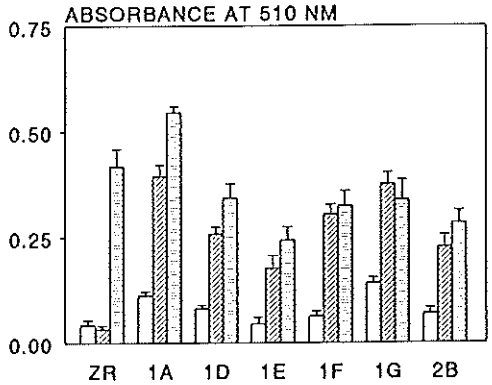

Fig. 3. Effect of EGF and estradiol in SFM on MTT reduction by ZR-75-1 cells (ZR) and ZR/HERc cell clones. Cells were plated in RPMI/BCS medium at a density of 2500 cells/well, on day 0; after 24 h, the medium was changed to SFM (□), SFM containing estradiol (1 nM, ■), or EGF (10 ng/ml, □). Cell stimulation was determined on day 6 using the MTT assay and is shown as the absorbance 510 nm reading. All values are the means of 8 wells (±SD).

PR expression, extracts prepared from these ZR/HERc cells contained high amounts of both ER and PR (Table 2). The presence of PR indicates that the lack of colony formation induction of EGF-treated ZR/HERc cells by estradiol was not caused by a defective ER machinery, since PR expression is regulated by ER (29).

EGF-mediated Growth Control. To address the question of whether EGF-mediated events are dominant over those of estradiol or *vice versa*, the effects of EGF and estradiol when supplied individually or simultaneously were investigated. The number of ZR/HERc cells in the estradiol-stimulated (10 or 100 pM) cultures was significantly reduced by the additional presence of 10 ng/ml EGF (Fig. 6), in spite of the growth stimulatory effects of EGF in the absence of estradiol. Inhibitory effects of EGF alone (up to 100 ng/ml) were not observed (not shown). Furthermore, the morphology of these ZR/HERc cells was similar to the cell cultures supplemented with EGF alone. Increase of cell population-doubling time of ZR/HERc cells was also observed in long-term cultures (20 days) stimulated with EGF and 1 nM estradiol (data not shown). Cultures

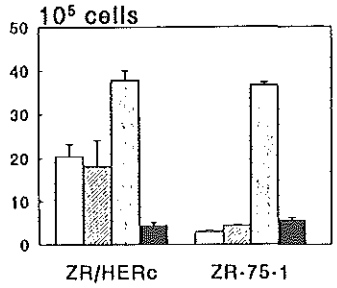

Fig. 4. Effect of OH-tamoxifen on EGF- or estradiol-mediated proliferation of ZR-75-1 and ZR/HERc cells. Cells (1.5×10^5) were plated in RPMI/BCS medium. After 24 h, the following factors were added: EGF (10 ng/ml, □), EGF + OH-tamoxifen (1 μM, □), estradiol (1 nM, ■), or estradiol + OH-tamoxifen (■). The means ± SD of triplicate incubations, harvested and counted on day 10, are plotted.

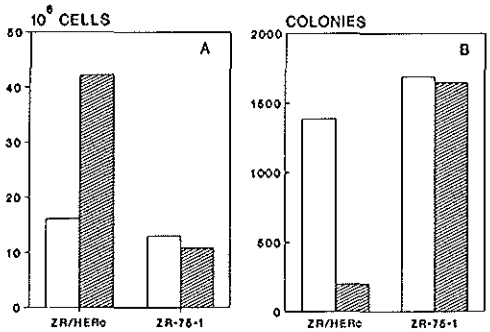

Fig. 5. Replating efficiency after preculture with EGF. ZR-75-1 and ZR/HERc cells (1×10^7) were plated on day 0 in medium plus 4.5% DCC-FCS, without (□) or with EGF (10 ng/ml, □) (in duplicate). The cells were harvested on day 12 and counted (*A*). Subsequently, single-cell suspensions containing 1×10^4 cells were seeded in 25-cm² flasks with medium containing estradiol (1 nM). After 12 days of culture, the number of cell colonies was determined (*B*).

supplemented with EGF and 100 nM ICI 164,384, which completely eliminates the stimulatory effect of 10 pM estradiol, showed slightly enhanced proliferation of the cells (Fig. 6; two additional independent experiments not shown). This may be explained by neutralization of remnant traces of estradiol in the DCC serum by the pure antagonist. Our results strongly suggest that simultaneous induction of ER-mediated gene activation and EGF receptor signal transduction in ZR/HERc cells is not tolerated.

Structural Alterations of ZR/HERc Cells Cultured with EGF. ZR/HERc cells could be maintained in culture for >14 weeks with EGF in the absence of estradiol, without apparent loss of viability (<5%), as determined by the trypan blue exclusion method and reduction of MTT in individual cells. Stimulation of ZR/HERc cells with EGF resulted in altered cell morphology. The cell volume increased significantly, and some cells contained many vacuoles (Fig. 7, *A* and *B*). In addition, the cells appeared more flattened, and a stronger adherence to the plastic culture flask was noted compared to ZR/HERc or ZR-75-1 cells cultured in the presence of estradiol. To determine whether EGF-induced maturation may explain these morphological alterations and the failure of secondary colony formation in estrogen-containing medium (Fig. 5), expression of various cytoskeletal proteins and the milk fat globule antigen (HMFG-2) membrane glycoprotein was studied using monoclonal antibodies. Intermediate filaments have been established as markers of cell differentiation and are well preserved during malignant transformation (30). ZR-75-1 cells expressed keratins 8, 18, and 19, typical for the luminal epithelial cell, in both estradiol- and EGF-supplemented cultures. EGF induced an increase of keratin 8, 18, and 19 structures in ZR/HERc cells after 10–12 days of culture, compared to control cultures with estradiol. Even more noticeable was the change in filament structure for keratins 8 and 18, which changed from a loose network of slender fibers to a very dense structure when stimulated with EGF (Fig. 7, *C* and *D*). No such changes were observed in ZR-75-1 cells. To exclude that this phenomenon was solely due to reduction of proliferation, ZR/HERc and ZR-75-1 cells incubated for 12 days in media without EGF or estradiol were analyzed. These cells did not show a change in cytoskeletal structure and displayed a moderate decrease in keratin expression levels. All studied cells were negative for keratin 7, desmin, and vimentin. Cell membrane HMFG-2 expression was strongly increased following treatment of ZR/HERc cells with EGF. Since HMFG-2 is a marker for mature cells (31), this result suggests that ZR/HERc cells progress to a more mature state following treatment with EGF.

Progression to Hormone Independence. We next determined whether ectopic expression of EGF receptors would effect the rate of progression to a completely hormone-independent phenotype. ZR/HERc cells and control ZR-75-1 cells were cultured in medium containing 10% BCS and excess OH-tamoxifen (1 μM). The growth of ZR-75-1 cells was completely

Table 2 *Stable receptor expression by ZR-75-1 and ZR/HERc cells*

Cells (1×10^7) were cultured in medium with 10% DCC-FCS containing no additives or EGF (10 ng/ml) as indicated. After 12 days, a small portion was used for the replating study (Fig. 5*B*), and the remaining cells were incubated in estradiol-supplemented medium for 2 days. After a further 4 h incubation in SFM, the cells were harvested with 0.5 mM EDTA and used for receptor determinations. Values are in fmol/mg protein.

Cell line	Addition	EGF receptor	ER	PR
ZR-75-1	NO	[a]	51	>500
ZR-75-1	EGF	[a]	55	>500
ZR/HERc	NO	232	34	134
ZR/HERc	EGF	108	85	>500

[a] No specific ^{125}I-EGF binding.

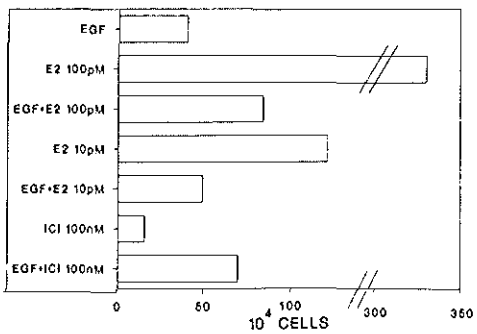

Fig. 6. Effect of EGF on estradiol-stimulated proliferation of ZR/HERc cells. Cells (2×10^5) were plated in 25-cm² flasks in medium containing 10% DCC-FCS supplemented with EGF (10 ng/ml), estradiol (E2, 10 or 100 pM), and ICI 164,384 (100 nM) as indicated. Cells were harvested and counted on day 12.

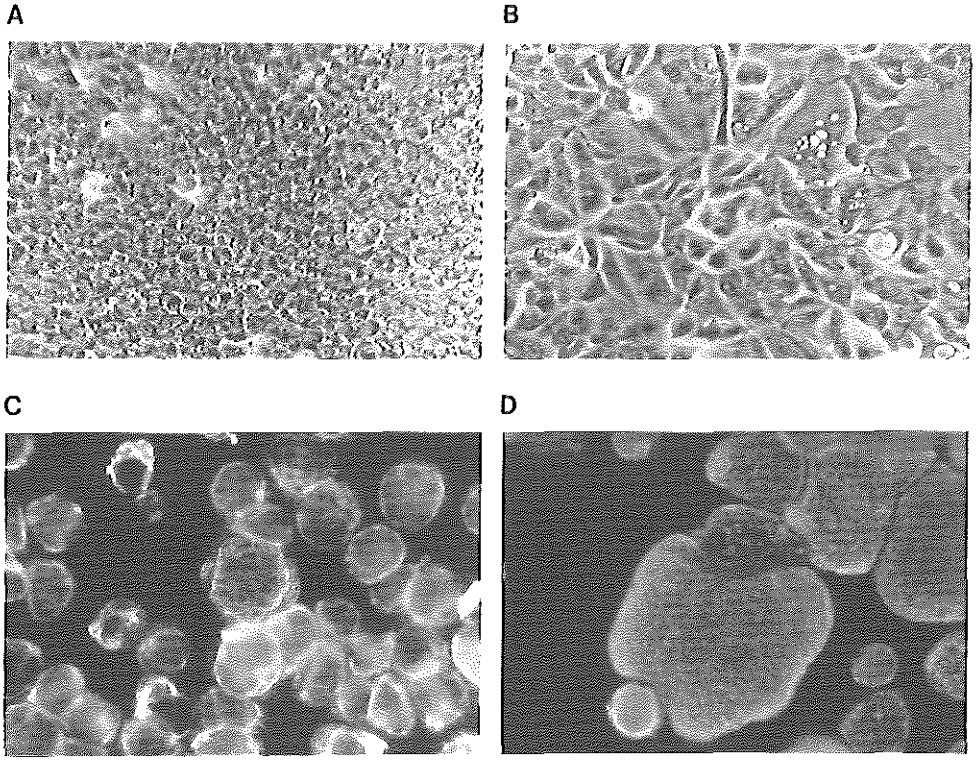

Fig. 7. EGF-induced changes in morphology and keratin expression of ZR/HERc cells. Phase-contrast photomicrographs of ZR/HERc cells cultured with 1 nM 17β-estradiol (*A*) and ZR/HERc cells cultured for 12 days in the presence of 10 ng/ml EGF (*B*). Immunofluorescent detection of keratin 8 expression in ZR/HERc cells cultured with 1 nM 17β-estradiol (*C*) and in the presence of 10 ng/ml EGF (*D*) for 12 days.

arrested under these conditions, but ZR/HERc cells started to proliferate after 3–4 weeks, showing many foci. Individual clones have been isolated, demonstrating stable growth in the presence of EGF. Receptor analysis showed that expression of ER was strongly reduced to 3–13 fmol/mg protein, and PR was no longer detectable. Apparently, ZR/HERc cells progressed to a hormone-independent, EGF-dependent phenotype (ER−/±, PR−, and EGF receptor positive) under selection of the antiestrogen.

DISCUSSION

Although many endocrine tumors respond to hormonal therapy, progression of the tumor to a hormone-independent phenotype is inevitable. ER and PR status of primary human breast tumors provide important markers for response to endocrine therapy. EGF receptor expression has been suggested as a prognostic factor for early progression during primary endocrine therapy (18, 19). Whether the EGF receptor plays a causal role in the biology and the clinical course of breast cancer is not known. However, the presence of transforming growth factor α and EGF in breast tumor tissue has been established (32) and, thus, could stimulate cell proliferation via the EGF receptor. In an attempt to obtain insight into the biological basis of antiestrogen resistance and to study this possible autocrine loop, the consequence of ectopic expression of the EGF receptor in a model human breast cancer cell line was investigated. This study demonstrates successful transduction of functional human EGF receptors in ZR-75-1 cells which are completely estrogen dependent and devoid of EGF receptors. Retroviral introduction of the EGF receptor cDNA under control of a heterologous promotor resulted in expression of high-affinity binding sites for EGF. Cell clones (ZR/HERc) have been obtained with different quantities of ^{125}I-EGF-binding sites (range, 43–251 fmol/mg membrane protein). ZR/HERc cells acquired a responsiveness to EGF, independently of the hormonal pathway in proliferation (serum containing) and MTT assays (serum free). These results indicate that ZR-75-1 cells possess all necessary intracellular properties for transduction of signals provided by EGF. No correlation was seen between the EGF-induced proliferation of individual clones and the levels of EGF receptor expression. Even clones containing low levels of EGF receptors responded to EGF with cell proliferation (Table 1 and Fig. 2) and striking morphological alterations within 1

week of culture. Estrogen-dependent growth of ZR-75-1 and ZR/HERc cells could be completely inhibited by OH-tamoxifen or ICI 164,384, in contrast to the responsiveness to EGF of ZR/HERc cells. Therefore, ZR/HERc cells exhibit a hormone- and EGF-responsive phenotype. ZR/HERc cells retained the capacity to form tumors in nude mice and to grow in soft agar in the presence of estradiol. Neither tumor formation nor colony formation was observed in the absence of estradiol with EGF supplementation. Prolonged *in vitro* culture in the presence of EGF resulted in a significantly increased cell population-doubling time of the ZR/HERc cells, but unlimited proliferation potential was retained. The altered cell morphology, the changed keratin expression, and the increased HMFG-2 expression suggest some maturation of the cells, which may explain the increased cell-doubling time. Preculture of ZR/HERc cells with EGF resulted in loss of estrogen-dependent proliferation, despite the presence of a functional hormonal pathway. Furthermore, we observed a negative interaction of EGF- and estradiol-mediated growth, when supplied simultaneously. Our results differ from the data of Valverius *et al.* (33), who failed to induce a hormone-independent phenotype by transfection of the EGF receptor cDNA in ZR-75-1 cells. The reason for this discrepancy is as yet unknown but might be explained by clonal differences in the ZR-75-1 cell line. The ZR-75-1 cells used in our studies are fully dependent on estradiol for growth and do not express detectable EGF receptors. Valverius *et al.* (33) used an estrogen-responsive subclone, which also expressed EGF receptors.

Little information is available concerning coexpression and function of ER and EGF receptors on individual cells in primary breast cancer using immunohistochemical techniques. Toi *et al.* (34) noted that in ER/EGF receptor-positive tumors, EGF receptor-stained cells were distributed inversely of ER-positive cells. It is not known whether functional EGF receptor and ER signal pathways are compatible in an individual cell. Both EGF and estrogen are important regulators of normal breast epithelial cell development but may not function simultaneously. The inverse correlation between ER/PR expression and EGF receptor expression in primary breast tumors agrees very well with the negative interaction of ER and EGF receptor stimulation on proliferation of ZR/HERc cells. Histochemical analysis of normal breast tissue as well as malignant tissue may establish whether coexpression of estrogen receptors and EGF receptors occurs in single cells and at what stage of epithelial cell differentiation. In this way, the hypothesis that these regulatory components function in separate stages of tissue development and thus give rise to different classes of breast tumors may be explored.

Our experiments in the ZR/HERc model show that prolonged culture in the presence of excess OH-tamoxifen results in antihormone-resistant subclones lacking expression of ER and PR. Similar conditions failed to induce proliferating subclones of the parental ZR-75-1 cells. These results suggest that as a consequence of antiestrogen therapy ER expression of initially hormone-responsive cells can be down modulated, resulting in cells with an EGF receptor-positive/ER-negative phenotype. Whether this observation reflects the clinical situation remains a matter of study. Further investigations in model systems and with immunohistochemical techniques to study individual cells in biopsy specimens obtained before and after therapy are needed to establish whether EGF receptor plays a role in progression to hormone independence during antiestrogen therapy of breast cancer.

In conclusion, the results presented in this paper show that ectopic expression of the EGF receptor, in the presence of its ligand, can bypass estrogen-dependent proliferation with concomitant change in differentiation of ZR-75-1 cells. Our results suggest that an incompatibility between active ER and EGF receptor signal transduction pathways exists, which may result in down-modulation of ER expression during antiestrogen therapy and, thus, could contribute to the failure of endocrine treatment. ZR-75-1 and the derived ZR/HERc cells provide an attractive model to study progression from hormone-dependent to hormone-independent growth and the interplay between ER and EGF receptor signal pathways.

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REFERENCES

1. Dickson, R. B., and Lippman, M. E. Estrogenic regulation of growth and polypeptide growth factor excretion in human breast carcinoma. Endocrinol. Rev., *8:* 29–43, 1987.
2. Ullrich, A., and Schlessinger, J. Signal transduction by receptors with tyrosine kinase activity. Cell, *61:* 203–212, 1990.
3. Van de Vijver, M. J., and Nusse, R. The molecular biology of breast cancer. Biochim. Biophys. Acta, *1072:* 33–50, 1991.
4. Jordan, V. C. Long-term adjuvant tamoxifen therapy for breast cancer. Breast Cancer Res. Treat., *15:* 125–136, 1990.
5. Santen, R. J., Manni, A., Harvey, H., and Redmond, C. Endocrine treatment of breast cancer in women. Endocr. Rev., *11:* 221–265, 1990.
6. Berry, M., Metzger, D., and Chambon, P. Role of the two activating domains of the oestrogen receptor in cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. EMBO J., *9:* 2811–2818, 1990.
7. Fawell, S. E., White, R., Hoare, S., Sydenham, M., Page, M., and Parker, M. G. Inhibition of estrogen receptor-DNA binding by the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization. Proc. Natl. Acad. Sci. USA, *87:* 6883–6887, 1990.
8. Gibson, M. K., Nemmers, L. A., Beckman, W. C., Jr., Davis, V. L., Curtis, S. W., and Korach, K. S. The mechanism of ICI 164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. Endocrinology, *129:* 2000–2010, 1991.
9. Clarke, R., Dickson, R. B., and Brunner, N. The process of malignant progression in human breast cancer. Ann. Oncol., *1:* 401–407, 1990.
10. Darbre, P. D. Steroids and steroid receptors in growth control of cultured breast cancer cells. Int. J. Cancer, *55:* 67–75, 1990.
11. Dorssers, L. C. J., Van Agthoven, T., and Sieuwerts, A. M. Genetic mechanisms involved in progression to hormone independence of human breast cancer. *In:* E. Berns, J. C. Romijn, and F. H. Schroeder (eds.), Mechanisms of Progression to Hormone-independent Growth of Breast and Prostatic Cancer, pp. 169–182. Carnforth, United Kingdom, Parthenon Publishing Group, Ltd., 1991.
12. King, R. B. Receptors, growth factors and steroid insensitivity of tumours. J. Endocrinol., *124:* 179–181, 1990.
13. Sluyser, M. Steroid/thyroid receptor-like proteins with oncogenic potential: a review. Cancer Res., *50:* 451–458, 1990.
14. Gullick, W. J. New developments in the molecular biology of breast cancer. Eur. J. Cancer, *26:* 509–510, 1990.
15. Davidson, N. E., Gelmann, E. P., Lippman, M. E., and Dickson, R. B. Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Mol. Endocrinol., *1:* 216–223, 1987.
16. Jensen, E. V. Hormone dependency of breast cancer. Cancer (Phila.), *47:* 2319–2326, 1981.
17. Klijn, J. G. M., Berns, P. M. J. J., Schmitz, P. I. M., and Foekens, J. A. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endocr. Rev., *13:* 1–15, 1992.
18. Nicholson, S., Halcrow, P., Sainsbury, J. R. C., Angus, B., Chambers, P., Farndon, J. R., and Harris, A. L. Epidermal growth factor receptor (EGFr) status associated with failure of primary endocrine therapy in elderly postmenopausal patients with breast cancer. Br. J. Cancer, *58:* 810–814, 1988.
19. Nicholson, S., Halcrow, P., Farndon, J. R., Sainsburyslamon, 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. Lancet, *1:* 182–185, 1989.
20. Engel, L. W., Young, N. A., Tralka, T. S., Lippman, M. E., O'Brien, S. J., and Joyce, M. J. Establishment and characterization of three continuous cell lines derived from human breast carcinomas. Cancer Res., *38:* 3352–3364, 1978.
21. Miller, A. D., and Buttimore, C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol., *6:* 2895–2902, 1986.
22. Von Rüden, T., and Wagner, E. F. Expression of functional human EGF receptor on murine bone marrow cells. EMBO J., *7:* 2749–2756, 1988.
23. Leibovitz, A., Stinson, J. C., McCombs, W. B., McCoy, C. E., Mazur, K. C., and Mabry, N. D. Classification of human colorectal adenocarcinoma cell lines. Cancer Res., *36:* 4562–4569, 1976.
24. Carmichael, J., De Graff, W. G., Gadzar, A. F., Minna, J. D., and Mitchell, J. B. Evaluation of a tetrazolium-based semi-automated colorimetric assay: assessment of chemosensitivity testing. Cancer Res., *47:* 936–941, 1987.
25. EORTC Breast Cancer Cooperative Group. Revision of the standards for the assessment of hormone receptors in human breast cancer. Eur. J. Cancer, *16:* 1513–1516, 1980.
26. Benraad, T. J., and Foekens, J. A. Hydroxylapatite assay to measure epidermal growth factor receptors in human primary breast tumors. Ann. Clin. Biochem., *27:* 272–273, 1990.
27. Koenders, P. G., Beex, L. V. A. M., Geurts-Moespot, A., Heuvel, J. J. T. M., Kienhuis, C. B. M., and Benraad, T. J. Epidermal growth factor receptor-negative tumors are predominantly confined to the subgroup of estradiol receptor-positive human primary breast cancers. Cancer Res., *51:* 4544–4548, 1991.
28. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature (Lond.), *309:* 418–425, 1984.
29. Horwitz, K. B., and McGuire, W. L. Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. J. Biol. Chem., *253:* 2223–2228, 1978.
30. Trask, D. K., Band, V., Zajchowski, D. A., Yaswen, P., Suh, T., and Sager, R. Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. Proc. Natl. Acad. Sci. USA, *87:* 2319–2323, 1990.
31. Baildam, A. D., Howell, A., Baruess, D. M., Redford, J., Healy, K., Swindell, R., and Sellwood, R. A. Expression of differentiation antigens within human mammary tumours is related to response to endocrine therapy and survival. Int. J. Cancer, *42:* 154–158, 1988.
32. Dotzlaw, H., Miller, T., Karvelas, J., and Murphy, L. C. Epidermal growth factor gene expression in human breast cancer biopsy samples: relationship to estrogen and progesterone receptor gene expression. Cancer Res., *50:* 4204–4208, 1990.
33. Valverius, E. M., Velu, T., Shankar, V., Ciardiello, F., Kim, N., and Salomon, D. S. Over-expression of the epidermal growth factor receptor in human breast cancer cells fails to induce an estrogen-independent phenotype. Int. J. Cancer, *46:* 712–718, 1990.
34. Toi, M., Hamada, Y., Nakamura, T., Mukaida, H., Suehiro, S., Wada, T., Toge, T., Nimoto, M., and Hattori, T. Immunocytochemical and biochemical analysis of epidermal growth factor receptor expression in human breast cancer tissues: relationship to estrogen receptor and lymphatic invasion. Int. J. Cancer, *43:* 220–225, 1989.

Induction of Estrogen Independence of ZR-75-1 Human Breast Cancer Cells by Epigenetic Alterations

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Antagonists of steroid hormones are clinically important in the management of breast cancer. However, the duration of response is limited due to the development of hormone-independent tumors in virtually all cases. In an attempt to obtain insight into the mechanisms underlying antiestrogen resistance, the consequences of epigenetic changes in gene expression were studied *in vitro*. Estrogen-dependent ZR-75-1 human breast cancer cells were treated with 5-azacytidine, an inhibitor of DNA methylation, and cultured in the absence of estradiol or in the presence of antiestrogens. Estrogen-independent cell colonies developed within 3 weeks at high frequency in 5-azacytidine-treated cultures (0.7×10^{-3}), in contrast to control cultures ($\leq 10^{-8}$). The derived cells (ZR/AZA) were resistant to 4-hydroxytamoxifen and ICI 164,384, independent of the selection protocol, but had lost the ability to grow anchorage-independent. Whereas expression of estrogen receptor, progesterone receptor, and pS2 were down-regulated, expression of epidermal growth factor (EGF) receptor and HER2/neu were increased in ZR/AZA cells. In contrast to the stable altered expression patterns of estrogen receptor and EGF receptor, transient keratin 7 expression was observed. Transforming growth factor-α mRNA was identified in ZR-75-1 cells and ZR/AZA cells and EGF-like peptides were secreted in the culture medium. Proliferation of ZR/AZA cells could be partially inhibited with an EGF receptor-blocking antibody. Presence of both growth factor receptors and possible ligands suggests the development of an autocrine growth mechanism. Our data show that epigenetic alterations of gene expression result in rapid progression of breast cancer cells to hormone independence. (Molecular Endocrinology 8: 1474–1483, 1994)

INTRODUCTION

Breast growth and development is regulated by a complicated set of interactions among hormones and polypeptide growth factors with their specific cellular receptors (1, 2). These regulators may be produced by the cell itself, by surrounding cells, or by cells at distant sites (3). Estrogen receptor (ER) and the progesterone receptor (PR), like other members of the steroid hormone receptor family, are ligand-inducible transcription factors (4, 5) which, among other functions, play an important role in the biology of the normal mammary gland (3). The ER and its ligand have been implicated in the development and progression of breast cancer (6–8). However, the presence of both ER and PR in primary breast tumors identifies patients with a lower risk of relapse and prolonged survival (6, 9). Furthermore, ER is a target for endocrine therapy of clinical breast cancer. The therapeutic strategies using antagonists of hormones are based on the dependence of breast cancer cells on estrogens for proliferation. These antagonists exert their inhibitory effects on cell proliferation via the hormone receptor system (10). Currently the nonsteroidal antiestrogen tamoxifen is established as the first choice of endocrine therapy of advanced breast cancer, in particular when the primary tumor is steroid hormone receptor-positive (11, 12). Approximately 50% of these patients with ER-positive primary tumors will have an objective response. However, the treatment is palliative and the majority of patients will experience a relapse (7, 13–16).

The mechanism of progression to hormone independence and loss of response to hormonal therapy is yet poorly understood. Changes in therapy response may be related to alterations in the ER function and signal cascade, alterations in the paracrine interactions, and changes in the drug pharmacology (see for review Refs. 3, 15, and 17–19). ER variants have been implicated in response failure (20–26) but do not appear to account for most tamoxifen-resistant breast tumors (27). Altered regulation of gene expression in breast cancer cells has been implicated in the development of estrogen-independent disease and loss of response to hormonal therapy (28, 29). Stable changes in gene expression can derive from both genetic and epigenetic mechanisms and may result in altered cell phenotypes. The cytosine analog 5-azacytidine (5-azaC) is a potent

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inhibitor of methylation of newly synthesized DNA and an inducer of cell differentiation. In many studies it has been shown that treatment with 5-azaC can result in activation of silent genes and in altered phenotypes of target cells (30–33). Whether methylation of DNA plays a causal role in repression of genes *in vivo* is not fully understood (34). Genes with tissue-specific expression often undergo *de novo* methylation in cultures of established cell lines. This may explain the loss of cell type-specific functions and repression of nonessential properties in culture (35). It has been shown that specific functions can be activated in cultured cancer cell lines by treatment with 5-azaC. Furthermore, subjection to specific selection protocols resulted in the identification of mechanisms and genes involved in specific processes of these cells (36–38).

The aim of this study was to establish the consequences of epigenetic changes on estrogen dependence of human breast cancer cells. The ZR-75-1 human breast cancer cell line is a well characterized *in vitro* model with which to study the progression of estrogen-dependent cells to estrogen independence (38, 39). These cells are entirely estrogen-dependent, and growth is completely inhibited with antiestrogens (39). We have treated ZR-75-1 cells with 5-azaC and determined the effects on estrogen dependence and gene expression of various ER-regulated genes *in vitro*.

RESULTS

Induction of Epigenetic Alterations

Human breast carcinoma ZR-75-1 cells maintained in our laboratory are strictly estrogen-dependent for proliferation (39). Culture in the absence of estradiol or the addition of the antiestrogens 4-hydroxy-tamoxifen or ICI 164,384 to estrogen-containing cultures resulted in a complete growth arrest (37, 39). ZR-75-1 cells were treated with different concentrations of 5-azaC (range 0.1–10 μM) for 3 days to induce epigenetic alterations. Subsequently the cells were exposed to antiestrogens or cultured in estrogen-depleted medium. Within 3 weeks of selection, proliferating cell clones were apparent in a background of nonproliferating cells. The number of 5-azaC-induced, estrogen-independent clones was dose dependent over a narrow effective 5-azaC concentration range. In Fig. 1 the results of 4-hydroxy-tamoxifen selection are shown. Maximal numbers of antiestrogen-resistant colonies were observed in cultures containing 5 μM 5-azaC during 3 days. This resulted in approximately 730 clones per 10^6 plated cells using 1 μM 4-hydroxy-tamoxifen selection. This pool of colonies was mass-cultured and designated ZR/AZA-TAM cells. Furthermore, single cell-derived subclones were generated using conditioned medium of murine CRIP cells (38) and hereafter referred to as ZR/AZA 1, 2, and 3. Experiments performed in the presence of 100 nM ICI 164,384 resulted in approximately the same number of colonies (designated ZR/AZA-ICI cells). In estrogen-deprived medium, the number of estrogen-independent colonies (designated ZR/AZA-DCC cells) was approximately 60% lower, compared to 4-hydroxy-tamoxifen or ICI 164,384 selection. Control cultures of ZR-75-1 cells not exposed to 5-azaC, but subjected to the same selection procedures, did not result in estrogen-independent growth. This is in agreement with our previously reported frequency ($\leq 10^{-6}$) of spontaneous development of estrogen independence of these ZR-75-1 cells (38).

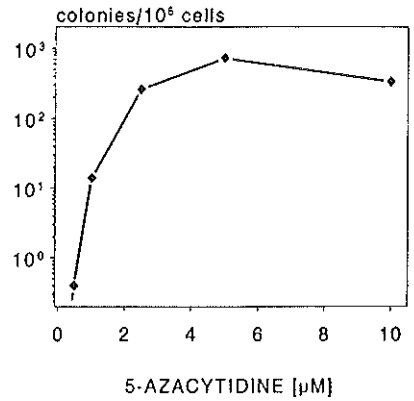

Fig. 1. Induction of Antiestrogen Resistance by Treatment with 5-Azacytidine

Frequency of colonies after treatment of ZR-75-1 cells with increasing doses of 5-azaC and culture in the presence of 1 μM 4-hydroxy-tamoxifen. Colony numbers were determined on day 17 after start of selection.

Down-Regulation of ER, PR, and pS2 Expression

We performed ribonuclease (RNase) protection analysis assays and Northern analysis to examine whether down-regulation of ER and ER-regulated genes in ZR/AZA cells had occurred and could explain antiestrogen resistance. RNase protection analyses using an antisense ER complementary DNA (cDNA) probe showed that ZR/AZA cells contained reduced ER mRNA levels in comparison with the wild type ZR-75-1 cells. In the single cell clone ZR/AZA 3, ER mRNA was barely detectable (Fig. 2). RNase protection assays (Fig. 3) with an antisense PR cDNA probe showed that the estrogen-induced PR mRNA expression was strongly reduced in ZR/AZA cells (range ~2–20% of the level observed in ZR-75-1 cells).

The expression of the pS2 gene is like the PR gene under strict control of ER in breast cancer cells and may reflect functional ER signal transduction (40–43). pS2 mRNA levels were determined by Northern blotting with subsequent hybridization to a pS2 probe. ZR-75-1 cells, which proliferate only in the presence of estradiol, showed very high expression of pS2 mRNA (Fig. 4). ZR/AZA-TAM, -ICI, and -DCC pools and ZR/AZA-TAM subclones were cultured in the presence and

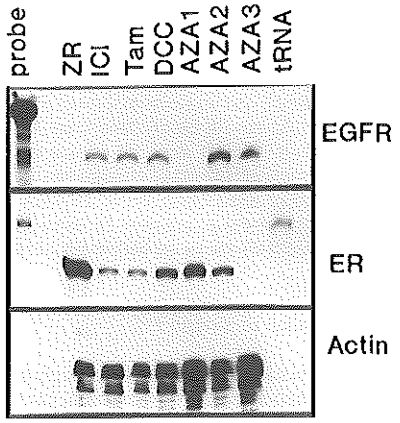

Fig. 2. RNase Protection Assay to Detect ER and EGF Receptor Expression in ZR-75-1 Cells and the Derived ZR/AZA Cells

Twenty micrograms of total RNA of ZR-75-1 (ZR), ZR/AZA-ICI (ICI), -TAM (Tam), -DCC (DCC), ZR/AZA subclones (AZA1-AZA3), and control tRNA were used. Positions of the protected fragments of ER, EGF receptor, and γ-actin mRNA are indicated. Undigested probes for ER, EGF receptor, and γ-actin are shown in the *left lane* (probe). Only the relevant parts of the autoradiographs are shown.

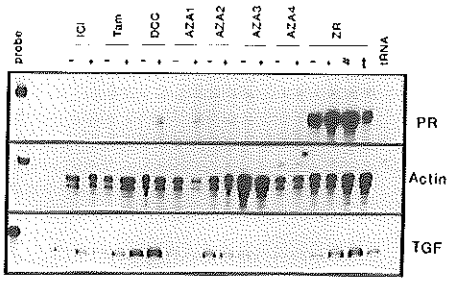

Fig. 3. RNase Protection Assay to Detect PR and TGF$_\alpha$ mRNA

Total RNAs of cells cultured for 5 days without estradiol (−), with 1 nM estradiol (+), with 4-hydroxy-tamoxifen (t), or in the absence of estradiol and subsequently stimulated for 5 h (#) (see also Fig. 2).

absence of estradiol. In the absence of estradiol, the ZR/AZA cells did not express pS2 mRNA. In the presence of estradiol, ZR/AZA-DCC cells showed efficient induction of pS2 mRNA, comparable with expression levels detected in the parental ZR-75-1 cells. In ZR/AZA-TAM cells, estrogen-induced pS2 expression was lower (~40% of the level observed in ZR-75-1 cells), whereas in ZR/AZA-ICI cells pS2 induction was almost completely lost (5%) compared to ZR-75-1 cells (Fig. 4). ZR/AZA subclone 1 showed high pS2 expression when stimulated with estradiol, comparable to ZR-75-1 cells. Subclone 2 contained very low inducible pS2

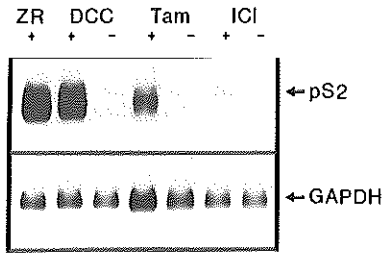

Fig. 4. Northern Blot Analysis of pS2 mRNA from ZR-75-1 Cells and ZR/AZA Cells

Pooled clones of ZR/AZA-DCC, -TAM, and -ICI, were cultured in the presence (+) or absence (-) of 1 nM estradiol. Total RNA (20 μg) was blotted and hybridized with a pS2 probe. The same blot was hybridized with a glyceraldehyde-3-phosphate dehydrogenase probe as an internal control.

Table 1. Expression of Estrogen, Progesterone, and EGF Receptors on ZR-75-1 and ZR/AZA Cells

Cell Line	ER	PR	EGFR
	$-E_2/+E_2$	$-E_2/+E_2$	
ZR-75-1	−/115	/625	ND
ZR/AZA-DCC	−/15	−/56	49
ZR/AZA-Tam	3/8	0/1	72
ZR/AZA-ICI	3/5	0/0	62
ZR/AZA-1	−/9	−/19	ND
ZR/AZA-2	−/2	−/2	20
ZR/AZA-3	−/5	−/1	28
ZR/AZA-4	4/5	1/3	27

For receptor determinations, ZR-75-1 cells were cultured continuously in the presence of 1 nM estradiol. ZR/AZA cells were cultured in the presence of 17β-estradiol (E_2) for 4 days, to allow measurement of ER-induced PR expression ($+E_2$). In addition, ER and PR of ZR/AZA-Tam, ICI, and clone 4 were determined in the absence of 17β-estradiol ($-E_2$). Values are in femtomoles/mg protein. ND, No specific [^{125}I]-EGF binding detected.

mRNA levels (4%) and in clone 3 no pS2 mRNA was detected (Northern blot not shown).

Subsequently, the amount of steroid receptors of ZR/AZA and ZR-75-1 cells was determined. Cytosolic extracts prepared from ZR-75-1 cells contained high levels of ER (115 fmol/mg cytosolic protein) and PR (625 fmol). In contrast, cytosolic extracts prepared from ZR/AZA pools as well as single cell clones contained reduced or hardly detectable ER protein levels (2–15 fmol). This was also apparent for estrogen-induced PR protein levels (0–56 fmol) (Table 1).

EGF Receptor Expression Is Induced in ZR/AZA Cells

The parental ZR-75-1 cells, used in our experiments, expressed ER and PR but contained no detectable EGF receptors (39). We have shown that treatment of ZR-

75-1 cells with 5-azaC and subsequent selection with antiestrogens resulted in down-regulation of ER expression. To examine whether the observed down-regulation of ER was possibly accompanied by an up-regulation of EGF receptor expression, RNase protection assays were performed on RNA preparations of ZR/AZA-TAM, -ICI, and -DCC cells and single cell clones, using an antisense EGF receptor cDNA probe. These analyses showed up-regulation of EGF receptor mRNA levels in all tested ZR/AZA cells as compared with ZR-75-1 cells, except for clone 1, in which we failed to detect EGF receptor mRNA (Fig. 2). Scatchard analysis using [^{125}I]EGF was performed on membrane preparations of ZR/AZA cells to determine EGF receptor expression at the protein level. ZR/AZA cell pools and subclones contained readily detectable, but variable, numbers of EGF binding sites (Table 1). Figure 5 shows an example of Scatchard plots of ZR/AZA cell pools selected in either 4-hydroxy-tamoxifen or ICI 164,384 medium.

Loss of HER2/neu Regulation by Estrogens in ZR/AZA Cells

The HER2/neu protooncogene is down-regulated by estrogen-activated ER in ER-positive breast cancer cell lines (44–46). ZR/AZA cells contained no, or low, levels of ER in comparison with parental ZR-75-1 cells. We determined HER2/neu expression levels in ZR-75-1 and ZR/AZA cells with different residual levels of ER, cultured in the presence and absence of estradiol. Figure 6 shows that HER2/neu is clearly down-regulated in estradiol-stimulated ZR-75-1 cells, as compared with control cells arrested with 4-hydroxy-tamoxifen. In contrast, no differences were observed in ZR/AZA cells cultured in the presence or absence of estradiol. All tested ZR/AZA cell lines contained high levels of HER2/neu mRNA.

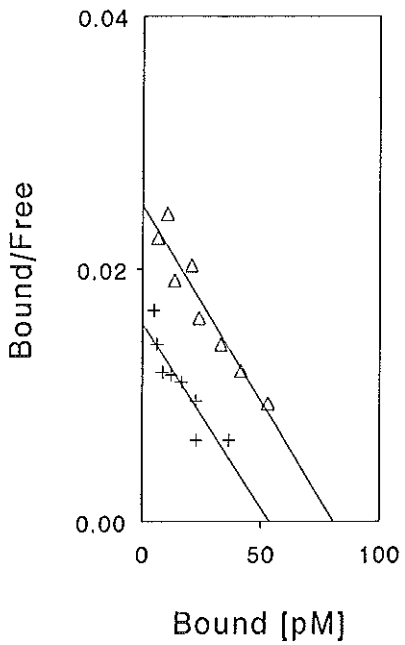

Fig. 5. Scatchard Analysis of EGF Binding
ZR/AZA cells were cultured to confluence in 225 cm^2 flasks in RPMI 1640 medium supplemented with 10% BCS. Specific binding of ZR/AZA-ICI (+) and ZR/AZA-TAM (Δ) is plotted. The amount of [^{125}I]EGF binding sites was 62 and 72 fmol/mg membrane protein with dissociation constant (K_D) of 0.7 and 0.6 nM, respectively. Parental ZR-75-1 cells contained no detectable EGF receptors.

Transient Activation of Keratin 7

ZR/AZA cells presented with marked morphological differences in comparison with the parental estrogen-stimulated ZR-75-1 cells or the nonproliferating control ZR-75-1 cultures in the presence of antiestrogens. These morphological differences presumably reflect alterations in differentiation state regulated by the expression of genes determining cell structure, extracellular matrix attachment, and cell-cell communication. In a previous study we showed structural changes and increased keratin expression in ZR-75-1 cells transfected with the EGF receptor cDNA (39). Changes in expression of keratins may indicate an alteration in maturation state of cells (47). To address the question whether ZR/AZA cells acquired altered differentiation characteristics, keratin and vimentin expression was studied, using various monoclonal antibodies. Parental ZR-75-1 cells expressed keratin 8, 18, and 19, and no vimentin expression was observed. In addition to keratin 8, 18, and 19, we detected large amounts of keratin 7 in ZR/AZA-TAM, -ICI, and -DCC cells. These experiments were repeated with available single cell ZR/AZA clones. Only sporadic cells expressed keratin 7 in these single cell clones. Furthermore, when ZR/AZA cell pools were cultivated over a prolonged time (3 months), keratin 7 expression was detected only in a subset (6–11%) of cells. ZR/AZA cells were negative for vimentin expression.

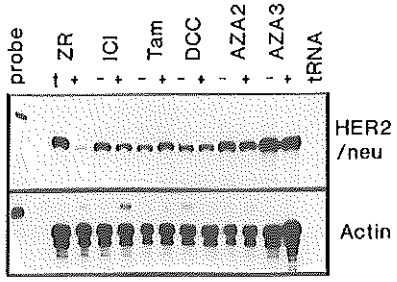

Fig. 6. RNase Protection Assay to Detect HER2/neu mRNA
ZR-75-1 cells were cultured in the presence of 1 μM 4-hydroxy-tamoxifen (t) or 1 nM estradiol (+) and ZR/AZA cells cultured in the presence (+) or absence (–) of estradiol.

Growth Characteristics of ZR/AZA Cells

ZR/AZA cells were completely antiestrogen-resistant and estradiol-independent for proliferation. Furthermore ZR/AZA cells exhibited resistance to 4-hydroxy-tamoxifen and ICI 164,384, independently of the selection strategy (data not shown). The growth characteristics of the ZR/AZA cells, compared to the parental estrogen-stimulated ZR-75-1 cells, were also changed. In contrast to ZR-75-1 cells, proliferation of ZR/AZA cells was extremely cell density-dependent. Initial attempts to subclone ZR/AZA pools, in order to obtain homogeneous single cell clones, failed probably due to this strong dependency on cell density. In contrast from the parental ZR-75-1 cells, ZR/AZA cells could be moderately stimulated by the addition of EGF to the culture medium in a dose-dependent manner (Fig. 7A). To evaluate the contribution of endogenous EGF-like proteins on basal proliferation of ZR/AZA cells, we used the murine hemopoietic cell line 32D, which is not responsive to EGF, and the EGF-responsive 32D/HERc cells carrying the human EGF receptor cDNA. In contrast to the parental 32D cells, the 32D/HERc cells were stimulated by conditioned media of ZR-75-1 cells and ZR/AZA cells, indicating the presence of EGF-like activity in the conditioned medium of these cells (Fig. 7B). RNase protection assays showed that both the ZR-75-1 cells and the ZR/AZA cells expressed transforming growth factor-α (TGFα) (Fig. 3). In ZR-75-1 cells the TGFα mRNA level was increased approximately 3-fold by estradiol, whereas ZR/AZA cells showed no gross differences between estradiol-stimulated or nonstimulated cultures. Expression of both functional EGF receptors and TGFα suggests an autocrine growth mechanism via the EGF receptor signaling pathway. To further assess this autocrine loop, we studied the effects of an EGF receptor-blocking antibody on the growth of ZR/AZA cells and ZR-75-1 control cells. Addition of 0.1 μg/ml of the EGF receptor-blocking monoclonal antibody to the cultures resulted in 23–30% inhibition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction by ZR/AZA cells (Fig. 7C). Further inhibition was observed when 0.5 or 1 μg/ml antibody was added to the culture medium, which resulted in up to 53% growth reduction. The estrogen-stimulated proliferation of the ZR-75-1 cells was not reduced with 0.1 or 0.5 μg/ml antibody. Only the addition of 1 μg/ml antibody to ZR-75-1 cultures resulted in a minor (5%) growth inhibition.

We determined whether down-regulation of ER in ZR/AZA cells had altered the anchorage-independent growth properties of these cells. ZR-75-1 cells showed efficient anchorage-independent growth in the presence of estradiol (1885 colonies per 10^4 plated cells) while no growth was observed in the absence of estradiol. In contrast to the parental cells, no proliferation of ZR/AZA pools and the subclones was observed in soft agar either in the presence or absence of estradiol.

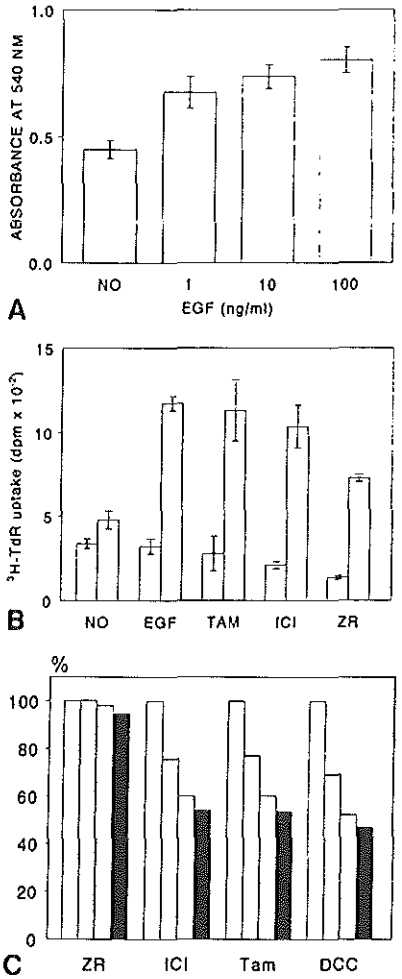

Fig. 7. Biological Characteristics of ZR/AZA Cells

A, Effect of EGF on MTT reduction by ZR/AZA cells. Cells (5×10^3) were plated in medium with 15% BCS. After adherence of the cells the medium was changed for FCS-DCC containing medium. Cell stimulation in response to EGF was measured on day 6. Data are the means of eight wells (±SD). B, EGF-like peptides in conditioned media of ZR/AZA cells. 32D (□) and EGF-responsive 32D/HERc cells (▨) were cultured overnight with or without 10 ng/ml of EGF or with addition of 15% conditioned medium of ZR/AZA-TAM pool cells, ZR/AZA-ICI pool cells, or ZR-75-1 cells. DNA synthesis was determined by measuring the uptake of [^{3}H]thymidine (^{3}H-TdR) as described (68). Data are the means of triplicate incubations (±SD). C, Effect of anti-EGF receptor-blocking antibody on MTT reduction by ZR-75-1 cells (ZR) and ZR/AZA ICI, -Tam, and -DCC cells. ZR/AZA cells were plated at a density of 10^4/well in RPMI/BCS medium containing no (□), 0.1 μg/ml (□), 0.5 μg/ml (▨), or 1 μg/ml (■) antibody. The culture medium of ZR-75-1 cells was supplemented with 10 pM estradiol to stimulate cell proliferation. MTT reduction without addition of anti-EGF receptor antibody is depicted as 100%. All values are the mean of eight wells (SD< 5%).

DISCUSSION

Antiestrogens are used extensively for the treatment of breast cancer (16). Although up to 50% of human breast tumors respond to endocrine therapy, progression of the tumor to a hormone-independent phenotype is inevitable. Insight into the mechanisms underlying antiestrogen resistance is a prerequisite for improvement of therapy. Cell line models have been used to identify genetic elements contributing to progression from hormone-dependent growth to hormone independence. Loss of hormone dependence was observed after long-term steroid depletion or prolonged selection with various types of antiestrogens and adriamycin (reviewed by Refs. 29 and 48) and appears associated with altered gene expression but not with major gene amplification (49). Ectopic expression of v-Ha-*ras* (50), fibroblast growth factor 4 (51), Her2/neu (52), and EGF receptor (39) in breast tumor cell lines resulted in altered hormone responsiveness and implicates these genes in progression. Transfection of random cDNA libraries (53) and insertional mutagenesis using retroviruses (38) could also transform the hormonal phenotype and may lead to the identification of different genes.

In this study we report the effects of epigenetic changes in gene expression as a possible mechanism of development of antiestrogen resistance. ZR-75-1 cells treated with the hypomethylating agent 5-azaC rapidly developed cell colonies in the absence of estradiol as well as in the presence of antiestrogens. Cell clones were obtained when selected with both the partial antagonist 4-hydroxy-tamoxifen and with the pure antiestrogen ICI 164,384. Furthermore, 5-azaC-treated cultures depleted of estrogens progressed to estrogen independence and antiestrogen resistance. ZR/AZA pools and subclones were cultured for several months and showed stable estrogen-independent phenotypes.

Proliferation of ZR/AZA cells could not be stimulated by estradiol. Furthermore, various ER-mediated responses were strongly reduced as a consequence of the down-regulation of ER. Transcription of PR is strictly controlled by ER in breast tissue (40) and is strongly diminished in the ZR/AZA cells at the mRNA and the protein level. Only low levels of PR were detected in the ZR/AZA cells and correlated with the residual ER protein levels. The expression of pS2 in malignant breast epithelial cells is also under strict control of activated ER (41). However, in contrast to PR, pS2 expression was still well induced by moderate or low ER levels. The expression of TGFα has also been shown to be modulated by ER (54). Our results demonstrated moderately increased levels of TGFα mRNA in estradiol-stimulated ZR-75-1 cells, but no significant changes were observed in various ZR/AZA pools and subclones when stimulated with estradiol. This indicates that TGFα is constitutively expressed in ZR/AZA cells independently of ER. Expression of HER2/neu is inhibited by estrogens in estrogen-responsive breast cancer cells, at both the mRNA and the protein level (44–46, 55). ZR/AZA cells contained high levels of HER2/neu mRNA, which expression was no longer down-regulated by residual ER as occurred in the parental cells. ZR-75-1 cells treated with 4-hydroxy-tamoxifen showed high expression levels of HER2/neu, which is down-regulated by the addition of estrogen, in agreement with published data (44–46, 55). ZR/AZA cells selected for estrogen-independent growth exhibit decreased ER levels and consequently have lost the major part of the ER-mediated transcription regulation of *trans*-activated genes. Our data suggest that the induction of PR expression requires a higher threshold level of ER than needed for pS2 induction and thus pS2 may provide a more sensitive monitor for residual ER function. Analogous to the regulation of PR, expression of HER2/neu and TGFα, estrogen-induced morphology, and anchorage-independent growth also appear to require high levels of ER expression.

ZR/AZA cells were extremely dependent on cell density for proliferation, which could be bypassed, at least partially, through the addition of EGF or conditioned medium of mouse fibroblasts. Furthermore, conditioned media obtained from ZR/AZA cells as well as ZR-75-1 cultures contained EGF-like peptides. Moreover, significant amounts of TGFα mRNA were detected in ZR-75-1 cells and ZR/AZA cells. TGFα is a potent mitogen for a variety of epithelial cells and has high affinity for the EGF receptor. Our results show that hypomethylation of ZR-75-1 cells resulted in up-regulation of EGF receptor gene expression. In contrast to the parental cells, ZR/AZA cells acquired a responsiveness to EGF, indicating the presence of a functional EGF receptor signal cascade. Simultaneous expression of TGFα and EGF receptor in ZR/AZA cells is suggestive of an escape from estrogen regulation via an autocrine growth mechanism. This suggestion is supported by the observation that an EGF receptor-blocking antibody does interfere with this autocrine loop (Fig. 7C).

We demonstrated down-regulation of ER expression when ZR-75-1 cells were treated with 5-azaC and selected for estrogen independence. Except for quantitative differences, this phenomenon was independent of the selection regimen and occurred in mass-cultured pools of colonies as well as in single cell clones. Apparently, the reduction or loss of ER expression and function is a prerequisite for estrogen-independent proliferation of ZR/AZA cells. In contrast to the parental cell line, ZR/AZA cells expressed EGF receptor mRNA and/or EGF binding sites. It is well established that EGF receptor expression is inversely related with ER expression in primary breast cancer tissues and in breast cancer cell lines (for a review, see Ref. 56). Furthermore, EGF receptor is expressed in tumors unresponsive to endocrine therapy (57, 58). ZR/AZA cells, generated for this study, also exhibited this inverse relation between ER and EGF receptor expression. In a previous paper we described the transduction of the EGF receptor cDNA in ZR-75-1 cells and showed that simultaneous stimulation of ER and EGF receptors resulted in interference of these signal pathways. Furthermore,

antiestrogen treatment resulted in rapid progression to estrogen independence and down-regulation of ER (39). Recently, we determined the expression patterns of ER and EGF receptors at the single cell level in a series of ER-positive primary breast tumors with dual-staining immunohistochemical techniques (59). Expression of ER and EGF receptors was detected in most biopsy specimens either in the tumor cells or in nonmalignant cells present in the tumor. However, coexpression of ER and EGF receptors in the same tumor cells was a rare event, in agreement with another study (60). In normal ducts adjacent to the tumor cells, we frequently observed coexpression of ER and EGF receptors in individual luminal epithelial cells. This phenomenon was also observed in normal breast tissue indicating a role in normal development (59). The inverse relationship of ER and EGF receptor gene expression observed in the development and progression of breast cancer may be explained by interference of simultaneous activation of these signal pathways prohibiting rapid proliferation.

The effect of 5-azaC on the differentiation state of ZR-75-1 cells was assessed by studying the intermediate filament expression pattern (47). Inverse relation between keratin and vimentin expression has been documented in a subset of ER-negative breast cancer cell lines (61). Association between vimentin expression and ER-independent breast cancer was also observed (62). A positive correlation between EGF receptor and vimentin expression in ER-negative breast tumors has been reported (63). In the present study, we show that ZR-75-1 and ZR/AZA cells expressed keratin 8, 18, and 19 and no vimentin expression was observed, illustrating that the estrogen-independent phenotype is not accompanied by a switch from keratin to vimentin expression. Treatment of ZR-75-1 cells with 5-azaC resulted in activation of keratin 7 expression. Keratin 7 is commonly expressed in luminal epithelial cells of the breast, but rarely at high levels in cultured breast cancer cell lines (64, 65). Interestingly, small amounts of keratin 7 were detected in ER-negative breast cancer cell lines and not in ER-positive cell lines with a sensitive assay (47). However, in ZR/AZA cells expression of keratin 7 was transient, which may be explained by *de novo* DNA methylation during prolonged culture, resulting in inactivation of the keratin 7 gene. Presumably, high expression levels of keratin 7 expression is not essential for continuous proliferation *in vitro*.

Our study shows that epigenetic alterations in gene expression can change the hormonal dependence of breast cancer cells. We demonstrated that altered involvement of growth factor and receptor may contribute to the transition from antiestrogen-responsive to antiestrogen-unresponsive breast tumor cell proliferation *in vitro*. Treatment with 5-azaC resulted in a greater than 4 log increase in estrogen-independent colony formation, compared to control cultures. This is extremely high in comparison with the frequency of classical mutations, which are changes in DNA sequence (point mutation, deletion, insertion, rearrangement). Heritable changes based on modulation of epigenetic changes in gene expression may therefore explain the acquisition of estrogen independence and short benefit of antiestrogen therapy.

MATERIALS AND METHODS

Cells and Culture Conditions

ZR-75-1 human breast cancer cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum (BCS) and 1 nM 17β-estradiol as described (39). The derived ZR/AZA cells were maintained in RPMI 1640 containing 16% BCS. The cytogenetic characteristics of the ZR-75-1 cell line maintained in our laboratory and the variant estrogen-independent ZR/AZA cells are similar to the published data for ZR-75-1 cells (66).

Induction of Estrogen Independence

ZR-75-1 cells (10^7) were exposed to 5-azaC (Sigma, St. Louis, MO) for 3 days (range 0.1–10 μM) in the presence of 1 nM estradiol to stimulate cell proliferation. Subsequently, the medium was changed and 5-azaC was omitted. After an additional 2 days of culture without 5-azaC, the cells were harvested, counted, and reseeded at 3×10^6 cells per 75 cm^2 flask. Selection for estrogen-independent cell growth was performed either in the presence of 1 μM 4-hydroxy-tamoxifen or 100 nM ICI 164,384, a generous gift of ICI (Macclesfield, UK) or in phenol red-free RPMI 1640 medium supplemented with 4.5% fetal calf serum, stripped by dextran-coated charcoal (FCS-DCC). ZR-75-1 cells, not treated with 5-azaC, served as control cultures. Estrogen-independent colonies were pooled and mass-cultured in selection medium for further analysis. Previous experiments showed marked growth advantage and improved attachment of hormone-independent ZR-75-1- derived cell clones in the presence of conditioned medium of murine CRIP fibroblastic cells (38). In a subsequent experiment, cells resistant to 4-hydroxy-tamoxifen were subcloned in medium supplemented with 1 μM 4-hydroxy-tamoxifen plus 10 μg/ml porcine insulin (Organon, Oss, The Netherlands) and 10% conditioned medium of CRIP cells. After two passages, clones were expanded in medium with 15% BCS. In total, eight subclones were isolated and three were randomly chosen for further analysis.

Assays of Cell Growth

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay was used to evaluate the effects of additives to the culture medium on cell proliferation (39, 67).

32D/HERc cells were used to detect EGF-like activities in conditioned media of ZR/AZA cells in a bioassay. 32D/HERc cells originated from 32D (interleukin-3-dependent myeloid leukemia cells) after introduction of the human EGF cDNA utilizing a retroviral vector in a similar manner as previously described (39, 68). In contrast to the parental 32D cells, 32D/HERc cells expressed EGF binding sites and exhibited a transient proliferative response to EGF. DNA synthesis-assay (^{3}H-TdR uptake) was used to determine proliferative response of 32D and 32D/HERc cells and was performed as described previously (68).

EGF receptor blocking studies were performed with EGF receptor (Ab-1) blocking monoclonal antibody from Oncogene Science, Inc. (Manhasset, NY).

Anchorage-independent growth was determined in the presence or absence of 1 nM estradiol, by plating 10^4 cells in RPMI medium containing 20% BCS and 0.3% agar on top of a solidified 0.6% agar layer in 35-mm Petri dishes. After 11 days

of culture the ability of the cells to form colonies was determined.

RNA Isolation and Northern Blot Analysis

RNA was isolated by lysis of the cells with guanidine thiocyanate salt and cesium chloride centrifugation as previously described (69, 70). Northern blot analysis was performed with 1.2% agarose-formaldehyde gels with 20 μg total RNA as described elsewhere (70). RNA was transferred to nitrocellulose membranes (Schleicher & Schull, Dassel, Germany). Probes were random primer labeled using α-^{32}P (Amersham, Buckinghamshire, UK). The probe used was a 528 base pair (bp) cDNA human pS2 probe (41). Hybridization was performed overnight in 9% dextran sulfate-50% formamide. Final washing was performed in 0.3 × sodium citrate-0.1% sodium dodecyl sulfate at 65 C. The efficiency of recovery and the integrity of the RNA were checked by hybridization with glyceraldehyde-3-phosphate dehydrogenase probe. Filters were exposed to Kodak X-Omat AR films at −70 C using intensifying screens.

RNase Protection Analysis

RNase protection was carried out according to standard procedures (70), using 20 μg total RNA. The following probes were used: ER, a 252 bp cDNA fragment [nucleotide positions 1248–1500 according to Green *et al.* (71)]; EGF receptor, a 340 bp EGF receptor cDNA fragment [nucleotide 2951–3291 according to Ullrich *et al.* (72)]; TGFα, a 600 bp NCO1 TGFα cDNA fragment (73); PR, a 123 bp cDNA fragment [nucleotide positions 3171–3294 according to Kastner *et al.* (74)]; HER2/neu, a 376 bp *Bam*HI/*Eco*RI cDNA fragment [nucleotide positions 1075-1451 according to Coussens *et al.*(75)]. In all experiments a probe for γ-actin (76) was used to measure RNA recovery.

Quantitative Assessment of ER, PR, and EGF Receptors

Immunoreactive cytosolic ER and PR were measured using commercially available enzyme immunoassay (EIA) kits, (Abbott Laboratories, Chicago, IL) as instructed by the manufacturer. ZR/AZA cells were cultured in medium supplemented with 1 nM estradiol for 4 days, to allow measurement of ER-induced PR expression. Cell pellets and cytosolic extracts were prepared as described (39), after a 4-h preculture in the absence of estradiol. EGF receptor expression was determined by Scatchard analyses of ^{125}I EGF binding and using hydroxylapatite to separate bound and free ligand as previously described (39, 77).

Immunofluorescence

Immunological detection of keratins was performed on cytocentrifuged cells and/or on a monolayer of cells growing in Leighton tubes (Costar Europe Ltd., Cambridge, MA), as described (39). The monoclonal antibodies used in this study were RCK 105 to keratin 7, M 20 to keratin 8, RCK 106 to keratin 18, and LP2K to keratin 19.

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REFERENCES

1. Osborne MP 1987 Breast development and anatomy. In: Harris JR, Hellman S, Henderson IC, Kinne W (eds) Breast Diseases. Lippincott Company, Philadelphia, pp 1–14
2. Mauvais-Jarvis P, Sitruk-Ware R, Kuttenn F 1988 The role of hormones in the pathogenesis of benign breast disease. In: Grundfest-Broniatowski S, Esselstyn CB (eds) Controversies in Breast Disease. Marcel Dekker, Inc., New York, pp 43–61
3. Clarke R, Dickson RB, Lippman ME 1992 Hormonal aspects of breast cancer. Growth factors, drugs and stromal interactions. Crit Rev Oncol Hematol 12:1–23
4. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
5. Beato R 1989 Gene regulation by steroid hormones. Cell 56:335–344
6. Jensen EV 1981 Hormone dependency of breast cancer. Cancer 47:2319–2326
7. Horwitz KB 1993 Mechanisms of hormone resistance in breast cancer. Breast Cancer Res Treat 26:119–130
8. King RJB 1993 Estrogen and progestin effects in human breast carcinogenesis. Breast Cancer Res Treat 27:3–15
9. McGuire WL, Clark GM, Dressler LG, Owens MA 1986 Role of steroid hormone receptors as prognostic factors in primary breast cancer. Natl Cancer Inst Monogr 1:19–23
10. Parker MG 1993 Action of "pure" antiestrogens in inhibiting estrogen receptor action. Breast Cancer Res Treat 26:131–137
11. Fisher B, Redmond C, Brown A, Wickerman DL, Wolmark N, Allegra J, Escher G, Lippman M, Savlov E, Wittclif J, Fisher ER 1983 Influence of tumor estrogen and progesterone receptor levels on the response to tamoxifen and chemotherapy in primary breast cancer. J Clin Oncol 1:227–241
12. McGuire WL 1987 Prognostic factors for recurrence and survival in human breast cancer. Breast Cancer Res Treat 10:5–9
13. Pritchard KI, Sutherland DJA 1989 The use of endocrine therapy. Hematol Oncol Clin North Am 3:765–805
14. Santen RJ, Manni A, Harvey H, Redmond C 1990 Endocrine treatment of breast cancer in women. Endocr Rev 11:221–265
15. Jordan VC, Murphy CS 1990 Endocrine pharmacology of antiestrogens as antitumor agents. Endocr Rev 11:578–610
16. Early Breast Cancer Trial Collaboration Group 1992 Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 Randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 339:1–15;71–85
17. Katzenellenbogen BS 1991 Antiestrogen resistance: mechanisms by which breast cancer cells undermine the effectiveness of endocrine therapy. J Natl Cancer Inst 83:1434–1435
18. King RJB 1992 Progression from steroid sensitive to

insensitive state in breast tumour. Cancer Surv 14:131–146
19. Johnston SRD, Dowsett M, Smith IE 1992 Towards a molecular basis for tamoxifen resistance in breast cancer. Ann Oncol 3:503–511
20. McGuire WL, Chamness GC, Fuqua SAW 1991 Estrogen receptor variants in clinical breast cancer. Mol Endocrinol 5:1571–1577
21. Scott GK, Kushner P, Vigne JL, Benz CC 1991 Truncated forms of DNA-binding estrogen receptors in human breast cancer. J Clin Invest 88:700–706
22. Murphy LC, Dotzlaw H, Hamerton J, Schwarz J 1993 Investigation of the origin of variant, truncated estrogen receptor-like mRNAs identified in some human breast cancer biopsy samples. Breast Cancer Res Treat 26:149–161
23. Fuqua SAW, Allred DC, Elledge RM, Krieg SL, Benedix MG, Nawaz Z, O'Malley BW, Greene GL, McGuire WL 1993 The ER-positive/PgR-negative breast cancer phenotype is not associated with mutations within the DNA binding domain. Breast Cancer Res Treat 26:191–202
24. Jiang SY, Parker CJ, Jordan VC 1993 A model to describe how a point mutation of the estrogen receptor alters the structure-function relationship of antiestrogens. Breast Cancer Res Treat 26:139–147
25. Miksicek RJ, Lei Y, Wang Y 1993 Exon skipping gives rise to alternatively spliced forms of the estrogen receptor in breast tumor cells. Breast Cancer Res Treat 26:163–174
26. Koehorst SGA, Jacobs HM, Thijssen JHH, Blankenstein MA 1993 Wild type and alternatively spliced estrogen receptor messenger RNA in human meningioma tissue and MCF7 breast cancer cells. J Steroid Biochem Mol Biol 45:227–233
27. Karnik PS, Kulkarni S, Liu XP, Budd GT, Bukowski RM 1994 Estrogen receptor mutations in tamoxifen-resistant breast cancer. Cancer Res 54:349–353
28. Dorssers LCJ, Van Agthoven T, Sieuwerts AM 1991 Genetic mechanisms involved in progression to hormone independence of human breast cancer. In: Berns PMJJ, Romijn JC, Schroeder FH (eds) Mechanisms of Progression to Hormone-Independent Growth of Breast and Prostatic Cancer. Parthenon Publishing Group Ltd, Carnforth, UK, pp 169–182
29. Clarke R, Thompson EW, Leonessa F, Lippman J, McGarvey M, Frandsen TL, Bruenner N 1993 Hormone resistance, invasiveness, and metastatic potential in breast cancer. Breast Cancer Res Treat 24:227–239
30. Taylor SM, Jones PA 1979 Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771–779
31. Avvedimento EV, Obici S, Sanchez M, Gallo A, Musti A, Gottesman ME 1989 Reactivation of thyroglobulin gene expression in transformed thyroid cells by 5-azacytidine. Cell 58:1135–1142
32. Hansen RS, Gartler SM 1990 5-Azacytidine-induced reactivation of the human × chromosome- linked PGK1 gene is associated with a large region of cytosine demethylation in the 5′ CPG island. Proc Natl Acad Sci USA 87:4174–4178
33. Lee SW, Tomasetto C, Swisshelm K, Keyomarsi K, Sager R 1992 Down-regulation of a member of the S100 gene family in mammary carcinoma cells and reexpression by azadeoxycytidine treatment. Proc Natl Acad Sci USA 89:2504–2508
34. Bird A 1992 The essentials of DNA methylation. Cell 70: 5–8
35. Antequera F, Boyes J, Bird A 1990 High levels of *de novo* methylation and altered chromatin structure at CpG islands in cell lines. Cell 62:503–514
36. Habets GGM, Van der Kammen R, Scholtes WHM, Collard JG 1990 Induction of invasive and metastatic potential in mouse T- lymphoma cells (BW5147) by treatment with 5-azacytidine. Clin Exp Metastasis 9:379–390
37. Van Agthoven T, Van Agthoven TLA, Foekens JA, Dorssers LCJ 1993 Regulation of gene expression involved in the progression of human breast cancer to hormone independence. Ann NY Acad Sci 684:250–252
38. Dorssers LCJ, Van Agthoven T, Dekker A, Van Agthoven TLA, Kok EM 1993 Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. Mol Endocrinol 7:870–878
39. Van Agthoven T, Van Agthoven TLA, Portengen H, Foekens JA, Dorssers LCJ 1992 Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. Cancer Res 52:5082–5088
40. Horwitz KB, McGuire WL 1978 Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. J Biol Chem 253:2223–2228
41. Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P 1982 Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. Nucleic Acids Res 10:7895–7903
42. Foekens JA, Rio M-C, Seguin P, Van Putten WLJ, Fauque J, Nap M, Klijn JGM, Chambon P 1990 Prediction of relapse and survival in breast cancer patients by pS2 protein status. Cancer Res 50:3832–3837
43. Foekens JA, Van Putten WLJ, Portengen H, De Koning HYW CM, Thirion B, Alexieva-Figusch J, Klijn JGM 1993 Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: multivariate analysis. J Clin Oncol 11:899–908
44. Read DR, Keith DK, Slamon DJ, Katzenellenbogen BS 1990 Hormonal modulation of HER-2/neu protooncogene messenger ribonucleic acid and p185 protein expression in human breast cancer cell lines. Cancer Res 50:3947–3951
45. Waerri AM, Laine AM, Majasuo KE, Alitalo KK, Haerkoenen PL 1991 Estrogen suppression of erbB2 expression is associated with increased growth rate of ZR-75-1 human breast cancer cells *in vitro* and in nude mice. Int J Cancer 49:616–623
46. Russell KS, Hung MC 1992 Transcriptional repression of the neu protooncogene by estrogen stimulated estrogen receptor. Cancer Res 52:6624–6629
47. Trask DK, Band V, Zajchowski DA, Yaswen P, Suh T, Sager R 1990 Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. Proc Natl Acad Sci USA 87:2319–2323
48. Wolf DM, Jordan VC 1993 Drug resistance to tamoxifen during breast cancer therapy. Breast Cancer Res Treat 27:27–40
49. Bruenner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R 1993 Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 53:283–290
50. Kasid A, Lippman ME, Papageorge AE, Lowy DR, Gelman EP 1985 Transfection of v-RasH DNA into MCF-7 human breast cancer cells bypasses dependence on estrogen for tumorgenicity. Science 228:725–728
51. McLeskey SW, Kurebayashi J, Honig SF, Zwiebel J, Lippman ME, Dickson RB, Kern FG 1993 Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. Cancer Res 53:2168–2177
52. Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, Shepard HM, Osborne CK 1992 Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat 24:85–95
53. Toi M, Harris AL, Bicknell R 1993 cDNA transfection followed by the isolation of a MCF-7 breast cell line

resistant to tamoxifen *in vitro* and *in vivo*. Br J Cancer 68:1088–1096
54. Bates SE, Davidson NE, Valverius EM, Freter CE, Dickson RB, Tam JP, Kudlow JE, Lippman ME, Salomon DS 1988 Expression of transforming growth factor alpha and its messenger ribonucleic acid in human breast cancer: Its regulation by estrogen and its possible functional significance. Mol Endocrinol 2:543–555
55. Antoniotti S, Maggiora P, Dati C, De Bortoli M 1992 Tamoxifen up-regulates c-erbB-2 expression in oestrogen-responsive breast cancer cells *in vitro*. Eur J Cancer 28:318–321
56. Klijn JGM, Berns PMJ J, Schmitz PIM, Foekens JA 1992 The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: A review on 5232 patients. Endocr Rev 13:3–17
57. Nicholson S, Halcrow P, Sainsbury JRC, Angus B, Chambers P, Farndon JR, Harris AL 1988 Epidermal growth factor receptor (EGFr) status associated with failure of primary endocrine therapy in elderly postmenopausal patients with breast cancer. Br J Cancer 58:810–814
58. Nicholson S, Halcrow P, Farndon JR, Sainsburyslamon JRC, Chambers P, Harris AL 1989 Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. Lancet 182–185
59. Van Agthoven T, Timmermans M, Foekens JA, Dorssers LCJ, Henzen-Logmans SC 1994 Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry. Am J Pathol 144:1238–1246
60. Sharma AK, Horgan K, Douglas-Jones A, McClelland R, Gee J, Nicholson R 1994 Dual immunocytochemical analysis of oestrogen and epidermal growth factor receptors in human breast cancer. Br J Cancer 69:1032–1037
61. Sommers CL, Walker-Jones D, Heckford SE, Worland P, Valverius E, Clark R, McCormick F, Stampfer M, Abularach S, Gelmann EP 1989 Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene-transformed mammary epithelial cells. Cancer Res 49:4258–4263
62. Domagala W, Lasota J, Bartkowiak J, Weber K, Osborn M 1990 Vimentin is preferentially expressed in human breast carcinomas with low estrogen receptor and high Ki-67 growth fraction. Am J Pathol 136:219–227
63. Cattoretti G, Andreola S, Clemente C, D'Amato L, Rilke F 1988 Vimentin and p53 expression on epidermal growth factor receptor-positive, oestrogen receptor-negative breast carcinomas. Br J Cancer 57:353–357
64. Moll R, Franke WF, Schiller DL, tumors, cultured cells 1982 The catalog of human cytokeratins: patterns of expression in normal epithelia. Cell 31:11–24
65. Ramaekers F, Huysmans A, Schaart G, Moesker O, Vooijs P 1987 Tissue distribution of keratin 7 as monitored by a monoclonal antibody. Exp Cell Res 170:235–249
66. Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ, Joyce MJ 1978 Establishment and characterization of three continuous cell lines derived from human breast carcinomas. Cancer Res 38:3352–3364
67. Carmichael J, De Graff WG, Gadzar AF, Minna JD, Mitchell JB 1987 Evaluation of tetrazolium-based semi-automated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47:936–941
68. Touw I, Van Gurp R, Schipper P, Van Agthoven T, Löwenberg B 1992 Introduction of the human interleukin-6 (IL-6) receptor in murine IL-3-dependent hematopoietic cells restores responsiveness to IL-6. Blood 79:2867–2872
69. MacDonald RJ, Swift GH, Przybyla AE, Chirgwin JM 1987 Isolation of RNA using guanidinium salts. In: Berger SL, Kimmel AR (eds) Guide to Molecular Cloning Techniques. Methods in Enzymology. Academic Press, San Diego, CA, vol 152:219–227
70. Sambrook J, Fritsch EF, Maniatis T 1989 Molecular Cloning: A Laboratory Manual, ed 2. Cold Spring Harbor Press, Cold Spring Harbor, NY
71. Green S, Walter P, Kumar V, Krust A, Bornert J-M, Argos P, Chambon P 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-*erb*-A. Nature 320:134–139
72. Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH 1984 Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309:418–425
73. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV 1984 Human transforming growth factor alpha: precursor structure and expression in *E. coli*. Cell 38:287–297
74. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P 1990 Two distinct estrogen-related promotors generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 9:1603–1614
75. Coussens L, Yang-Feng TL, Liao Y, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, Levinson A, Ullrich A 1985 Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 230:1132–1138
76. Enoch T, Zinn K, Maniatis T 1986 Activation of the human β-interferon gene requires an interferon inducible factor. Mol Cell Biol 6:801–810
77. Benraad TJ, Foekens JA 1990 Hydroxylapatite assay to measure epidermal growth factor receptor in human primary breast tumours. Ann Clin Biochem 27:272–273

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Differential Expression of Estrogen, Progesterone, and Epidermal Growth Factor Receptors in Normal, Benign, and Malignant Human Breast Tissues using Dual Staining Immunohistochemistry

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Distribution of estrogen (ER), progesterone (PR) receptors, and epidermal growth factor (EGF) receptors was assayed by dual staining immunohistochemistry on 28 selected cytosolic ER-positive breast carcinomas and 22 nonmalignant breast tissues. ER-positive tumor cells were detected in 26 (93%) and EGF receptor positive tumor cells were detected in 7 (25%) carcinomas. In five tumors both ER and EGF receptors were detected but localized in distinct tumor cells. Only in one case of ductal carcinoma in situ co-expression was observed in a subset of tumor cells. In contrast, simultaneous expression of ER/PR and EGF receptors was observed in non-neoplastic ductal remnants in the majority of the carcinomas and the fibroadenomas. In addition, double-positive cells were occasionally detected in luminal epithelial cells of normal breast tissue and mastopathies. This study shows that ER/PR and EGF receptors in breast tumor cells are inversely related at the single cell level. However, demonstration of ER/PR and EGF receptors in individual normal luminal cells shows that expression is not mutually exclusive. (Am J Pathol 1994, 144:1238–1246)

The estrogen receptor (ER) and the progesterone receptor (PR) belong to the steroid hormone receptor family[1,2] of ligand inducible transcription factors that play a role in the development and progression of breast cancer.[3,4] Significant amounts of ER have been detected in over 50% of primary human breast cancers.[5] Presence of ER in primary tumors identifies patients with a lower risk of relapse and better overall survival.[5,6] Furthermore, response to endocrine therapy largely depends on the presence of ER and PR, the latter indicating functional ER.[7–9] As a consequence, ER determination has become an established procedure in the management of patients with breast cancer.[10] Approximately 50% of the patients with recurrent disease who had ER-positive primary tumors will respond to hormonal manipulation. However, duration of the response is limited because of progression to an estrogen-independent state of the tumor.[3,11] Although little is known about the mechanisms underlying this progression,[12,13] some prognostic factors predicting failure of endocrine therapy have been identified.[14] The presence of EGF receptors has been reported to be indicative of poor prognosis and to correlate with lack of response to endocrine therapy in recurrent breast cancer.[15,16] However, there is no agreement on the prognostic value of EGF receptor expression in breast cancer.[17]

It has been well established that expression of EGF receptor is inversely related with ER expression in primary breast tumors and in breast cancer cell lines, both on the protein and the mRNA level.[17] However, approximately 50% of ER-positive tumors contain EGF receptors.[18] In nonmalignant breast tissue, presence of both receptors was also demonstrated.[19] Until now most determinations of ER and EGF receptors

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have been performed in extracts of homogenates of tumor and nonmalignant breast tissue, which does not allow for the identification of the cellular origin of these receptors. Consequently, little is known on whether in breast tissues both receptors are expressed in different or in the same cells.[20] Furthermore, assays on tissue extracts do not allow for the assessment of the possible heterogeneity of ER and EGF receptors among cells or regions within a tumor specimen. In addition, the presence of normal cells in a tumor specimen may further complicate these analyses. Until now immunohistochemical analyses have been performed on tumor cells only for either receptor separately, thus solely excluding the possible contribution of normal cells.[20]

It would be of interest to determine the relationship of ER and EGF receptor expression at the single cell level using immunohistochemical techniques. We have studied whether in breast tumors with known ER status EGF receptors are inversely or co-expressed in individual cells. Furthermore, we have determined the expression patterns in normal tissues and of benign breast lesions. We have established a double immunohistochemical staining method to determine steroid hormone receptors and EGF receptor expression patterns in breast biopsies. Double immunohistochemical analysis for PR and EGF receptors was performed to determine whether the ER is functional in tumors expressing both ER and EGF receptors. The presence of PR indicates functional ER signal transduction, because PR expression is regulated by ER.[7] The lack of breast cancer lines expressing both ER or PR and EGF receptors prompted us to use transfected cell lines co-expressing these receptors to develop a reliable assay.

Materials and Methods

Cell Lines

ZR/HERc cells were derived from ZR-75-1 cells after introduction of the human EGF receptor cDNA,[21] under control of the herpes simplex thymidine kinase promotor, using a retroviral vector[22] as previously described.[23] MDA/HEGO cells were derived from MDA-MB-231 cells by co-transfection with 10 µg of the human ER cDNA expression vector HEGO containing the early SV40 promoter[24] and 1 µg of the pLN expression vector for the neomycin resistance gene[25] by cationic liposomes (Lipofectin Reagent, GIBCO-BRL, Life Technologies Ltd., Paisley, UK). Colonies surviving 1 mg/ml of the neomycin analogue Geneticin (GIBCO-BRL) were expanded to stable cell lines. MDA/HEGO cell lines were selected for ER expression by Northern (RNA) analysis and cytosolic ER determination.

Culture Conditions

ZR-75-1 and the derived ZR/HERc cells were maintained in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% heat-inactivated bovine calf serum (BCS) (HyClone Laboratories Inc., Logan, UT) and 1 nmol/L 17β-estradiol (Merck, Darmstadt, Germany) as described.[23] The RPMI 1640 culture medium of the MDA-MB-231 and the MDA/HEGO cells was supplemented with 10% BCS.

Quantitative Assessment of ER, PR, and EGF Receptors

Cytosolic ER and PR was determined using a commercially available enzyme immunoassay (EIA) kit (ER-EIA, PgR-EIA; Abbott Laboratories, Chicago, IL). EGF receptor expression was determined by Scatchard analyses of [^{125}I]EGF binding and using hydroxylapatite to separate bound and free ligand as previously described.[23,26]

Tissue Specimens and Cell Pellets

Cell lines for immunohistochemical analyses were cultured in 225-cm^2 flasks in the appropriate medium. At 90 to 100% confluence, cells were harvested by using 1 mmol/L EDTA in phosphate-buffered saline (PBS). The cells were pelletted by centrifugation for 10 seconds in a microcentrifuge and were snap-frozen in liquid nitrogen and stored at −80 C until use. Surgical biopsy specimens of normal breast tissue, benign breast lesions, and 28 mammary carcinomas (26 primary breast tumors and 2 with recurrent disease) were examined. The carcinomas were comprised of 21 invasive ductal carcinomas (6 of these contained a predominant intraductal component), 4 invasive lobular carcinomas (1 of these contained an *in situ* component), 2 mixed invasive (ductal/lobular) carcinomas (1 contained an *in situ* component), and 1 colloid carcinoma.

Nonneoplastic breast tissues were analyzed in 22 cases: 2 noninvolved tissue specimens adjacent to the tumor, 4 normal breast tissues from women who underwent mammoplastic surgery, and 8 mastopathies. In addition, eight fibroadenomas were analyzed. All tissues were snap-frozen and stored in liquid nitrogen. Histological diagnosis was assessed

according to the World Health Organization (1981). For double immunohistochemical analysis tumors were selected containing cytosolic ER using the ER-EIA assay, the lowest positive value was 9 fmol/mg protein. No EGF receptor data were available for selection of the tumors.

Immunohistochemical Procedures

Immunohistochemical detection of EGF receptor was performed with monoclonal antibody (MAb) EGFR1 (Amersham International plc, Amersham, UK) and MAb clone 2E9 (Monosan, Uden, The Netherlands). The latter MAb was included for its strong reactivity with EGF receptor-positive cells. Both MAbs recognize a protein determinant of the extracellular domain of the EGF receptor and do not cross-react with blood group A antigen.[27,28] ER and PR detection was performed with commercially available immunocytochemical (ICA) kits (ER-ICA, PgR-ICA; Abbott Laboratories).

Cell pellets and tumor samples were mounted using Tissue Tec (OCT compound; Miles Diagnostics Division, Elkhart, IN). Frozen tissue samples and cell pellets were cut at 6 μ in a cryostat (at −20 C) and thaw-mounted on glass slides. Slides were air-dried for 30 minutes and fixed in 3.7% formaldehyde-PBS solution for 10 minutes followed by immersion in −20 C ethanol for 5 minutes and in −20 C acetone for 3 minutes. Each slide was immersed in 1% bovine serum albumin (BSA) in PBS for 30 minutes before a 60-minute room temperature incubation with the primary MAbs. Specific binding of either of the EGF receptor MAbs was visualized by incubation with rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) for 30 minutes, followed by incubation with mouse alkaline phosphatase anti-alkaline phosphatase (APAAP; Sigma) for 30 minutes and subsequent color development with Fast Blue BB salt (Sigma) creating a blue reaction product. Subsequently, the immunohistochemical assay for ER or PR was performed as described by the manufacturer (Abbott Laboratories) with the following modification. Primary ER or PR antibody incubation was prolonged to overnight at ambient temperature in a moist chamber. Diaminobenzidine (DAB) was used as chromogen, resulting in a brown nuclear localized reaction product.

Control incubations were done by replacing the primary antibody with PBS or normal rat antibody. Slides were not counterstained to obtain maximum sensitivity. In all analyses serial sections cut from a single MDA/HEGO cell pellet were included in each slide to monitor the immunohistochemical staining procedure. For each experiment separate ER-ICA and EGFR-ICA were performed on the cell pellets and the tumor biopsies as a control. In all cases, these ER-ICAs and EGFR-ICAs showed identical results as the dual ICAs, with respect to the number of ER- or EGF receptor-positive cells in cell line preparations and in tumors. Simultaneous detection of PR and EGF receptors was performed on the cell lines and a selection of biopsies using the same procedure as the double ER and EGF receptor ICA. In these analyses sections cut from a ZR/HERc pellet were included as control. The presence of myoepithelial cells was confirmed with an anti-muscle Actin IgG (ENZO Diagnostics, Inc., New York) on a serial section.

Results

Simultaneous Immunohistochemical Detection of Steroid Hormone and EGF Receptors

Cell Lines

The combination of an anti-ER rat IgG (H222) and anti-EGF receptor mouse IgG (EGFR-1 or 2E9) allowed for double staining, as described in Materials and Methods. In a panel of cell lines, simultaneous immunohistochemical detection of ER and EGF receptor yielded clear labeling in contrasting colors of both receptors (Figure 1). In Table 1, the results obtained with the double immunohistochemical assay on two established human breast cancer cell lines and their transfected derivatives are summarized. ZR-75-1 cells showed moderate expression of ER in approximately 40% of the nuclei and no EGF receptor was detected with either MAb EGFR1 or 2E9. ZR/HERc cells (ZR-75-1 cells transfected with the EGF receptor cDNA) expressed ER in 70% of the cells and EGF receptor was detected in all cells with both antibodies. All MDA-MB-231 cells showed strong EGF receptor expression but no ER staining (Figure 1A). In contrast, approximately two-thirds of the MDA/HEGO cells (MDA-MB-231 cells transfected with the ER cDNA) also expressed ER (Figure 1B).

The same panel of cell lines was used to establish the feasibility of double immunohistochemical detection of PR and EGF receptors. The combination of an anti-PR rat IgG and anti-EGF receptor mouse IgG (EGFR-1 or 2E9) allowed for double staining, similarly as described for ER and EGF receptor. As shown in Table 1 and Figure 1, C and D, PR was readily detected in combination with EGF receptor. ZR-75-1 cells expressed PR in 67% of the cells and no EGF receptors were detected. The ZR/HERc cell line

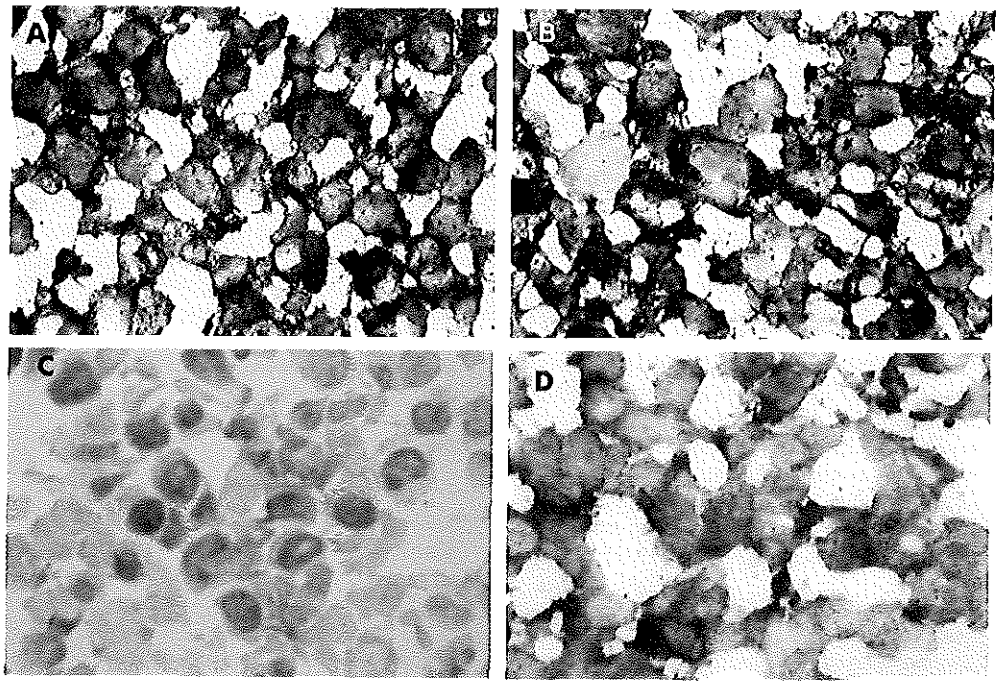

Figure 1. *Dual staining immunohistochemical analysis of ER and EGF receptor expression and PR and EGF receptor expression on breast cancer cell lines.* A: *High magnification of MDA-MB-231 cells revealing EGF receptor-positive membrane staining and ER-negative nuclei.* B: *MDA/HEGO cells showing strong EGF receptor membrane staining and positive reaction of the nuclei with the anti-ER MAb, indicating co-expression of ER and EGF receptors.* C: *PR expression in the nuclei of ZR-75-1 cells, no reaction for EGF receptor is observed.* D: *ZR/HERc cells showing brown nuclear staining of PR and blue membrane staining of EGF receptor with MAb 2E9, indicating co-expression of PR and EGF receptor (×100). Similar results were obtained with MAb EGFR1 but with approximately five times reduced staining intensity (data not shown).*

Table 1. *Expression of ER and EGF Receptor Determined on Breast Cancer Cell Lines*

Cell Line	ER-EIA	PR-EIA	EGFR	ER-ICA	PR-ICA	EGFR-ICA
ZR-75-1	95	>500	ND	+ (40%)	++ (67%)	–
ZR/HERc	100	>500	35	+ (70%)	++ (68%)	+ (100%)
MDA-MB-231	0	0	879	–	–	+++ (100%)
MDA/HEGO	202	0	1012	++ (70%)	–	+++ (100%)

Receptor concentration determined with biochemical assays is expressed as fmol/mg protein. ND, no EGFR detectable. Dual immunohistochemical assay was scored as follows. Staining intensity: –, no staining; +, weak positive staining; ++, moderately positive staining; +++, strong positive staining. The percentage of positive cells is shown.

expressed PR in 68% of the cells and EGF receptors in all cells. PR was not detected in the ER-negative MDA-MB-231 cell line, nor in the ER-positive MDA/HEGO cells with either the PR-ICA or the PR-EIA assay (Table 1). Introduction of the ER cDNA into hormone-independent MDA-MB-231 cells (MDA-HEGO) resulted in ER expression and 50% reduction of cell proliferation on estradiol addition to the culture medium. However, various ER-regulated genes were not expressed in these cells (unpublished observations in agreement with other studies).[29,30]

Tumor Biopsies

In Table 2, the results obtained with 28 human breast cancer biopsies are summarized.

ER Expression. As expected from the selection criteria (only ER-EIA positive tumors were included) the majority of the 28 (92.9%) carcinomas examined showed brown nuclear immunoreactivity in the tumor cells, indicating ER expression (Figure 2A). The percentage of immunoreactive tumor cells varied from 12 to 95%. In addition, ER was also detected in luminal epithelial cells of nonneoplastic ductal remnants in

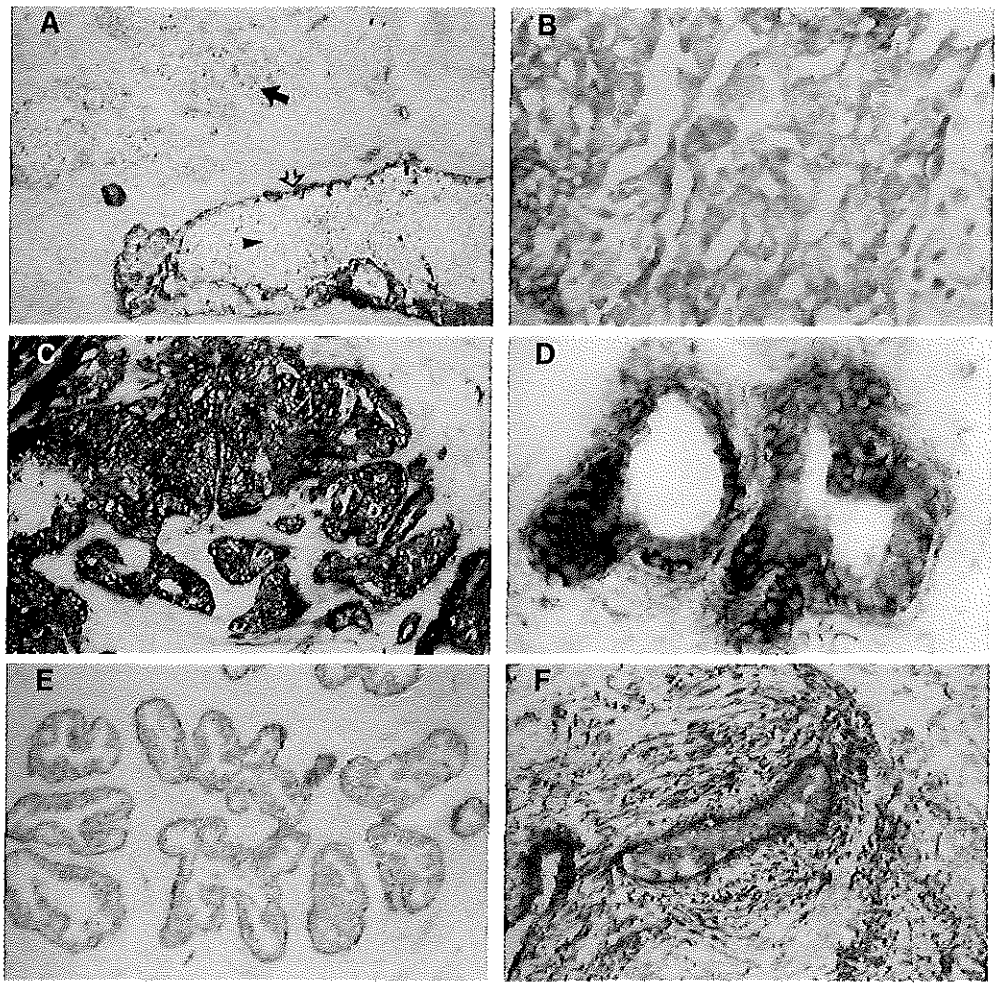

Figure 2. *Dual immunohistochemical staining of ER, PR, and EGF receptor on breast tissues.* A: *Case 27 invasive ductal carcinoma/DCIS with ER-positive invasive cells* (arrow) *and DCIS cells* (arrowhead) *and EGF receptor-positive myoepithelial cells* (open arrow). Original magnification ×16. B: *High magnification of a subset of DCIS cells from case 27 showing co-expression of ER and EGF receptors and cells expressing only one receptor type* (×63). C: *Dual immunohistochemical staining of a carcinoma (case 21) with strong EGF receptor expression in most of the tumor cells* (×16). D: *Normal duct adjacent to the tumor (case 11) with co-expression of ER and EGF receptors in luminal epithelial cells and EGF receptor expression in the myoepithelial cells* (×63). E: *Example of mastopathy with PR expression in luminal cells and EGF receptor-positive myoepithelial cells* (×16). F: *Fibroadenoma with EGF receptor-positive stromal cells and duct with PR-positive luminal cells and EGF receptor expression in the myoepithelial cells* (×16).

Table 2. *Expression of ER, PR, and EGF Receptors in Breast Carcinomas Using Dual Staining*

Case	Carcinoma Type	ER/PR-EIA*	CIS		Invasive	Stroma†	Non-neoplastic Ductal Remnants
			Luminal	Myoepithelial			
1	Invasive ductal	601			O		O/EGFR
2	Invasive ductal	19/36			EGFR		O/EGFR‡
3	Invasive ductal	204			O		O + EGFR
4	Invasive ductal	592			O	EGFR	O/EGFR
5	Invasive ductal	546			O	EGFR	
6	Invasive ductal	238			O	EGFR	O + EGFR
7	Invasive ductal	36/532			O,P + EGFR†		O,P/EGFR
8	Invasive ductal	61			O		EGFR
9	Invasive ductal	65			O	EGFR	O/EGFR
10	Invasive ductal	51			O		O/EGFR
11	Invasive ductal	62			O	EGFR	O/EGFR
12	Invasive ductal	14/8			EGFR		
13	Invasive ductal	22			O	EGFR	O/EGFR
14	Invasive ductal	142			O		O/EGFR
15	Invasive ductal/DCIS	305	O	EGFR	O	EGFR	
16	Invasive ductal/DCIS	193	O	EGFR	O	EGFR	O/EGFR
17	Invasive ductal/DCIS	98/559	O,P + EGFR	EGFR	O,P	EGFR	O,P/EGFR
18	Invasive ductal/DCIS	13	O	EGFR	O		O/EGFR
19	Invasive ductal/DCIS	38/64	O,P + EGFR	EGFR	O,P	EGFR	O,P/EGFR
20	Recurrent invasive ductal	760			O		EGFR
21	Recurrent invasive ductal/DCIS	9/1	EGFR	EGFR	O + EGFR	EGFR	O,P/EGFR
22	Invasive lobular	656			O		O/EGFR
23	Invasive lobular	62			O	EGFR	O/EGFR
24	Invasive lobular	38	O		O		O/EGFR
25	Invasive lobular/DCIS	52	O	EGFR	O		O/EGFR
26	Invasive lobular/ductal	101			O		O/EGFR
27	Invasive lobular/duct/DCIS	153/23	O/EGFR	EGFR	O,P	EGFR	O,P/EGFR
28	Colloid	271			O	EGFR	O/EGFR

The tumor biopsies were divided in carcinoma *in situ* (CIS), with luminal cells and myoepithelial cells, the invasive component, stroma, and nonneoplastic ductal remnants. The abbreviations used are: O, ER expression; EGFR, EGFR expression; O + EGFR, ER and EGF receptor expression on different subsets of cells; O/EGFR, expression of ER and EGF receptors in the same cells and cells expressing only one receptor type; P, PR expression; DCIS, ductal carcinoma *in situ*.

* PR-EIA data are only given for the biopsies with EGFR-positive tumor cells (cases 2, 7, 12, 17, 19, 21, and 27) that were investigated in the double PR/EGFR ICA. Receptor concentration determined with ER-EIA and PR-EIA is expressed as fmol/mg protein.

† Cells reactive with MAb 2E9 but not reactive with MAb EGFR1 were detected.

‡ No normal ducts present in section for PR/EGF receptor analysis.

the tumor biopsy present in 25 of the 28 (89.3%) tumors (Figure 2D). No ER expression was observed in the stroma or in myoepithelial cells.

EGF Receptor Expression. Of the 28 carcinomas 7 (25%) showed immunoreactivity of tumor cells with MAbs EGFR1 and 2E9, indicating EGF receptor expression (Figure 2C, Table 2, cases 2, 7, 12, 17, 19, 21, and 27). Of these seven EGF receptor-positive carcinomas, immunoreactivity was confined to the carcinoma *in situ* (CIS) component in three cases. Both membrane and cytoplasmic staining of the tumor cells was observed with MAb 2E9 and EGFR1. In addition, nonneoplastic ductal remnants in the tumor biopsy and normal breast ducts in adjacent breast tissue showed strong staining of the myoepithelial cells and moderate staining of some of the luminal cells (Figure 2D). Furthermore, EGF receptor expression of the surrounding myoepithelial cells of ductal carcinomas *in situ* (DCIS) was observed (Figure 2A, Table 2). In most cases MAb 2E9 exhibited a similar pattern but a more intense staining when compared with MAb EGFR1. Apart from reactivity with tumor cells and myoepithelial cells, MAb 2E9 but not MAb EGFR1 showed weak to moderate staining with parts of the stroma containing actin-positive myofibrillic elements (Table 2).

Co-expression of ER, PR, and EGF receptors. Our study revealed that all biopsies contained EGF receptor-positive cells. However, only in 7 of the 28 biopsies were EGF receptors expressed in the tumor cells. Five (17.9%) of the 28 tumors showed mosaic expression of ER and EGF receptor, ie, ER-positive tumor cells were distinct from EGF receptor-positive cells in the section. Only in a mixed invasive ductal

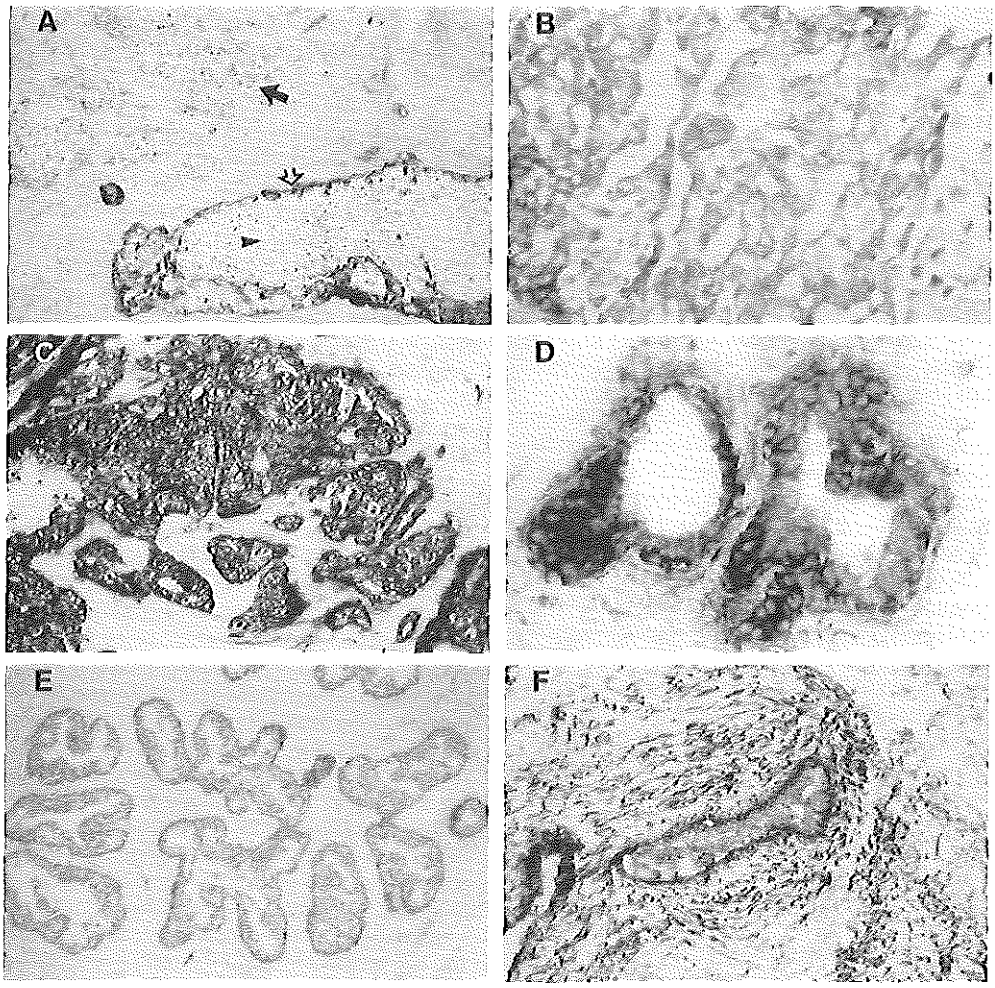

Figure 2. *Dual immunohistochemical staining of ER, PR, and EGF receptor on breast tissues.* A: *Case 27 invasive ductal carcinoma/DCIS with ER-positive invasive cells* (arrow) *and DCIS cells* (arrowhead) *and EGF receptor-positive myoepithelial cells* (open arrow). Original magnification ×16. B: *High magnification of a subset of DCIS cells from case 27 showing co-expression of ER and EGF receptors and cells expressing only one receptor type* (×63). C: *Dual immunohistochemical staining of a carcinoma (case 21) with strong EGF receptor expression in most of the tumor cells* (×16). D: *Normal duct adjacent to the tumor (case 11) with co-expression of ER and EGF receptors in luminal epithelial cells and EGF receptor expression in the myoepithelial cells* (×63). E: *Example of mastopathy with PR expression in luminal cells and EGF receptor-positive myoepithelial cells* (×16). F: *Fibroadenoma with EGF receptor-positive stromal cells and duct with PR-positive luminal cells and EGF receptor expression in the myoepithelial cells* (×16).

carcinoma, DCIS (case 27) co-expression of ER and EGF receptors (MAb 2E9) was observed in a subset of cells. In the DCIS 70% of the tumor cells expressed ER; 60% EGF receptors and approximately 30% contained both receptors (Figure 2, A and B). Co-expression of ER and EGF receptor in individual tumor cells was not observed with MAb EGFR1 because of reduced staining intensity.

PR is regulated by the ER and often expressed at considerable higher levels than the latter. Double staining for PR and EGF receptor was performed on the EGF receptor-positive tumors to confirm the absence of hormone receptors in EGF receptor-expressing tumor cells. Analogous to ER, PR-expressing tumor cells were distinct from EGF receptor-expressing cells. The CIS component, con-

taining ER and EGF receptor double-positive cells, was not present in the remaining specimen of case 27 for analysis of PR and EGF receptor co-expression.

Expression of ER, PR, and EGF Receptor in Nonmalignant Breast Tissue

Double-positive nonneoplastic ductal epithelial cells were observed in 21 of 28 tumor biopsies with ER MAb and the EGF receptor MAbs. In addition, in two samples of noninvolved breast tissue adjacent to the tumor, we noticed strong co-expression of ER and EGF receptor in up to 10% of the luminal epithelial cells (case 11 and 22, Figure 2D).

Analysis of another 22 nonmalignant tissue samples showed the following results. Four normal breast tissue specimens showed EGF receptor expression in the myoepithelial cells and the luminal epithelial cells. Focal and heterogeneous expression of ER and PR was observed in the luminal epithelial cells. Co-expression of ER, PR, and EGF receptor was noticed in a small percentage of the luminal cells. In eight cases of mastopathy, weak to moderate expression of ER and PR in the majority of the luminal epithelial cells was observed and moderate expression of EGF receptors in the surrounding myoepithelial cells (Figure 2E). Co-expression of ER, PR, and EGF receptors was observed in a small number of luminal epithelial cells. In eight cases of fibroadenoma the luminal cells expressed ER and the myoepithelial cells EGF receptors. Frequently, luminal cells showed strong co-expression of ER and EGF receptors. Similar observations were made for PR expression. In the stromal cells of all the fibroadenomas we noticed strong reactivity with MAb 2E9 (Figure 2F). EGF receptor was also visualized with MAb EGFR1 in the fibroadenomas, indicating high expression levels.

Discussion

In this study the expression patterns of ER, PR, and EGF receptor in individual cells of human breast carcinomas and nonmalignant breast tissues were determined using dual staining immunohistochemical techniques. We have developed a method for the simultaneous detection of ER or PR and EGF receptor on frozen sections. Using a panel of transfected human breast cancer cell lines the procedure was optimized and verified for specificity and sensitivity. Our results show that low levels of hormone and EGF receptors can be detected simultaneously. ER levels determined in cytosolic preparations of cell lines (range 40 to 202 fmol/mg protein) were readily detectable in the tumor cells with the double immunohistochemical assay (range 25 to 70% positive cells). ER levels determined in cytosolic preparations of tumor homogenates (range 9 to 760 fmol/mg protein) were readily detected with the immunohistochemical assay (range 12 to 95% positive tumor cells). Correlation of ER-EIA and ER-ICA was 92.9%. Only in two ER-EIA-positive cases (cases 2 and 12) ER-ICA failed to detect ER- or PR-positive tumor cells. However, one of these biopsies (case 2) contained ER-positive ductal remnants, which may explain this difference. Discrepancies may also arise due to sample differences for the EIA and ICA analysis.

We detected 40 to 70% ER-positive cells in the cell lines in contrast to EGF receptor expression, which was observed in all cells of the EGF receptor-positive cell lines. Heterogeneity of ER expression by tumor cells is well established[31,32] and the failure to detect ER in all cells may be attributed to either low sensitivity of detection, mosaicism of tumor cells, or cell cycle regulation of ER protein levels. Using a MAb directed against PR, which is often expressed at considerably higher levels than ER, we found mostly similar numbers of positive cells. Immunohistochemistry with a different anti-ER mouse IgG (Dako) on the cell lines and a subset of the biopsies gave increased staining intensity, yet the degree of staining heterogeneity was not significantly altered (data not shown). The ZR-75-1 cell line and clonal derivative are fully estrogen dependent and exhibit a homogeneous phenotype.[13,23] Expression of ER in the clonal MDA/HEGO cells is controlled by a heterologous promotor and is never detectable in all cells. These results show that neither the sensitivity nor the clonal state of these cell lines can explain the heterogeneous staining for ER. This suggests that ER and PR protein levels may be strongly regulated during cell cycle progression, in contrast to EGF receptors that were detected in all cells of the EGF receptor-expressing cell lines. In the tumor biopsies EGF receptor expression levels were also less heterogeneous compared with ER expression. In CIS components of the tumors we detected up to 95% ER-positive cells, possibly reflecting the different proliferation kinetics in the tumor in comparison to the cell lines.

Screening of 28 breast carcinomas has shown that all possible expression patterns occur in individual tumor cells. Tumor cells expressing either ER or EGF receptors were readily detected. In addition, tumor areas without detectable ER or EGF receptors were observed, indicating absence or expression below our detection level. Thus, alternative signal pathways may be used for regulation of proliferation of these breast cancer cells. Alternatively, variant ER proteins

missing exon 5 may have escaped detection by the ER antibody used,[33,34] but analysis of PR did not reveal more double-positive cases. The fourth phenotype, ER and EGF receptors on the same tumor cell was only observed in one case of DCIS with MAb 2E9, indicating a low expression level of EGF receptors. In the majority of the tumors containing both ER and EGF receptors, these were inversely expressed in different subsets of tumor cells or in normal components present in the tumor biopsy.

Our study shows that expression of ER and EGF receptors is heterogeneous with respect to tumor biopsy compound (invasive/CIS/stroma/nonneoplastic ductal remnants) and thus this study further documents the heterogeneous nature of primary breast tumors. Consequently, in addition to biochemical assays, immunohistochemical analysis of ER and EGF receptor expression may help to give better understanding concerning the biology, prognosis and the tumor response to hormonal therapy. ER and PR expression was exclusively restricted to actin-negative cells of the epithelial lineage but absent in actin-positive myoepithelial breast cells. In contrast, EGF receptor expression was not only demonstrated in luminal and myoepithelial cells but also in stromal cells surrounding the breast carcinoma cells and in all cases of fibroadenomas studied.

In malignant breast tissues ER and EGF receptor are inversely related. Even in tumors expressing both receptors, ER and EGF receptor are rarely expressed simultaneously in the same cells. Apparently both receptor signal pathways become uncoupled during malignant progression. This may be explained by the negative interaction between ER and EGF receptor signal pathways observed in artificial cell lines.[23,29,30] It was demonstrated that in ER-positive cells HER2/neu becomes down-regulated on estrogen stimulation.[35–37] Similar regulation mechanisms for ER and EGF receptor may exist in breast epithelial cells. However, our observations show that ER and EGF receptor are frequently co-expressed in nonmalignant breast tissue and thus the interplay of both receptor signal pathways must play an as yet unidentified role in the growth and differentiation of normal mammary ducts.

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References

1. Evans RM: The steroid and thyroid hormone receptor superfamily. Science 1988, 240:889–895
2. Beato R: Gene regulation by steroid hormones. Cell 1989, 56:335–344
3. Horwitz KB: Mechanisms of hormone resistance in breast cancer. Breast Cancer Res Treat 1993, 26:119–130
4. King RJB: Estrogen and progestin effects in human breast carcinogenesis. Breast Cancer Res Treat 1993, 27:3–15
5. Jensen EV: Hormone dependency of breast cancer. Cancer 1981, 47:2319–2326
6. McGuire WL, Clark GM, Dressler LG, Owens MA: Role of steroid hormone receptors as prognostic factors in primary breast cancer. Natl Cancer Inst Monogr 1986, 1:19–23
7. Horwitz KB, McGuire WL: Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. J Biol Chem 1978, 253:2223–2228
8. Fisher B, Redmond C, Brown A, Wickerman DL, Wolmark N, Allegra J, Escher G, Lippman M, Savlov E, Wittcliff J, Fisher ER: Influence of tumor estrogen and progesterone receptor levels on the response to tamoxifen and chemotherapy in primary breast cancer. J Clin Oncol 1983, 1:227–241
9. McGuire WL: Prognostic factors for recurrence and survival in human breast cancer. Breast Cancer Res Treat 1987, 10:5–9
10. DeSombre ER, Carbone PP, Jensen EV, McGuire WL, Wells SA, Wittliff JL, Lipsett MB: Special report: Steroid receptors in breast cancer. N Engl J Med 1980, 301:1011–1012
11. McGuire WL, Chamness GC, Fuqua SAW: Estrogen receptor variants in clinical breast cancer. Mol Endocrinol 1991, 5:1571–1577
12. Dorssers LCJ, Van Agthoven T, Sieuwerts AM: Genetic mechanisms involved in progression to hormone independence of human breast cancer: mechanisms of progression to hormone-independent growth of breast and prostatic cancer. Edited by Berns PMJJ, Romijn JC, Schroeder FH. Camforth, UK, Parthenon Publishing Group Ltd, 1991, pp 169–182
13. Dorssers LCJ, Van Agthoven T, Dekker A, Van Agthoven TLA, Kok EM: Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. Mol Endocrinol 1993, 7:870–878
14. Klijn JGM, Berns EMJJ, Dorssers LCJ, Foekens JA: Molecular markers of resistance to endocrine treatment of breast cancer: drug and hormonal resistance in breast cancer: cellular and molecular mechanisms. Edited by Dickson RB, Lippman ME. Chichester, Ellis Horwood Ltd, 1994, in press

15. Sainsbury JRC, Farndon JR, Needham GK, Malcolm AJ, Harris AL: Epidermal growth factor receptor as predictor of early recurrence of and death from breast cancer. Lancet 1987, 1:1398–1402
16. Nicholson S, Halcrow P, Farndon JR, Sainsbury JRC, Chambers P, Harris AL: Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. Lancet 1989, 1:182–185
17. Klijn JGM, Berns PMJJ, Schmitz PIM, Foekens JA: The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endocr Rev 1992, 13:3–17
18. Koenders PG, Beex LVAM, Geurts-Moespot A, Heuvel JJTM, Kienhuis CBM, Benraad TJ: Epidermal growth factor receptor-negative tumors are predominantly confined to the subgroup of estradiol receptor-positive human primary breast cancers. Cancer Res 1991, 51: 4544–4548
19. Barker S, Panahy C, Puddefoot JR, Goode AW, Vinson GP: Epidermal growth factor receptor and oestrogen receptor in the non-malignant part of the cancerous breast. Br J Cancer 1989, 60:673–677
20. Toi M, Hamada Y, Nakamura T, Mukaida H, Suehiro S, Wada T, Toge T, Nimoto M, Hattori T: Immunocytochemical and biochemical analysis of epidermal growth factor receptor expression in human breast cancer tissues: relationship to estrogen receptor and lymphatic invasion. Int J Cancer 1989, 43:220–225
21. Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH: Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 1984, 309:418–425
22. Von Rüden T, Wagner EF: Expression of functional human EGF receptor on murine bone marrow cells. EMBO J 1988, 7:2749–2756
23. Van Agthoven T, Van Agthoven TLA, Portengen H, Foekens JA, Dorssers LCJ: Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. Cancer Res 1992, 52:5082–5088
24. Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I, Chambon P: The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. EMBO J 1989, 8:1981–1986
25. Miller AD, Rosman GJ: Improved retroviral vectors for gene transfer and expression. Biotechniques 1989, 7:980–990
26. Benraad ThJ, Foekens JA: Hydroxylapatite assay to measure epidermal growth factor receptors in human primary breast tumors. Ann Clin Biochem 1990, 27: 272–273
27. Defize LHK, Mummery CL, Moolenaar WH, De Laat SW: Antireceptor antibodies in the study of EGF-receptor interaction. Cell Differ 1987, 20:87–102
28. Gerdin E, Juhlin C, Malmgren M, Gerdin B: Immunohistochemical identification of receptors for epidermal growth factor in tumor endothelium may be affected by cross-reactivity to blood group A antigen. Am J Clin Pathol 1993, 99:28–31
29. Jiang SY, Jordan VC: Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst 1992, 84:580–591
30. Garcia M, Derocq D, Freiss G, Rochefort H: Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of the cells. Proc Natl Acad Sci USA 1993,
31. Fritz F, Posey YF: Discrepancies of the biochemical and immunohistochemical estrogen receptor assays in breast cancer. Hum Pathol 1988, 19:960–966
32. Robertson JFR, Bates K, Pearson D, Blamey RW, Nicholson RI: Comparison of two oestrogen receptor assays in the prediction of the clinical course of patients with advanced breast cancer. Br J Cancer 1992, 65: 727–730
33. Greene GL, Sobel NB, King WJ, Jensen EV: Immunochemical studies of estrogen receptors. J Steroid Biochem 1984, 20:51–56
34. Fuqua SAW, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, O'Malley BW, McGuire WL: Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res 1991, 51:105–109
35. Read DR, Keith DK, Slamon DJ, Katzenellenbogen BS: Hormonal modulation of HER-2/neu protooncogene messenger ribonucleic acid and p185 protein expression in human breast cancer cell lines. Cancer Res 1990, 50:3947–3951
36. Waerri AM, Laine AM, Majasuo KE, Alitalo KK, Haerkoenen PL: Estrogen suppression of erbB2 expression is associated with increased growth rate of ZR-75-1 human breast cancer cells in vitro and in nude mice. Int J Cancer 1991, 49:616–623
37. Russell KS, Hung MC: Transcriptional repression of the neu protooncogene by estrogen stimulated estrogen receptor. Cancer Res 1992, 52:6624–6629

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EXPRESSION OF ESTROGEN, PROGESTERONE AND EPIDERMAL GROWTH FACTOR RECEPTORS IN PRIMARY AND METASTATIC BREAST CANCER

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The prognostic value of epidermal growth factor receptor (EGFR) expression and its biological role in estrogen receptor-positive (ER^+) and ER-negative (ER^-) primary breast cancer is controversial. In this study, distributions of ER, progesterone receptor and EGFR have been established using immunohistochemistry in both primary breast tumors and their matched axillary lymph node metastases of 26 patients or their matched distant metastases of 2 patients. In addition, 5 patients with bilateral breast cancer were studied. ER^+ tumor cells were detected in 22 (69%) and $EGFR^+$ tumor cells were detected in 11 (34%) primary breast carcinomas. Expression of ER and EGFR was inverse regarding the individual tumor cells in both primary tumors and metastases. Relationship of EGFR expression with poorly differentiated and large breast tumors was observed. Furthermore, primary tumors with a predominant lobular component were ER^+ and, with one exception, $EGFR^-$. Invasive ductal carcinomas were more frequently $EGFR^+$. No apparent differences in receptor expression were observed between primary tumors and lymph node metastases or chronously or metachronously occurring bilateral breast cancers. Only one ER^+ primary tumor showed a switch to EGFR expression in the involved lymph node. Our study shows that a shift in receptor phenotype between primary tumors and lymph node metastases is a rare event and, thus, additional analyses of involved lymph nodes will not likely serve as a better predictor for response to anti-estrogen therapy. We conclude that expression of EGFR is not a prerequisite for development of metastases. © *1995 Wiley-Liss, Inc.*

Multiple factors contribute to the development and progression of breast cancer. Among these factors estrogens play a crucial role. Estrogens bind to the estrogen receptor (ER), resulting in an activated complex that acts as a transcription factor through binding to target genes (Evans, 1988; Beato, 1989; Parker *et al.*, 1993). Estrogens can stimulate tumor cells directly or indirectly by an autocrine growth mechanism (Clarke *et al.*, 1992). Estrogen-dependent growth can be blocked by anti-estrogens, which compete for binding to the ER (Pritchard and Sutherland, 1989; Jordan and Murphy, 1990; Watts *et al.*, 1994). Consequently, the presence of ER in primary tumors identifies breast cancer patients responsive to anti-estrogen therapy. However, the presence of ER is no warrant for response to endocrine therapy because half of the patients with ER^+ primary tumors do not respond (Jaiyesimi *et al.*, 1995). Furthermore, almost all patients will ultimately develop metastases refractory to treatment. The mechanisms underlying hormone resistance for the majority of cases remain unclear (Dorssers *et al.*, 1991; Katzenellenbogen, 1991; King, 1992; Johnston *et al.*, 1992; Clarke *et al.*, 1992; Horwitz, 1993; Osborne and Fuqua, 1994; Van Agthoven *et al.*, 1994*b*).

Since the actions of estrogens and anti-estrogens are mediated *via* the ER, the development of ER^- tumors would ultimately result in hormone resistance. Expression of ER is inversely related to expression of the epidermal growth factor receptor (EGFR) in breast cancer (reviewed by Klijn *et al.*, 1992). Patients with breast tumors containing high levels of EGFR fail to respond to endocrine therapy (Nicholson *et al.*, 1989). In a previous report, we described the expression patterns of ER, progesterone receptor (PR) and EGFR in a series of cytosolic ER^+ primary breast carcinomas (Van Agthoven *et al.*, 1994*a*). Dual-staining immunohistochemistry allowed simultaneous detection of the steroid hormone receptors and EGFR at the single-cell level. Expression of ER/PR and EGFR was detected in most biopsy specimens in either cancer cells or non-malignant cells present in the tumor. Co-expression of hormone receptors and EGFR in the same tumor cells was rarely observed (Van Agthoven *et al.*, 1994*a*; Sharma *et al.*, 1994). The purpose of the present work was to study further the biology of tumor progression by comparing the expression patterns of ER/PR and of EGFR in a series of primary breast tumors with their matched metastases.

MATERIAL AND METHODS

Selection of breast tumors

Frozen tumor specimens of both primary breast cancer and axillary lymph node metastasis were available from 26 patients. Furthermore, 5 patients with either synchronously (n = 3) or metachronously (n = 2) occurring bilateral breast cancer and 2 patients with distant metastasis (one of the patients with synchronously occurring bilateral breast cancer) were analyzed. Patients were chosen at random. Histological diagnosis was assessed according to the World Health Organization (Azzopardi *et al.*, 1982). Tumors were graded according to the Bloom and Richardson system with minor modifications as described by Page and Anderson (1987). The age of the patients at the time of surgery, size of the primary tumor and number of involved lymph nodes were recorded as well.

Statistic analysis

Statistic analysis was performed using Fisher's exact tests.

Immunohistochemical procedures

Expressions of ER, PR and EGFR were individually assessed in duplicate serial sections of snap-frozen tissues stored in liquid nitrogen as described (Van Agthoven *et al.*, 1994*a*). In addition, a part of the cases were ER/EGFR double-stained (Van Agthoven *et al.*, 1994*a*) to confirm the interpretations of the individually stained sections. Immunohistochemical detection of ER and PR was performed with commercially available immunocytochemical (ICA) kits, (ER-ICA, PgR-ICA; Abbott, Chicago, IL). Detection of EGFR was performed with a monoclonal antibody (MAb) clone 2E9 (Monosan, Uden, The Netherlands) and MAb EGFR1 (Amersham, Aylesbury, UK). In all analyses, serial sections of positive and negative control cell lines were included in each slide to monitor the immunohistochemical staining procedure. The presence of myoepithelial cells was confirmed with an anti-muscle actin IgG (Enzo, New York, NY). Furthermore, a serial section was used to test the presence of keratin 8- or 18-positive epithelial cells using MAb CAM 5.2 (Becton Dickinson, Erebodegem, Belgium). All slides were analyzed by 3 investigators (T.V.A., M.T. and S.C.H.-L.).

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TABLE I – EXPRESSION OF ER, PR AND EGFR AND CHARACTERISTICS OF PRIMARY BREAST TUMORS AND METASTASES

Case	Age (year)	Tumor type	Grade	Size (cm)	CIS		Invasive	Stroma	NNDR	META		
					Luminal	Myo				Number of involved nodes	Tumor	Stroma
1	52	Invasive ductal	II	2.4			OP	E	E	1	OP	E
2	45	Invasive ductal	II	4			OP	E	EOP	4	O	E
3	56	Invasive ductal	II	3			OP	E	EOP	6	OP	E
4	58	Invasive ductal	II	3			E		EOP	7	E	
5	61	Invasive ductal	III	5			OP			11	OP	
6	63	Invasive ductal	III	3			O		EOP	2	O	
7	53	Invasive ductal	III	5			E		EOP	9	E/E	
8	43	Invasive ductal	III	8			E	E	EP	7	E	E
9	55	Invasive ductal	III	16			E		E	1	E	
10	23	Invasive ductal	III	5			E			>8	E	
11	66	Invasive ductal/DCIS	II	4	O	E	O		EOP	3	OP	
12	62	Invasive ductal/DCIS	II	1.5	OP	E	OP	E	EOP	4	OP	E
13	41	Invasive ductal/DCIS	II	1.3	E	E	E		EOP	6	E	E
14	49	Invasive ductal/DCIS	III	6	E	E	E	E	EP	7	E	E
15	52	Invasive ductal/DCIS	III	2.2	OP	E	OP	E	EOP	4	OP	E
16	47	Invasive lobular	I	4			OP	E	EOP	4	OP	E
17	45	Invasive lobular	I	4			OP	E	EOP	14	OP	E
18	45	Invasive lobular	I	5			OP	E	EO	7	OP	E
19	45	Invasive lobular	II	4.5			OP	E	EOP	8	OP	E
20	49	Invasive lobular	II	7			OP	E	E	6	OP	E
21	50	Invasive lobular/ductal	II	3			OP		EOP	6	OP	
22	46	Invasive lobular/ductal	II	3			OP	E	E	5	OP	E
23	43	Invasive lobular/ductal	II	2.7			OP	E	EOP	1	OP	E
24	43	Invasive lobular/ductal	III	2.5			OP	E	E	2	OP	E
25	48	Invasive lobular/ductal DCIS	II	3	OP	E	OP			8	E	E
26	45	Invasive lobular/ductal DCIS	I	4.5	OP	E	OP	E	EOP	4	OP	E
27L	49	Invasive ductal	III	7			E	E		28	E	
27R	49	Invasive ductal	III	7			E	E				
28L	49	Invasive ductal	III	ND*			E	E		ND		
28R	49	Invasive ductal	III	ND*			E	E		0		
28	50	Skin metastasis									E	
29L	68	Colloid	I	1.5			OP		EOP	0		
29R	68	Colloid	I	0.8			OP		EOP	0		
30R	56	Invasive lobular/DCIS	I	1.5	OPE	E	OP		EOP	1	ND	
30L	59	Invasive lobular	I	4			OPE	E	EOP	0		
31L	46	Invasive lobular/CIS	I	3	OP		OP		EOP	10	ND	
31R	49	Invasive lobular	I	ND			OP	E	EOP	0		
32	60	Invasive ductal	III	4			E			0		
32	63	Thorax meta	III								E	

Distribution of receptor expression was subdivided in case of primary tumors in carcinoma *in situ* (CIS) with luminal tumor cells and myoepithelial (myo) cells, the invasive tumor component, stromal and non-neoplastic ductal remnants (NNDR). Metastases were divided in tumor cells and stroma. Further abbreviations used are: O, ER expression; P, PR expression; E, EGFR expression; L, tumor in left breast; R, tumor in right breast; DCIS, ductal carcinoma *in situ;* ND, not determined; ND*, not determined, large tumor diffuse growth.

RESULTS

The clinical, histological and immunohistochemical data of the 32 patients are listed in Table I and summarized in Table II.

Estrogen receptor

Expression of ER was restricted to malignant epithelial cells and non-malignant luminal epithelial cells of non-neoplastic ductal remnants. Of the 32 primary breast carcinomas analyzed, ER was detected in the tumor cells of 22 (69%) patients. ER was observed both in the invasive tumor cells and in the tumor cells of the carcinoma *in situ* (CIS) component (7 cases). Of the 26 cases studied with lymph node metastases, the tumor cells of 19 (73%) primary breast cancers and 18 (69%) lymph node metastases were ER^+. In one case (25) with an ER^+ primary tumor, no ER^+ cells in the lymph node metastasis were detected.

In all 8 cases with ER^- primary breast tumors, lymph node metastases were ER^- as well. Staining of ER was heterogeneous with respect to intensity and percentage of positive cells. The number of ER^+ tumor cells varied between 7 and 152/40× high-power field in the primary tumors and between 15 and 160 in the lymph node metastases. However, no gross differences were observed between primary tumors and metastases.

Progesterone receptor

Expression patterns of PR were similar to those of ER. The number of positive tumor cells varied between 2 and 139 in the primary tumors and between 2 and 150 in the lymph node metastases. PR was detected in the tumor cells of 20 of the 32 (63%) primary tumors and in 16 of the 26 (62%) lymph nodes. In one case (2), PR expression was not detected in the involved lymph node, which cannot be explained by hormone status of the patient because both biopsy specimens were removed at the same time. In another primary tumor (11), we observed PR^- tumor cells and PR^+ non-neoplastic ductal remnants and clear PR^+ staining of the tumor cells in the lymph node.

Epidermal growth factor receptor

EGFR expression was detected in tumor cells, myoepithelial cells of ductal carcinoma *in situ* (DCIS) and myoepithelial cells and luminal cells of non-neoplastic ductal remnants with both MAbs EGFR1 and 2E9 (Table I). Stromal components often reacted with the MAb 2E9 to EGFR both in primary tumors

TABLE II - RELATIONSHIP BETWEEN EGFR EXPRESSION AND CHARACTERISTICS OF PRIMARY TUMORS

Variable	n	EGFR⁺	EGFR⁻	p-value
Total	32 (100%)	11 (34%)	21 (66%)	
Tumor type				
Ductal	18 (56%)	10 (56%)	8 (44%)	
Lobular	13 (41%)	1 (8%)	12 (92%)	<0.006
Colloid	1 (3%)	0 (0%)	1 (100%)	
Tumor size				
T_1 ($\leq$ 2 cm)	4 (13%)	1 (25%)	3 (75%)	
T_2 (> 2–5 cm)	22 (69%)	4 (18%)	18 (82%)	
$T_{3,4}$ (> 5 cm)	6 (19%)	5 (83%)	1 (17%)	<0.006
Grade				
I	7 (22%)	1 (17%)	6 (86%)	
II	13 (41%)	2 (15%)	11 (85%)	
III	12 (38%)	8 (67%)	4 (33%)	<0.001
Nodal status				
1–3	7 (24%)	1 (14%)	6 (86%)	
> 3	22 (76%)	7 (32%)	15 (68%)	N.S.
Estrogen receptor				
ER-ICA⁺	22 (69%)	1 (5%)	21 (95%)	
ER-ICA⁻	10 (31%)	10 (100%)	0 (0%)	<0.001
Progesterone receptor				
PR-ICA⁺	20 (63%)	0 (0%)	20 (100%)	
PR-ICA⁻	12 (38%)	10 (83%)	2 (17%)	<0.001

and metastases. The 2E9 MAb was included in this study for its high sensitivity compared with the EGFR1 MAb and specific detection of EGFR⁺ cells (Van Agthoven *et al.*, 1994*a*). Staining of the tumor cells with the 2E9 MAb and EGFR1 was in general more homogeneous in comparison with ER and PR. Similar to ER and PR, no apparent differences between primary tumors and metastases were observed.

With respect to tumor cells, EGFR was detected in 11 (34%) primary tumors both in the invasive and the CIS component (13, 14) or only in the CIS component (30). Of the 26 cases studied with involved lymph node metastases, 8 (31%) primary tumors and 9 (35%) metastases were EGFR⁺. EGFR was also detected in 2 cases with distant metastases both in the primary tumor and the metastases (2 and 16).

Relationships of receptor expression with tumor characteristics

The relationship between EGFR expression, tumor classification and steroid receptor expression is shown in Table II.

Expression of EGFR was inverse, with ER and PR ($p < 0.001$) both in the primary tumors and the metastases. One case (30) with ER/PR⁺ and EGFR⁺ tumor cells was observed. However, expression was on different tumor cells, which was confirmed with ER, PR and EGFR double-staining.

More than half of the cases with invasive ductal carcinoma were EGFR⁺ and ER⁻. A completely different distribution was observed in the cases with a predominant invasive lobular component ($p < 0.006$). All cases (n = 13) were ER⁺ and PR⁺ and, with one exception, EGFR⁻. This case of invasive lobular carcinoma with a DCIS component showed EGFR expression in the DCIS part of the biopsy (30).

With respect to tumor size, we observed that large tumors were more often ER⁻ and EGFR⁺ (83%) as compared with the smaller T1 (18%) and T2 (25%) tumors ($p < 0.006$). Similar observations were made with respect to degree of tumor differentiation. More than half of the high-grade tumors (67%) belonged to the ER⁻/EGFR⁺ group ($p < 0.001$). No ER⁻, EGFR⁺ tumors were observed in the grade I group and only 15% in the grade II group (Table II).

No clear relationship with the number of involved lymph nodes was observed, though patients with more than 3 involved nodes tended to be more frequently EGFR⁺. Interestingly, all patients with a tumor in the contralateral breast had identical receptor status, for both tumors. Furthermore, both tumors were of identical histological type, *i.e.*, invasive ductal carcinoma (27 and 28), invasive lobular carcinoma (30 and 31) and colloid carcinoma (29; Table I).

DISCUSSION

Our study shows a relationship between degree of tumor differentiation and inverse expression of ER and EGFR. Most tumors expressing EGFR belonged to the high-grade, poorly differentiated malignancies. Furthermore, large tumors more frequently showed expression of EGFR. More than half of the invasive ductal carcinomas were ER⁻ and EGFR⁺. In contrast, all primary breast tumors with a predominant lobular component expressed ER and, with one exception, were EGFR⁻. Interestingly, in normal breast tissues the highest frequency and expression levels of ER are found in the lobules compared to the ducts (Peterson, 1987). The published data about the EGFR⁻ phenotype of lobular carcinoma are controversial, as is the association of EGFR expression with differentiation grade and tumor size (reviewed in Klijn *et al.*, 1992). These discrepancies are at present not clear; however, some may be explained by the different assay systems used. In tumor extracts, the presence of EGFR⁺ non-malignant cells in the biopsy may complicate the interpretation of results.

In this study, we have confirmed our earlier observation (Van Agthoven *et al.*, 1994*a*) that expression of ER and EGFR is completely inverse in individual cancer cells. Most samples contained both ER⁺ and EGFR⁺ cells, but one receptor type was always expressed in a normal component of the tumor specimen. This result is in agreement with previous observations of inverse expression of EGFR and ER in breast tumor cell lines and primary breast tumors (for a review see Klijn *et al.*, 1992), indicating that functional co-expression of these signal cascades is not favorable for breast tumor cells (Van Agthoven *et al.*, 1992, 1994*b*). This inverse relation between ER and EGFR expression has now also been demonstrated for breast tumor metastases in untreated patients.

With only one exception, no switching of receptor type (ER/EGFR) was observed between primary tumors and involved lymph nodes or distant metastases or between synchronously and metachronously occurring bilateral breast cancers. It can be concluded that expression of EGFR is not a prerequisite for development of metastases, though patients with high numbers of axillary lymph node metastases were more often EGFR⁺, which may be explained by the aggressive phenotype of these tumors. Previously, we have shown, with dual-staining immunohistochemistry, that primary tumors may consist of both EGFR⁺ tumor cells and distinct ER⁺ tumor cells (Van Agthoven *et al.*, 1994*a*). Although only one mixed ER⁺, EGFR⁺ primary tumor and no mixed metastases were observed in this series, it cannot be excluded that a minor population of tumor cells with a different phenotype has been missed in our analyses. Such tumor cells could result from biological selection in metastasis with another receptor phenotype. However, our study shows that a shift in receptor phenotype between primary tumors and lymph node metastases is a rare event and, thus, analyses of involved lymph nodes will not serve as a better predictor for response to anti-estrogen therapy.

Studies using breast cancer cell lines have indicated that switching from ER expression to EGFR expression may occur during anti-estrogen treatment (Long *et al.*, 1992; Van Agthoven *et al.*, 1992, 1994*a*; Dorssers *et al.*, 1993). Thus far little evidence for this mechanism is available for development of acquired anti-estrogen resistance in clinical breast cancer. Although anti-estrogen therapy may select for a switch in

receptor expression in the tumor cells (Chrysogelos *et al.*, 1994), this does not explain the intrinsic response failure to anti-estrogen therapy in half of the patients with metastatic disease and ER$^+$ primary tumors. Our study strongly suggests that lymph node metastases of ER$^+$ primary tumors are also ER$^+$. Since the lymph node metastases analyzed were surgically removed at the same time as the primary tumor, the receptor expression may reflect only a confined, possibly early, stage in tumor progression. We have analyzed the 2 cases with distant metastasis, which were removed, respectively, 1 and 3 years after primary surgery. Both the primary tumor and the metastasis were EGFR$^+$ and, thus, not informative for the existence of a possible switch in receptor expression.

In conclusion, no evident differences in expression of ER and EGFR were observed between primary tumors and metastases in our study. An interesting difference was observed between ductal and lobular carcinomas, the latter being almost exclusively EGFR$^-$ and all expressing ER. Our study shows that a shift in receptor expression between primary tumors and lymph node metastasis is rare and, thus, receptor analysis of involved lymph nodes will most likely not be a better predictor for response to anti-estrogen therapy.

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REFERENCES

AZZOPARDI, J.G., CHEPICK, O.F. and HARTMANN, W.H., The World Health Organization histological typing of breast tumors. *Amer. J. clin. Pathol.*, **78**, 806–816 (1982).

BEATO, R., Gene regulation by steroid hormones. *Cell*, **56**, 335–344 (1989).

CHRYSOGELOS, S.A., YARDEN, R.I., LAUBER, A.H. and MURPHY, J.M., Mechanisms of EGF receptor regulation in breast cancer cells. *Breast Cancer Res. Treat.*, **31**, 227–236 (1994).

CLARKE, R., DICKSON, R.B. and LIPPMAN, M.E., Hormonal aspects of breast cancer. Growth factors, drugs and stromal interactions. *Crit. Rev. Oncol. Hematol.*, **12**, 1–23 (1992).

DORSSERS, L.C.J., VAN AGTHOVEN, T., DEKKER, A., VAN AGTHOVEN, T.L.A. and KOK, E.M., Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. *Mol. Endocrinol.*, **7**, 870–878 (1993).

DORSSERS, L.C.J., VAN AGTHOVEN, T. and SIEUWERTS, A.M., Genetic mechanisms involved in progression to hormone independence of human breast cancer. *In:* P.M.J.J. Berns, J.C. Romijn and F.H. Schroeder (eds.), *Mechanisms of progression to hormone-independent growth of breast and prostatic cancer*, pp. 169–182, Parthenon, Carnforth, UK (1991).

EVANS, R.M., The steroid and thyroid hormone receptor superfamily. *Science*, **240**, 889–895 (1988).

HORWITZ, K.B., Mechanisms of hormone resistance in breast cancer. *Breast Cancer Res. Treat.*, **26**, 119–130 (1993).

JAIYESIMI, I.A., BUZDAR, A.U., DECKER, D.A. and HORTOBAGYI, G.N., Use of tamoxifen for breast cancer: twenty-eight years later. *J. clin. Oncol.*, **13**, 513–529 (1995).

JOHNSTON, S.R.D., DOWSETT, M. and SMITH, I.E., Towards a molecular basis for tamoxifen resistance in breast cancer. *Ann. Oncol.*, **3**, 503–511 (1992).

JORDAN, V.C. and MURPHY, C.S., Endocrine pharmacology of antiestrogens as antitumor agents. *Endocr. Rev.*, **11**, 578–610 (1990).

KATZENELLENBOGEN, B.S., Antiestrogen resistance: mechanisms by which breast cancer cells undermine the effectiveness of endocrine therapy. *J. nat. Cancer Inst.*, **83**, 1434–1435 (1991).

KING, R.J.B., Progression from steroid sensitive to insensitive state in breast tumour. *Cancer Surv.*, **14**, 131–146 (1992).

KLIJN, J.G.M., BERNS, P.M.J.J., SCHMITZ, P.I.M. and FOEKENS, J.A., The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr. Rev.*, **13**, 3–17 (1992).

LONG, B., MCKIBBEN, B.M., LYNCH, M. and VAN DEN BERG, H.W., Changes in epidermal growth factor receptor expression and response to ligand associated with acquired tamoxifen resistance or oestrogen independence in the ZR-75-1 human breast cancer cell line. *Brit. J. Cancer*, **65**, 865–869 (1992).

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. *Lancet*, **i**, 182–185 (1989).

OSBORNE, C.K. and FUQUA, S.A.W., Mechanisms of tamoxifen resistance. *Breast Cancer Res. Treat.*, **32**, 49–55 (1994).

PAGE, D.L. and ANDERSON, T.J., *Diagnostic histopathology of the breast*, Churchill Livingstone, New York (1987).

PARKER, M.G., ARBUCKLE, N., DAUVOIS, S., DANIELIAN, P. and WHITE, R., Structure and function of the estrogen receptor. *Ann. N.Y. Acad. Sci.*, **684**, 119–126 (1993).

PRITCHARD, K.I. and SUTHERLAND, D.J.A., The use of endocrine therapy. *Hematol. Oncol. Clin. North. Amer.*, **3**, 765–805 (1989).

SHARMA, A.K., HORGAN, K., DOUGLAS-JONES, A., MCCLELLAND, R., GEE, J. and NICHOLSON, R., Dual immunocytochemical analysis of oestrogen and epidermal growth factor receptors in human breast cancer. *Brit. J. Cancer*, **69**, 1032–1037 (1994).

VAN AGTHOVEN, T., TIMMERMANS, M., FOEKENS, J.A., DORSSERS, L.C.J. and HENZEN-LOGMANS, S.C., Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry. *Amer. J. Pathol.*, **144**, 1238–1246 (1994*a*).

VAN AGTHOVEN, T., VAN AGTHOVEN, T.L.A., DEKKER, A., FOEKENS, J.A. and DORSSERS, L.C.J., Induction of estrogen independence of ZR-75-1 human breast cancer cells by epigenetic alterations. *Mol. Endocrinol.*, **8**, 1474–1483 (1994*b*).

VAN AGTHOVEN, T., VAN AGTHOVEN, T.L.A., PORTENGEN, H., FOEKENS, J.A. and DORSSERS, L.C.J., Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res.*, **52**, 5082–5088 (1992).

WATTS, C.K.W., SWEENEY, K.J.E., WARLTERS, A., MUSGROVE, E.A. and SUTHERLAND, R.L., Antiestrogen regulation of cell cycle progression and cyclin D1 gene expression in MCF-7 human breast cancer cells. *Breast Cancer Res. Treat.*, **31**, 95–105 (1994).

Induction of Antiestrogen Resistance in Human Breast Cancer Cells by Random Insertional Mutagenesis Using Defective Retroviruses: Identification of *bcar*-1, a Common Integration Site

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Duration of response to antiestrogen therapy in metastatic breast cancer is limited due to the development of antiestrogen-resistant tumors. The mechanisms involved are not understood but could originate from (epi)genetic alterations within the tumor cells. We have applied *in vitro* random insertional mutagenesis with replication defective retroviruses to identify those genes playing a key role in development of antiestrogen resistance in human breast cancer cells. Eighty antiestrogen-resistant cell clones were isolated from 7 × 10^8 estrogen-dependent ZR-75-1 cells, mass-infected with defective retroviruses and subjected to 4-OH-tamoxifen selection. Integration site-specific DNA probes were made by inverse polymerase chain reaction techniques and used to search for common integration sites. Six cell clones were identified with retroviral genome integrations in the same orientation in a single locus, designated breast cancer antiestrogen resistance locus-1 (*bcar*-1). These *bcar*-1 cell clones had lost estrogen receptor expression and had become estrogen independent. Our results strongly suggest that alteration of the *bcar*-1 locus is responsible for development of antiestrogen resistance in human breast cancer cells *in vitro*. In addition, we have shown that *in vitro* insertional mutagenesis using defective retroviruses can be applied for gene tagging in human cells. (Molecular Endocrinology 7: 870–878, 1993)

INTRODUCTION

Normal breast epithelial cells depend on estrogen for their proliferation and differentiation. Endocrine therapy, involving estrogen ablation or administration of antiestrogens, has been shown to be beneficial in early and metastatic breast cancer (1–3). With respect to metastatic disease, objective responses occur in about 50% of the patients with estrogen receptor (ER)-positive primary tumors. However, the response duration is rather limited due to the development of hormone-independent tumors in virtually all cases (1, 4, 5). The mechanism of action of antiestrogens is based on their ability to compete with estrogens for binding to the estrogen receptor and thus block cell proliferation (2, 6). Although prognostic factors which predict a lack of response to endocrine therapy have been described (4, 7), the mechanisms of progression to hormone independence of an initially responsive tumor are still not understood. It is speculated that the tumor is heterogeneous and that hormone-independent clones may be selected by the therapy applied. Alternatively, hormone-dependent tumor cells may acquire (epi)genetic alterations, which result in resistance to the endocrine therapy (8–15). Previous studies have shown that introduction of either an activated *ras* or insulin-like growth factor-II gene into breast tumor cell lines resulted in hormone-independent proliferation of the transfected cells (16, 17). In addition, we have presented evidence that ectopic expression of epidermal growth factor (EGF) receptor in human breast cancer cells can bypass the estrogen dependence (18). Furthermore, epigenetic alterations induced by demethylation with 5-azacytidine of ZR-75-1 cells also resulted in hormone-independent proliferation (11; Van Agthoven, T., T. L. A. Van Agthoven, A. Dekker, J. A. Foekens, and L. C. J. Dorssers, manuscript in preparation). These results suggest that dominant genetic alterations play a role in hormone-independent progression of breast cancer cells.

In order to identify specific genes involved in progression to hormone independence, we started a random insertion mutagenesis study on human breast cancer cells *in vitro* applying defective retroviruses. Retroviruses randomly integrate in the host genome as part of

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their life cycle (19) and may thus disturb local genome structure and gene expression (20, 21). Furthermore, the integrated viral genome provides a tag to the gene locus of interest and can be used to isolate and characterize the gene involved. Insertional mutagenesis with replication-competent murine retroviruses has been used to identify rodent (onco)genes *myc*, *Tpl*-1, *bml*-1, *pal*-1, *bla*-1, and *pim* involved in lymphoma (22–27), *int* in breast cancer (28–30), and *Evi*, *Spi*-1, *Fli*, and *Vin*-1 in leukemia (31–36) *in vivo* and *in vitro*. Replication-defective retroviruses can be generated by use of amphotropic packaging cell lines and provide a safe and elegant procedure to mutate human cells without risk of secondary infections (19, 37). Our results document that defective retroviral integration in the host genome transformed the estrogen dependency of ZR-75-1 human breast cancer cells and allowed for the identification of a locus, designated breast cancer antiestrogen resistance locus-1 (*bcar*-1), involved in this process.

RESULTS

Generation of Antiestrogen-Resistant Human Breast Cancer Cell Lines

In order to use retroviral insertional mutagenesis for linkage of a specific gene to a biological function *in vitro*, an extremely sensitive cell selection system is imperative. The widely used MCF-7 cells and the strongly hormone-dependent subline MCF-7/McGrath (38) were not useful, since about 5–10% of the colonies, although smaller in size, were retained in the presence of 1 μM of 4-OH-tamoxifen (OH-TAM), the active metabolite of tamoxifen (2). Subcloning of these MCF-7 cells or application of selection protocols to isolate a highly OH-TAM-sensitive subclone (39, 40) were not successful. ZR-75-1 cells (41) were completely dependent on estrogen for cell proliferation (18) and failed to produce surface colonies in the presence of 1 μM OH-TAM (data not shown).

To infect these human breast cancer cells with retroviruses, we have generated amphotropic producers of N2- and LN-defective murine retroviruses (37) using the PA317 and CRIP packaging cell lines (42, 43). These producers of defective viruses were selected for efficient infection of ZR-75-1 cells (more than 10^5 G418-resistant colony forming units/ml) and absence of replication competent virus and used in infection experiments to generate antiestrogen-resistant cell clones. In a typical experiment, 20–150 × 10^6 ZR-75-1 cells were treated for 2 days with conditioned medium (CM) of the amphotropic producers containing the defective virus and subsequently plated for selection with OH-TAM. About 30–50% of the cells showed resistance to the neomycin analog G418 in a surface colony assay, indicating stable integration of the defective retrovirus. Proliferating colonies of cells were detected by visual inspection within 3–5 weeks in the infected cultures subjected to antiestrogen selection (Fig. 1), picked, and expanded. In control experiments involving more than 2 × 10^6 ZR-75-1 cells, only one antiestrogen-resistant cell clone was rescued. This result demonstrates the low spontaneous frequency ($\leq 10^{-6}$) of progression to OH-TAM resistance of ZR-75-1 cells under these selective culture conditions.

Expansion of antihormone-resistant clones appeared to be tedious, since many proliferating colonies (up to 80%) were lost after picking and plating in microtiter plates in the early series of experiments. A considerable growth advantage was noted during the infection culture in comparison to the untreated cells, suggesting the presence of growth-stimulatory substances in the CM of the mouse fibroblast packaging cell line. The CRIP cell CM strongly enhanced attachment and growth of tamoxifen-resistant cell clones plated at low density (see also Fig. 4). Thus, in subsequent infection experiments picked clones were expanded in medium supplemented with 10% CRIP-CM and OH-TAM with a success rate of 87%.

Southern analyses of *Hin*dIII-, *Bgl*II-, and *Bam*HI-digested DNA of all OH-TAM-resistant cell clones using an LN virus or *Neo*R probe were performed to establish the number of viral integrations. These restriction enzymes cut outside the viral DNA and the number of bands correlates directly with the number of integrations (an example is shown in Fig. 3a). Most of the cell clones tested comprised 1–10 copies of the viral genome (median of two copies). Occasionally, restriction fragments hybridizing to a viral long terminal repeat (LTR) probe were observed which failed to hybridize to the *Neo*R probe. The size of these fragments was often too small to comprise a full-length viral genome, suggesting that part of the viral genome had been eliminated before or after integration in the host genome (44).

Overall we have now generated 80 OH-TAM-resistant cell lines with integrated viral genomes (primarily LN virus) from a total of about 7 × 10^8 ZR-75-1 cells subjected to retroviral infection and antihormone selection. This cell panel has been used to identify common integration sites, which indicate the presence of genes involved in development of tamoxifen resistance.

Generation of Integration Site-Specific Probes

In order to identify common integration sites of retroviral genomes in independently arisen antiestrogen-resistant cell clones, probes flanking the provirus are required. We have used the inverse DNA amplification procedure (45), which employs outward directed primers located within the known viral LTR sequence (Fig. 2) after recircularization of digested genomic DNA. We presently use a large panel of enzymes with six base recognition sites on cell clones with only one or two viral integrations, since restriction enzymes with four base recognition sequences resulted mostly in very short stretches of genomic sequences. So far we have tried to obtain border probes from six different antiestrogen-resistant cell clones. In one case with a single integration, no fragments have been amplified with the panel of enzymes used so far. In two cases only very

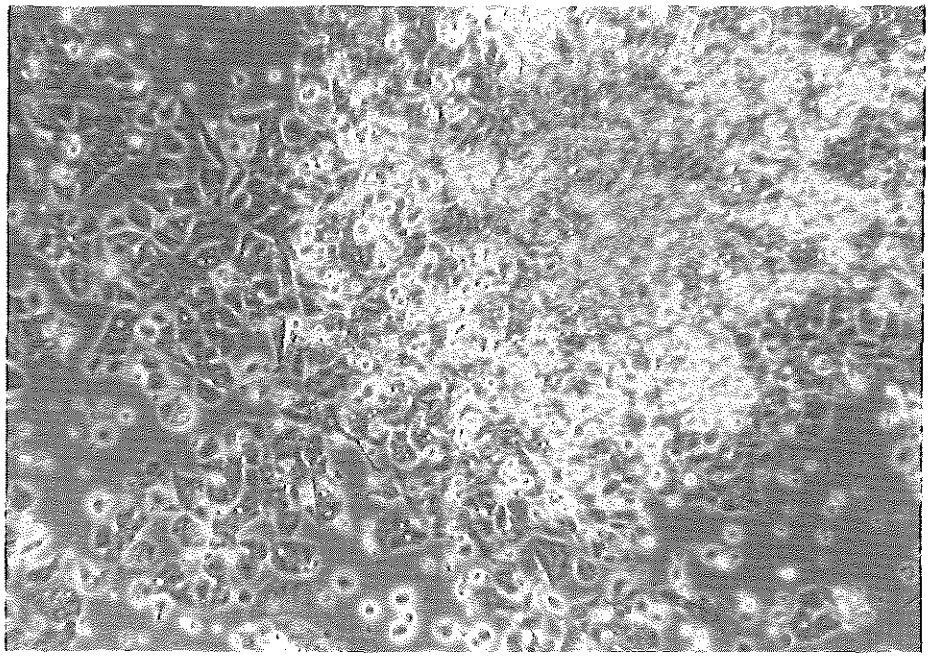

Fig. 1. Surface Colony of OH-TAM-Resistant, Infected ZR-75-1 Cells
Phase-contrast photomicrograph of infected ZR-75-1 cells subjected to 5 weeks of OH-TAM selection. A colony of proliferating, antiestrogen-resistant cells is shown in a background of nondividing cells.

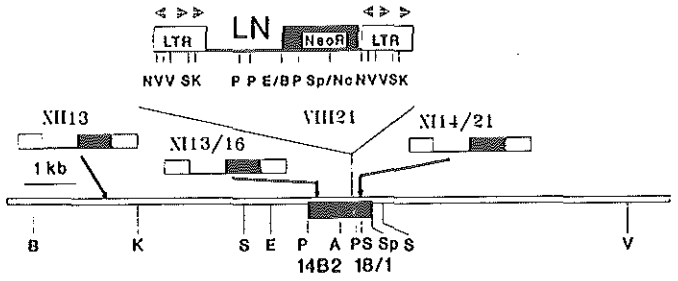

Fig. 2. Genomic Map and Defective Retrovirus Integrations in the *bcar*-1 Locus
Restriction sites are located with respect to the LN provirus (*top*) integration in cell clone VIII21. Positions of primers used for inverse PCR are shown above the LTRs of the LN virus. Integration site-specific probes 18/1 and 14B2(*Apa*LI-*Pst*I) are *hatched*. The *black box* denotes the Alu sequence within the cloned PCR fragment 14B2. Integration sites in the *bcar*-1 locus of other cell clones (*roman numerals*) are indicated. Restriction enzyme abbreviations are: A, *Apa*LI; B, *Bcl*I; E, *Eco*RI; V, *Eco*RV; K, *Kpn*I; Nc, *Nco*I; N, *Nhe*I; P, *Pst*I; S, *Sac*I; and Sp, *Sph*I.

small parts of genomic sequences consisting mainly of repetitive Alu sequences were obtained (not shown). From cell clone VIII24 containing two integrations, two border clones have been prepared with *Nco*I [2D, 300 base pairs (bp)] and the mixture of *Bam*HI, *Bgl*II, and *Bcl*I (2E, 1000 bp), which both recognize the same viral integration locus (not shown). From cell clone VIII20 carrying two integrated copies of the virus, a single 650-bp probe (3F) located downstream of the virus was recovered with the restriction enzyme *Nhe*I. Cell clone VIII21 DNA (one viral genome and a 4-kilobase (kb) LTR fragment lacking *Neo*R sequences) digested with *Pst*I or *Sph*I supplied three clones (14B2, 18/1, and 14B9). Clone 14B2 is located upstream of the virus and carries part of an Alu sequence, but an upstream approximately 500-bp *Apa*LI-*Pst*I fragment detected the

integration locus (Figs. 2 and 3f). Probe 18/1 was derived from the 3′-LTR and comprises a 450-bp *Sph*I fragment free of repetitive sequences (Fig. 3, b–e). Probe 14B9 represents a *Pst*I fragment also contained in clone 18/1 (Fig. 2).

Identification of a Common Integration Site

All blots with *Hin*dIII-, *Bgl*II-, and *Bam*HI-digested genomic DNA were subjected to screening with the integration site-specific probes derived from infection experiment VIII. In addition, blots were made from DNA digested with *Eco*RV and *Sac*I, which cut within the viral LTR (Fig. 2). Probes 2D and 2E (clone VIII24) and 3F (clone VIII20) only detected germ line restriction fragments in all cell clones tested so far, indicating the absence of retroviral integrations in the neighborhood of these probes (data not shown). In contrast, probes 18/1 and 14B2 derived from cell clone VIII21 did recognize restriction fragments, with altered mobility in five additional cell clones (XI13, XI14, XI16, XI21, and XII13). *Bgl*II (Fig. 3b) and *Bam*HI restriction fragments with a reduced mobility, corresponding to the integration of a 3-kb viral genome, were observed. The weak intensity of these altered fragments in comparison to the germ line band is explained by the presence of multiple copies of many chromosomes in the ZR-75-1 cells (18, 41). Blots carrying *Eco*RV digests of these DNA samples confirmed these results and further suggested that the integrations were in close proximity (Fig. 3, e and f). Detailed characterization of the integration locus by Southern hybridization suggested that clones XI14 and XI21 are derived from an infected cell of which one daughter cell acquired an additional viral copy during a subsequent infection cycle (Fig. 3a; *Bam*HI and *Hin*dIII blots not shown). The integration site in these clones falls within the probe 18/1, as both the left and right *Bcl*I and *Eco*RV fragments are recognized (Fig. 3, c and e, lane 2). Similarly, clones XI13 and XI16 originate from a single cell with two viral integrations, from which one daughter gathered two additional viral DNA copies. The *Bgl*II doublet at approximately 6 kb in clone XI16 (Fig. 3a, lane 5) is resolved in two bands by *Hin*dIII and *Bam*HI digestions (not shown). The integration site in clones XI13/16 is located upstream of the VIII21 integration and falls within the probe 14B2. The 2.2-kb *Sac*I germ line fragment is split into two bands (1 and 1.8 kb), both hybridizing with the 14B2 probe (not shown), the smaller one hybridizing with the 18/1 probe (Fig. 3d, lane 4). The single viral DNA integration in cell clone XII13 is located approximately 3–4 kb upstream of the other integrations within the *Bcl*I and *Eco*RV restriction fragments (Fig. 3, c and e, lane 6) without interrupting the *Sac*I germ line band (2.2 kb) recognized by the 14B2 (not shown) and 18/1 probes (Fig. 3d). The orientation of the provirus was determined with the *Neo*R probe on *Bcl*I- and *Eco*RI-digested DNA and was found to be identical to VIII21 for XI13/16, XI14/21, and XII13. The large *Bcl*I fragments hybridizing to 18/1 probe (Fig. 3c) also hybridized to the *Neo*R probe (data not shown). This common integration site identified by probes derived from cell clone VIII21 is designated *bcar*-1.

Loss of Estrogen Dependence of Cell Clones with Integrations in the *bcar*-1 Locus

Cell clones with integrations in the *bcar*-1 locus have been characterized with respect to their response to

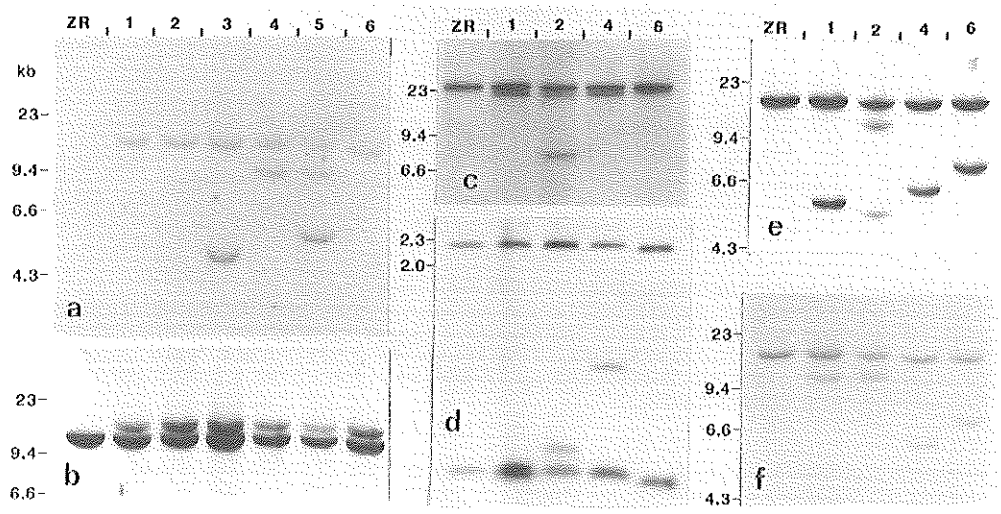

Fig. 3. Southern Analysis of ZR-75-1 and Derived Antiestrogen-Resistant Cell Clones

Blots carrying genomic DNA digested with *Bgl*II (a and b), *Bcl*I (c), *Sac*I (d), and *Eco*RV (e and f) were hybridized with an *Neo*R (a), 18/1 (b–e), or 14B2 (f) probe. Genomic DNA was prepared from ZR-75-1 cells (ZR) and antiestrogen-resistant clones VIII21 (1), XI21 (2), XI14 (3), XI13 (4), XI16 (5), and XII13 (6). Fragment sizes (in kilobases) of λ-DNA digested with *Hin*dIII are denoted on the *left*.

known stimulators and inhibitors of proliferation using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (MTT) assay (46). In this assay, the response is mostly proportional to the number of viable cells (18). Proliferation of the parental cell line ZR-75-1 is completely dependent on estradiol supplementation to the culture (18) and is not influenced in the MTT assay by addition of either EGF or the pure antagonist ICI164,384 (Fig. 4). ZR-75-1 cells pretreated with RPMI medium supplemented with 10% bovine calf serum (R/BCS medium) show a response to OH-TAM (Fig. 4), which is probably due to the initial intrinsic agonistic effect of OH-TAM (47). This response is masked when pretreatment is omitted (not shown) and does not support ZR-75-1 cell proliferation to a significant extent (18). Contrary to the parental cells, the *bcar*-1 cell clones were selected for proliferation in R/BCS medium plus OH-TAM. Increase in MTT reduction thus represents stimulation above basal cell proliferation in R/BCS (representative examples VIII21 and XI21 shown in Fig. 4). No significant change in MTT reduction was observed at day 5 (Fig. 4) or day 10 for all *bcar*-1 cell clones after addition of either estradiol, ICI164,384, or OH-TAM. RNase protection studies (Fig. 5a) using labeled antisense RNA copies of ER showed a strong reduction of ER mRNA in these antiestrogen-resistant cell clones in comparison to the parental ZR-75-1 cells. Similarly, ER- and estradiol-induced progesterone receptor (PgR) protein, determined by enzyme-linked immune assays ER-EIA and PgR-EIA, were very low (between 0–8 fmol/mg protein) in all cell clones with integrations in the *bcar*-1 locus. These assays in complete medium show that the *bcar*-1 cell clones have lost their response to estrogen and display a hormone-independent phenotype.

In contrast to the parental cells, clones VIII21, XI13, XI14, and XII13 showed some increase in MTT reduction upon supplementation of EGF (Fig. 4). A more pronounced stimulatory effect of EGF addition (2.1 ± 0.1-fold) was observed after plating of 2500 VIII21 cells and MTT assay at day 10. Low levels of EGF receptor

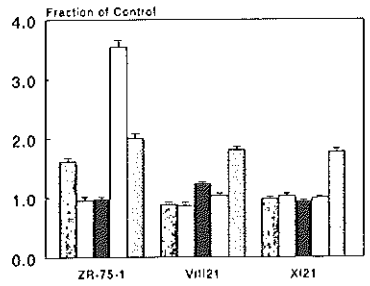

Fig. 4. Biological Characterization of *bcar*-1 Cell Clones

MTT reduction was determined at day 5 after plating of 5000 antiestrogen-resistant cells or 2500 parental ZR-75-1 cells, pretreated in R/BCS medium for 24 h, in R/BCS medium supplemented with 1 μM OH-TAM (□), 100 nM ICI164,384 (▨), 10 ng/ml EGF (▩), 1 nM estradiol (□), or 10% CRIP-CM (▩). Response is given as the fraction of the R/BCS control culture.

mRNA were detected in RNase protection experiments in all *bcar*-1 cell clones in contrast to the parental cell line (Fig. 5b). In addition, Scatchard analysis demonstrated specific [^{125}I]EGF binding to membrane preparations of cell clones XI13 and XI14 (40 and 15 fmol EGF receptor/mg protein, respectively). All ZR-75-1-derived cell lines were stimulated by the addition of 10% CRIP-CM (Fig. 4), indicating the presence of undefined accessory components in the fibroblast medium.

DISCUSSION

Linkage of a specific gene to a biological function by retroviral insertional mutagenesis has been achieved mainly in rodents *in vivo* and lead to the identification of genes involved in malignant processes (21). Mutagenesis *in vitro* was shown to be feasible (32, 33, 48) using replication-competent viruses in murine factor-dependent cell lines. Defective retroviruses lack the capability to spread into the culture after integration and thus provide a safe and elegant way to perform mutagenesis (19, 37). The frequency of retroviral integration in a specific locus has been estimated in the order of 10^{-6}–10^{-8} (20), thus requiring very low frequencies of spontaneous progression to the selected phenotype. Initial control experiments indicated that, in contrast to the MCF-7 cell line tested, the spontaneous rate of ZR-75-1 cells to form an antiestrogen-resistant cell clone was below 10^{-6}. The virus-induced progression rate to tamoxifen resistance is calculated to be about 10^{-7} (range of 0.6–3 × 10^{-7} in six experiments), clearly above the spontaneous frequency. This figure represents a 2- to 3-fold underestimation of the actual value since efficiencies in infection have been disregarded.

Using integration site-specific probes from three independently arisen antiestrogen-resistant cell clones, we have identified a common integration site (*bcar*-1) in six cell clones of our panel. Detailed analysis showed that these cell clones were derived from four independent integration events within ±4 kb total distance. Further screening of this chromosomal region may identify additional distant integrations in our panel of antiestrogen-resistant clones. The occurrence of six integrations in the *bcar*-1 locus in 80 cell clones carrying a total of approximately 190 retroviral integrations highly exceeds the reported frequencies (2–5 × 10^{-4}) of base-specific integrations in genomic hot spots (49). Under the model that in surviving cell clones the location of the integrated virus is completely random, based on two integrations per cell the probability to find a retroviral genome in a given 10-kb genomic DNA segment is 6.6 × 10^{-6}. As a consequence, the probability of identifying three additional independent integration events in this locus, among the remaining clones of the cell line panel, is extremely low (2 × 10^{-11}, binomial distribution). This implies that the model of complete randomness has to be rejected, *i.e.* it strongly suggests that integration within the *bcar*-1 locus is tightly linked

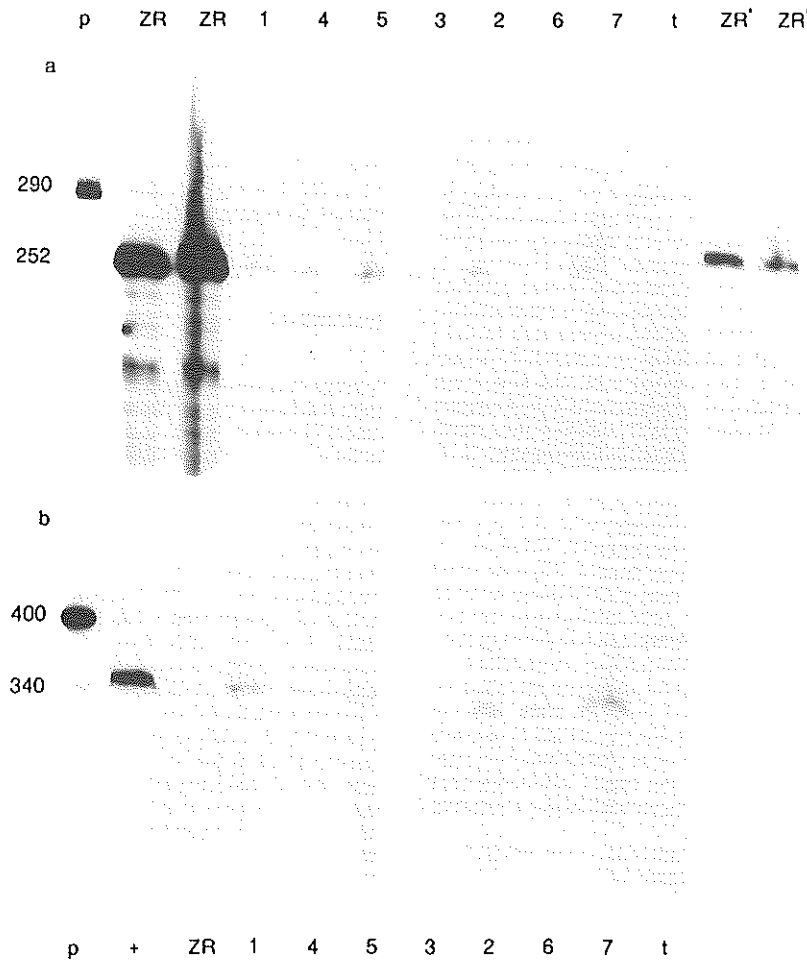

Fig. 5. RNase Protection of ER and EGF Receptor mRNA

Autoradiograph of protected radiolabeled RNA probes (p) of ER (a) and EGF -receptor (b) after hybridization to 20 μg total cell RNA as indicated, digestion with RNases, and fractionation on a denaturing 6% polyacrylamide-urea gel. Exposure was for 12 days or 16 h (') with intensifying screens at −80 C. Only the relevant parts of the autoradiographs are shown. No other protected fragments (>70 bases) were detected. RNA samples were from ZR-75-1 cells (ZR) and antiestrogen-resistant clones VIII21 (1), XI21 (2), XI14 (3), XI13 (4), XI16 (5), XII13 (6), VIII24 (7), an EGF receptor-positive derivative of ZR-75-1 (+), and tRNA (t).

to the selected biological phenotype of antiestrogen resistance. Dominant activation of a gene within the *bcar*-1 locus by either promotor/enhancer insertion, removal of negative regulatory sequences, or mRNA destabilizing sequences allowed for cell proliferation independent of antiestrogen from day 1 onward. Activation by enhancer insertion is mostly independent of orientation and position, whereas 3'-LTR promotion is often accompanied by deletions in the viral genome (21). The identical orientation of intact and functional viral integrates (all *bcar*-1 clones exhibit G418 resistance) within the *bcar*-1 locus suggests that either 5'-LTR promotion or removal of mRNA destabilizing sequences is the most likely activation mechanism (21). Alternatively, gene inactivation cannot be excluded but is rather unlikely in view of the presence of multiple copies of chromosomes in the ZR-75-1 cell line. Identification of the expressed sequences within the *bcar*-1 locus and their level of expression in antiestrogen-resistant cell clones may answer these questions.

The biological properties of the cell clones with integrations within the *bcar*-1 locus are quite similar. All clones show a virtually complete loss of response to estradiol, which is in agreement with the very low levels of wild type ER mRNA (Fig. 5a) and ER- and estradiol-induced PgR protein. The strongly reduced ER expres-

sion and function explains the lack of inhibition or stimulation of estrogen antagonists (OH-TAM and ICI164,384) on *bcar*-1 cell proliferation and excludes the possibility of acquired OH-TAM dependence (50). Production of constitutive transcriptionally active ER variants (lacking exon 5 sequences) is rather unlikely because of the absence of PgR expression in these cells (51). In addition, no aberrant protected transcript was seen in ZR-75-1 cells or in the tamoxifen-resistant cell lines. Variants lacking exon 4 sequences would have been missed in this analysis, but deletion of the basic region flanking the second zinc finger has been shown to impair DNA binding (52). Similarly, inactive fusion proteins involving the N-terminal part of ER (53) would not have been detected in our mRNA and protein analysis. Variants lacking exon 7-encoded sequences and capable of interfering with normal ER function (54) would have been detected as wild type protein and protected RNA fragment. Our analysis thus strongly suggests that the cell clones with integrations in the *bcar*-1 locus do not produce significant levels of a functional ER (variant) and are truly estrogen independent. ER expression may have been down-modulated as a result of alteration of the *bcar*-1 locus and/or the stringent selection with antihormone (18). The latter seems plausible, because clone VIII24 also showed a strong decrease in ER expression and an increase in EGF receptor expression (Fig. 5) without evidence of an integration within the *bcar*-1 locus. In contrast, one other OH-TAM-resistant cell clone obtained using this antiestrogen-selection protocol showed abundant ER and PgR expression (data not shown). Cell clones with integrations in the *bcar*-1 locus showed increased levels of EGF receptor mRNA expression in comparison to ZR-75-1 cells (Fig. 5b). EGF receptor protein expression in cell clones XI13 and XI14 was clearly increased compared to the EGF receptor-negative ZR-75-1 parental cells but lower than the expression level observed in the EGF-responsive ZR/HERc cells ectopically expressing the EGF receptor cDNA (18). The moderate response to EGF in complete medium of some cell clones (Fig. 4) suggests that this pathway is functional. The magnitude of this signal transduction pathway in proliferation of *bcar*-1 cell clones needs further analysis under serum-free conditions.

Clinical progression of human breast cancer to hormone independence appears to occur frequently during antiestrogen therapy and is unlikely to depend on a single gene mechanism. Breast tumors are heterogenous and may evade antihormone elimination via different pathways, some allowing for second-line endocrine treatment (5, 7, 55). We have provided strong evidence that development of antiestrogen resistance *in vitro* is directly linked to disruption of the *bcar*-1 locus by retroviral integration. It remains to be established whether *bcar*-1 has a role in clinical breast cancer development or progression. Additional genes possibly tagged in our cell panel and contributing to alternative ways to escape hormonal regulation await identification. Our experiments also demonstrate that gene tagging in human cells by *in vitro* insertional mutagenesis using defective retroviruses is feasible.

MATERIALS AND METHODS

Cell Culturing and Virus Infection

ZR-75-1 cells were obtained from Dr. R. J. B. King (Imperial Cancer Research Fund Laboratories, London, United Kingdom) and maintained in RPMI medium (Gibco BRL, Life Technologies Ltd., Paisly, United Kingdom) supplemented with 10% heat-inactivated bovine calf serum (R/BCS medium) and 1 nM estradiol for a maximum of 10 passages (18). Amphotropic producers of N2 (56) and LN (37) virus were established by infection of CRIP (43) and PA317 (42) packaging cells with ecotropic defective virus transiently produced by CRE cells (43) transfected with the respective plasmid construct. CM from subconfluent producer cells containing the amphotropic virus was centrifuged (5000 × *g*) and filtered through 0.45-μm filters, adjusted to 8 μg polybrene (Sigma, St. Louis, MO)/ml (20), and added undiluted to the ZR-75-1 cells. Approximately 2×10^7 ZR-75-1 cells per 175-cm^2 flask were infected during a period of 2-3 days with addition of fresh virus-containing supernatant twice a day. After infection, ZR-75-1 cells were trypsinized, counted, and seeded in 75-cm^2 flasks at a density of 4×10^6 cells in R/BCS with 1 μM 4-OH-TAM (ICI, Meccles-field, United Kingdom). The culture medium was changed twice a week, and surface colonies were scored and picked within 5 weeks after seeding and expanded in the same medium. In following experiments, expansion of picked clones was performed in medium additionally supplemented with 10% filtrated CM of nonproducer CRIP cells to improve attachment and survival. Infection frequencies of ZR-75-1 cells were estimated by plating 2×10^4 cells after infection in 25-cm^2 flasks in the absence or presence of 1 mg/ml G418 (Gibco BRL) in complete medium. Surface colonies were scored 10-14 days later. The presence of replication-competent virus was assayed by applying the undiluted culture medium of mass-infected ZR-75-1 cells to Balb3T3 or ZR-75-1 cells and subsequent selection for G418 resistance. MTT assay in R/BCS medium, which measures the number of viable cells capable of reducing the tetrazolium compound to a blue formazan product (46), and measurements of hormone receptors (ER-EIA and PgR-EIA; Abbott Laboratories, Chicago, IL) and EGF receptors were performed as described (18).

Analysis of Nucleic Acids

DNA was prepared from the antiestrogen-resistant cell clones using proteinase K and high salt (57), digested with *Hin*dIII, *Bam*HI, *Bgl*II, *Sac*I, and *Eco*RV (Pharmacia P-L Biochemicals Inc., Milwaukee, WI), fractionated on 0.6% agarose gels, and alkali blotted onto Hybond N+ (Amersham International plc, Amersham, United Kingdom) according to standard procedures (58). DNA probes purified on low-melting point agarose were melted and radiolabeled with [α-^{32}P]deoxy-ATP (Amersham) using the random primer procedure (59). Hybridization was carried out overnight at 65 C in 3× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 0.1% sodium dodecyl sulfate, 10× Denhardt's, 9% dextran sulfate, and 50 μg/ml denatured herring sperm DNA. Filters were washed in 0.3× SSC/0.1% sodium dodecyl sulfate at 65 C and autoradiographed using intensifying screens. Total RNA was isolated using the guanidinium isothicyanate/CsCl procedure and subjected to RNase protection assays according to described procedures (58). Radiolabeled RNA probes from the *Hin*dIII-*Bgl*II fragment of ER [nucleotides (nt) 1248-1500)] (60) and the *Bgl*II-*Sac*I fragment of EGF receptor (nt 2951-3291) (61) were generated with T7-RNA polymerase as specified by the supplier (Promega, Madison, WI).

Inverse Polymerase Chain Reaction (PCR)

Sequences adjacent to the integrated viral genome were amplified as described (45) with minor modifications. Briefly, genomic DNA (1 μg) was digested with a panel of restriction enzymes (*Sac*I, *Pst*I, *Eco*RI, *Nco*I, *Sph*I, *Nhe*I, and *Bam*HI +

*Bgl*II + *Bcl*I), heat treated (65–85 C) to inactivate the enzymes, diluted to approximately 10 μg/ml, and religated with T4-DNA ligase. A small aliquot (15–50 ng) was subjected to 30 cycles of DNA amplification with outward directed primers [nt 204–182 (5'gggatccgCATGCCTTGCAAAATGGCGTTAC) and 2833–2854 (5'ggaagcttGACCTGAAATGACCCTGTGCCT)] located on the LTR of the LN virus (37) using Taq polymerase (Promega) in the presence of radiolabeled deoxy-ATP. Annealing was at 62–65 C for 2 min, and extensions were carried out at 72 C for 2 min. Products were identified after gel electrophoresis by either ethidium bromide staining or autoradiography, isolated, reamplified using one internal primer [nt 688–709 (5'ggaagcttGGTCTCCTCTGAGTGATTGACT)], and cloned in either T-tailed *Hin*cII-digested pTZ18R DNA (62) or pTZ18R DNA digested with *Bam*HI and *Hin*dIII. Transformed JM109 bacteria were characterized, and flanking sequences were acquired using the T7-DNA polymerase sequencing kit (Pharmacia) on double-stranded templates. Probes were obtained after appropriate digestion with restriction enzymes to remove plasmid and viral sequences and separated on low-melting point agarose gels. Before large-scale screening, all potential flanking probes were tested for the presence of repetitive sequences (using ^{32}P-labeled low COT DNA; GIBCO BRL) and evaluated on Southern blots of the corresponding cell line DNA to verify the linkage to the integrated viral DNA.

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REFERENCES

1. Pritchard KI, Sutherland DJA 1989 The use of endocrine therapy. Hematol/Oncol Clin North Am 3:765–805
2. Jordan VC, Murphy CS 1990 Endocrine pharmacology of antiestrogens as antitumor agents. Endocr Rev 11:578–610
3. Early Breast Cancer Trialists' Collaborative Group 1992 Systemic treatment of early breast cancer by hormonal, cytotoxic or immune therapy. Lancet 339:1–15, 71–85
4. Nicholson S, Halcrow P, Farndon JR, Sainsbury JRC, Chambers P, Harris AL 1989 Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. Lancet i:182–185
5. Santen RJ, Manni A, Harvey H, Redmond C 1990 Endocrine treatment of breast cancer in women. Endocr Rev 11:221–265
6. Reese JC, Katzenellenbogen BS 1992 Examination of the DNA-binding ability of estrogen receptor in whole cells: implications for hormone-independent transactivation and the actions of antiestrogens. Mol Cell Biol 12:4531–4531
7. Klijn JGM, Berns EMJJ, Dorssers LCJ, Foekens JA, Molecular markers of resistance to endocrine treatment o breast cancer. In: Dickson RB, Lippman ME (eds) Drug and Hormonal Resistance in Breast Cancer: Cellular and Molecular Mechanisms. Ellis Horwood Ltd., Chichester in press
8. Dickson RB, Lippman ME 1987 Estrogenic regulation o growth and polypeptide growth factor excretion in human breast carcinoma. Endocr Rev 8:29–43
9. King RB 1990 Receptors, growth factors and steroid insensitivity of tumours. J Endocrinol 124:179–181
10. Sluyser M 1990 Steroid/thyroid receptor-like proteins with oncogenic potential: a review. Cancer Res 50:451–458
11. Dorssers LCJ, Van Agthoven T, Sieuwerts AM 199 Genetic mechanisms involved in progression to hormone independence of human breast cancer. In: Berns PMJJ Romijn JC, Schroeder FH (eds) Mechanisms of Progression to Hormone-Independent Growth of Breast and Prostatic Cancer. Parthenon Publishing Group Ltd., Carnforth UK, pp 169–182
12. Fuqua SA 1992 Where is the lesion in hormone-independent breast cancer? J Natl Cancer Inst 84:554–555
13. Johnston SRD, Dowsett M, Smith IE 1992 Towards molecular basis for tamoxifen resistance in breast cancer Ann Oncol 3:503–511
14. Bruenner N, Boulay V, Fojo A, Freter CE, Lippman ME Clarke R 1993 Acquisition of hormone-independen growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 53:283–290
15. Clarke R, Thompson EW, Leonessa F, Lippman J, Mc Garvey M, Frandsen TL, Bruenner N 1993 Hormone resistance, invasiveness, and metastatic potential i breast cancer. Breast Cancer Res Treat 24:227–239
16. Kasid A, Lippman ME, Papageorge AE, Lowy DR, Gelmar EP 1985 Transfection of v-RasH DNA into MCF-7 human breast cancer cells bypasses dependence on estrogen fo tumorgenicity. Science 228:725–728
17. Cullen KJ, Lippman ME, Chow D, Hill S, Rosen N, Zwiebe J 1992 IGF-II overexpression in MCF-7 cells induces phenotypic changes associated with malignant progression. Mol Endocrinol 6:91–100
18. Van Agthoven T, Van Agthoven TLA, Portengen H, Foe kens JA, Dorssers LCJ 1992 Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75–1 human breast cancer cells. Cancer Res 52:5082–5088
19. Varmus H 1988 Retroviruses. Science 240:1427–1435
20. Goff SP 1987 Gene isolation by retroviral tagging. Methods Enzymol 152:469–481
21. Kung HJ, Boerkoel C, Carter TH 1991 Retroviral mutagenesis of cellular oncogenes: a review with insights into the mechanisms of insertional activation. Curr Top Microbiol Immunol 171:1–25
22. Selten G, Cuypers HT, Zijlstra M, Melief C, Berns A 198 Involvement of c-*myc* in MuLV induced T cell lymphomas in myce: frequency and mechanisms of activation. EMBO J 3:3215–3222
23. Selten G, Cuypers HT, Berns A 1985 Proviral activation of the putative oncogene *pim*-1 in MuLV induced T-cell lymphomas. EMBO J 4:1793–1798
24. Bear SE, Bellacosa A, Lazo PA, Jenkins NA, Copeland NG, Hanson C, Levan G, Tsichlis PN 1989 Provirus insertion in *Tpl*-1, an *Ets*-1-related oncogene, is associated with tumor progression in Moloney murine leukemia virus induced rat thymic lymphomas. Proc Natl Acad Sci USA 86:7495–7499
25. Breuer ML, Cuypers HT, Berns A 1989 Evidence for the involvement of *pim*-2, a new common proviral insertion site, in progression of lymphomas. EMBO J 8:743–747
26. Van Lohuizen M, Verbeek S, Scheijen B, Wientjes E, Van der Gulden H, Berns A 1991 Identification of cooperating oncogenes in Eμ-*myc* transgenic mice by provirus tagging. Cell 65:737–752

27. Haupt Y, Alexander WS, Barri G, Klinken SP, Adams JM 1991 Novel zinc finger gene implicated as *myc* collaborator by retrovirally accelerated lymphomagenesis in Eμ-*myc* transgenic mice. Cell 65:753–763
28. Nusse R, Van Ooyen A, Cox D, Fung YKT, Varmus H 1984 Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15. Nature 307:131–136
29. Peters G, Lee AE, Dickson C 1986 Concerted activation of two potential proto-oncogenes in carcinomas induced by mouse mammary tumor virus. Nature 320:628–631
30. Nusse R 1991 Insertional mutagenesis in mouse mammary tumorigenesis. Curr Top Microbiol Immunol 171:44–65
31. Moreau-Gachelin F, Tavitian A, Tambourin P 1988 *Spi*-1 is a putative oncogene in virally induced murine erythroleukemias. Nature 331:277–280
32. Morishita K, Parker DS, Mucenski ML, Jenkins NA, Copeland NG, Ihle JN 1989 Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. Cell 54:831–840
33. Buchberg AM, Bedigian HB, Jenkins NA, Copeland NG 1990 *Evi*-2, a common integration site involved in murine myeloid leukemogenesis. Mol Cell Biol 10:4658–4666
34. Ben-David Y, Giddens EB, Bernstein A 1990 Identification and mapping of a common proviral integration site *Fli*-1 in erythroleukemia cells induced by Friend murine leukemia virus. Proc Natl Acad Sci USA 87:1332–1336
35. Ben-David Y, Bani MR, Chabot B, De Koven A, Bernstein A 1992 Retroviral insertions downstream of the heterogeneous nuclear ribonucleoprotein A1 gene in erythroleukemia cells: evidence that A1 is not essential for cell growth. Mol Cell Biol 12:4449–4455
36. Tremblay PJ, Kozak CA, Jolicoeur P 1992 Identification of a novel gene, *Vin*-1, in murine leukemia virus-induced T-cell leukemias by provirus insertional mutagenesis. J Virol 66:1344–1353
37. Miller AD, Rosman GJ 1989 Improved retroviral vectors for gene transfer and expression. BioTechniques 7:980–990
38. Osborne CK, Hobbs K, Trent JM 1987 Biological differences among MCF-7 human breast cancer cell lines from different laboratories. Breast Cancer Res Treat 9:111–121
39. Cook PW, Swanson KT, Edwards CP, Firestone GL 1988 Glucocorticoid receptor-dependent inhibition of cellular proliferation in dexamethasone-resistant and hypersensitive rat hepatoma cell variants. Mol Cell Biol 8:1449–1459
40. Herweijer H, Stokdijk W, Visser JWM 1988 High-speed photodamage cell selection using bromodeoxyuridine/Hoechst 33342 photosensitized cell killing. Cytometry 9:143–149
41. Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ, Joyce MJ 1978 Establishment and characterization of three continuous cell lines derived from human breast carcinomas. Cancer Res 38:3352–3364
42. Miller AD, Buttimore C 1986 Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol 6:2895–2902
43. Danos O, Mulligan RC 1988 Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc Natl Acad Sci USA 85:6460–6464
44. Lichtenauer-Kaligis EGR, Van der Velde-van Dijke I, Den Dulk H, Van de Putte P, Giphart-Gassler M, Tasseron-de Jong JG 1993 Genomic position influences spontaneous mutagenesis of an integrated retroviral vector containing the hprt cDNA as target for mutagenesis. Hum Mol Genet 2:173–182
45. Silver J, Keerikatte V 1989 Novel use of polymerase chain reaction to amplify cellular DNA adjacent to an integrated provirus. J Virol 63:1924–1928
46. Carmichael J, De Graff WG, Gadzar AF, Minna JD, Mitchell JB 1987 Evaluation of a tetrazolium-based semi-automated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47:936–941
47. Cormier EM, Jordan VC 1989 Contrasting ability of antiestrogen to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. Eur J Cancer Clin Oncol 25:57–63
48. Stocking C, Löliger C, Kawal M, Suciu S, Gough N, Ostertag W 1988 Identification of genes involved in growth autonomy of hematopoietic cells by analysis of factor-independent mutants. Cell 53:869–879
49. Shih C-C, Stoyce JP, Coffin JM 1988 Highly preferred targets for retrovirus integration. Cell 53:531–537
50. Gottardis MM, Jordan VC 1988 Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res 48:5183–5187
51. Fuqua SAW, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, O'Malley BW, McGuire WL 1991 Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res 51:105–109
52. Gronemeyer H 1991 Transcription activation by estrogen and progesterone receptors. Annu Rev Genet 25:89–123
53. Dotzlaw H, Alkhalaf M, Murphy LC 1992 Characterization of estrogen receptor variant mRNAs from human breast cancers. Mol Endocrinol 6:773–785
54. Fuqua SAW, Fitzgerald SD, Allred DC, Elledge RM, Nawaz Z, McDonnell DP, O'Malley BW, Greene GL, McGuire WL 1992 Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors. Cancer Res 52:483–486
55. Klijn JGM, Berns PMJJ, Bontenbal M, Alexieva-Figusch J, Foekens JA 1992 Clinical breast cancer, new developments in selection and endocrine treatment of patients. J Steroid Biochem Mol Biol 43:211–221
56. Keller G, Paige G, Gilboa E, Wagner EF 1985 Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. Nature 318:149–154
57. Miller SA, Dykes DD, Polesky HF 1986 Simple and efficient method for isolating high molecular weight DNA from mammalian sperm. Nucleic Acids Res 16:1215
58. Sambrook J, Fritsch EF, Maniatis T 1989 Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
59. Feinberg AP, Vogelstein B 1983 A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13
60. Green S, Walter P, Kumar V, Krust A, Bornert J-M, Argos P, Chambon P 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-*erb*-A. Nature 320:134–139
61. Ulrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH 1984 Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309:418–425
62. Marchuk D, Drumm M, Saulino A, Collins FS 1991 Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res 19:1154

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Identification of *BCAR3* by a random search for genes involved in antiestrogen resistance of human breast cancer cells

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The antiestrogen tamoxifen is important in the treatment of hormone-dependent breast cancer, although development of resistance is inevitable. To unravel the molecular mechanisms of antiestrogen resistance, a search for involved genes was initiated. Retrovirus-mediated insertional mutagenesis was applied to human ZR-75-1 breast cancer cells. Infected cells were subjected to tamoxifen selection and a panel of resistant cell clones was established. Screening for a common integration site resulted in the identification of a novel gene designated *BCAR3*. Transfer of this locus by cell fusion or transfection of the *BCAR3* cDNA to ZR-75-1 and MCF-7 cells induces antiestrogen resistance. BCAR3 represents a putative SH2 domain-containing protein and is partly homologous to the cell division cycle protein CDC48.

Keywords: breast cancer/CDC48/estrogen receptor/SH2 domain/tamoxifen

Introduction

Growth and differentiation of the mammary gland is regulated by complex interactions between hormones and polypeptide growth factors with their specific receptors (Osborne, 1987; Mauvais-Jarvis *et al.*, 1988; Clarke *et al.*, 1992). Disruption of these strictly controlled signaling pathways may result in the development of cancer. It is widely accepted that breast tumors initially are dependent upon estrogens for establishment and progression (Jensen, 1981; Horwitz, 1993). Treatment of hormone-dependent breast cancer with antiestrogens like tamoxifen can delay recurrence or induce remissions of the disease (Early Breast Cancer Trial Collab Group, 1992). Antiestrogens compete with estrogen for the estrogen receptor (ER) (Jordan and Murphy, 1990; Jordan, 1994; Wolf and Fuqua, 1996), resulting in blocking of hormone signaling and growth inhibition of the tumor cells (Jordan and Murphy, 1990; Musgrove *et al.*, 1993). Unfortunately, nearly all patients who initially respond to antiestrogen therapy experience a relapse due to the development of antiestrogen-resistant metastases. Understanding the mechanisms of drug resistance may lead to the development of new treatment strategies. A number of possibilities for tamoxifen resistance have been postulated (reviewed by King, 1990; Johnston *et al.*, 1992; Horwitz, 1993; Wolf and Jordan, 1993; Clarke *et al.*, 1994; Jordan, 1994; Klijn *et al.*, 1994; Osborne and Fuqua, 1994; Clarke and Brünner, 1995), including loss of ER, alterations in structure and/or function of the ER, tamoxifen-stimulated growth, altered drug metabolism and overexpression of different genes. However, the general mechanisms underlying the resistance for the majority of breast cancers are still not known. Progression of breast cancer to hormone insensitivity may be the result of a mutational event, followed by clonal selection (Dorssers *et al.*, 1991; Clarke *et al.*, 1993). Alternatively, the altered phenotype may be the result of epigenetic mechanisms involving stable changes in a large number of tumor cells (Van Agthoven *et al.*, 1994).

A random search for genes involved in antiestrogen resistance was initiated. Due to the inability to cultivate primary breast tumor cells in large quantities for prolonged times, the human breast cancer cell line ZR-75-1 (Engel *et al.*, 1978) was selected as a model for hormone-dependent breast cancer. This cell line is strictly estrogen dependent for proliferation and can be inhibited fully by antiestrogens (Van Agthoven *et al.*, 1992; Dorssers *et al.*, 1993). Previously, we have shown that up-regulation of expression of a single gene is sufficient to induce estrogen independence. Transduction of estrogen-dependent ZR-75-1 cells with an expression vector carrying the epidermal growth factor (EGF) receptor resulted in proliferation independent of estradiol (Van Agthoven *et al.*, 1992). Furthermore, in different experiments, we showed that random changes in the methylation pattern of the DNA, induced by 5-azacytidine, resulted in a high frequency of antiestrogen-resistant cell clones (Van Agthoven *et al.*, 1994). These results indicate that several escape mechanisms of hormone dependence are possible. However, these strategies do not readily allow for the isolation of the involved genes. Hence, identification of (novel) genes connected with the development of antiestrogen resistance was approached by retrovirus-mediated insertional mutagenesis (Dorssers *et al.*, 1993). Random integration of retroviruses may lead to alteration of expression or truncation of genes in the vicinity of the integration site. Consequently, insertional mutagenesis can mimic changes leading to tumor progression. The provirus, carrying long terminal repeats (LTRs) and a neomycin resistance (NeoR) gene, serves as a unique tag to identify genes in the proximity of the integration site. This approach has been successful in the identification of transforming genes in the mouse (Habets *et al.*, 1994; Jonkers and Berns, 1996), and preliminary results show that this strategy may be

useful for the identification for drug resistance genes involved in human disease (Dorssers *et al.*, 1993; Dorssers and Veldscholte, 1997). ZR-75-1 cells (8×10^8) were infected with defective retroviruses and subsequently selected for growth in the presence of 4-hydroxy-tamoxifen, the active metabolite of tamoxifen. Eighty 4-hydroxy-tamoxifen-resistant cell clones have been isolated and established as stable cell lines. Many of the resistant clones carry more than one integrated provirus. The presence of a retroviral genome integrated in a particular locus of several cell clones which have arisen independently (common integration site) strongly suggests that a specific gene function is altered by the viral integration and contributes to the development of antiestrogen resistance. Therefore, a search for common sites of integration in the established panel of resistant cell clones was performed to identify the loci of interest. This has resulted in the identification of three chromosomal loci, designated breast cancer antiestrogen resistance (*BCAR*) locus 1 (Dorssers *et al.*, 1993), 2 (Dorssers and Veldscholte, 1997) and 3. This report describes the cloning and characterization of the *BCAR3* gene, involved in antiestrogen resistance, identified by retrovirus-mediated insertional mutagenesis.

Results

Common site of integration BCAR3

To identify genes involved in antiestrogen resistance, cell clone X-3-6 was selected from our panel of 80 cell clones resistant to 4-hydroxy-tamoxifen. Cell clone X-3-6 contains only one integrated provirus which is expected to induce the resistant phenotype. Furthermore, cell clone X-3-6 does not belong to the clones which contain a viral integration in the *BCAR1* or *BCAR2* loci. To screen for additional clones with an insertion in this region, an integration site-specific probe was created by inverse PCR (Figure 1A). Chromosomal DNA of clone X-3-6, digested with a panel of restriction enzymes, was analyzed by Southern blotting techniques. Hybridization with a NeoR probe, specific for the retroviral integration, showed that a combination of *Bam*HI and *Bgl*II digestion resulted in a restriction fragment of ~6.5 kb, convenient for isolation of a large fragment adjacent to the viral integration (Figure 1A). DNA of cell clone X-3-6 cleaved with *Bam*HI and *Bgl*II restriction enzymes was circularized by ligation and amplified using outward-directed primers located within the NeoR and viral *gag* sequences. Subsequently, this inverse PCR product was amplified with two sets of nested primers located within the LTRs (Figure 1A). This approach resulted in a 3.2 kb PCR fragment of genomic sequences adjacent to the viral integration site. Alu-repetitive sequences were removed from this fragment by subcloning, which resulted in a 1.6 kb probe (Figure 1A). Southern analysis of X-3-6 and parental ZR-75-1 DNA digested with a panel of restriction enzymes and hybridized with the integration probe and a NeoR probe confirmed the specificity of this probe for the X-3-6 integration site. Subsequently, the cell line panel was screened for the occurrence of a common site of integration. Southern blots of the 80 antiestrogen-resistant cell lines containing DNA, digested with either *Bam*HI, *Bgl*II or *Hin*dIII, were hybridized with the integration-specific probe of clone

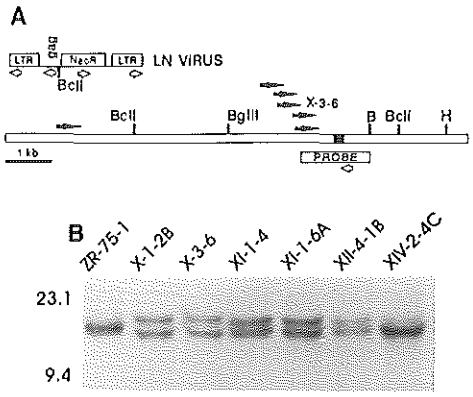


Fig. 1. (A) Genomic analysis of the *BCAR3* integration locus. The primers used for inverse PCR to obtain an integration site-specific probe are indicated below the LN virus (open arrows). The position and orientation of the different retroviral integrations are indicated with an arrow. Mapping of the different integrations was performed by Southern blotting techniques and PCR analysis with primers located on the 3′ LTR and on the integration probe (open arrows). The positions of the integrations are mapped with respect to the retroviral integration in cell clone X-3-6. The integration site-specific probe is marked. The exon detected near the integrations is denoted as a black box. Restriction sites *Bcl*I and *Bgl*II, *Bam*HI (B) and *Hin*dIII (H) and the *gag* region are indicated. (B) Common integration site. A Southern blot with genomic DNA of cell lines digested with the restriction enzyme *Hin*dIII was hybridized with the *BCAR3* integration probe. Roman numerals indicate the different *BCAR3* cell clones. In the six independently derived *BCAR3* cell lines of a total panel of 80 cell lines, an additional larger restriction fragment is detected as a result of the integration of the provirus. The intensity of the additional band in cell clone XIV-2-4C is less in comparison with the other cell lines. This may be explained by contamination with a different clone during isolation. Clone XIV-2-4C was omitted from further (RNA) analysis.

X-3-6. Figure 1B shows that this probe identified a 15 kb *Hin*dIII restriction fragment in the parental ZR-75-1 cells and in the derived integration cell clones. In the X-3-6 cell clone, an additional fragment (~18 kb) was observed, resulting from integration of the LN retrovirus (~3 kb) in the other allele (Figure 1B). This analysis revealed five additional cell clones with a provirus integrated in this region. In total, a region of ~25 kb surrounding the X-3-6 integration site was analyzed. Additional integrations further up- or downstream of this region cannot be excluded. Detailed mapping analysis of the six integration sites demonstrated that they were distinct and thus confirmed the independent origin of the clones (Figure 1A). In addition, Southern analysis and PCR mapping showed that all clones have a similar 5′ to 3′ direction of transcription of the integrated retrovirus. Five viral integrations have occurred in a 1 kb region and one, in clone X-1-2B, is integrated 5 kb upstream. The occurrence of six retroviral insertions in six cell lines which have arisen independently in a panel of 80 cell lines in a region of 5 kb is unlikely to be the result of a random process (2.9×10^{-19}, binomial distribution) (Dorssers *et al.*, 1993). This common site of integration is thus tightly linked with antiestrogen resistance *in vitro* and is termed the breast cancer antiestrogen resistance 3 (*BCAR3*) locus.

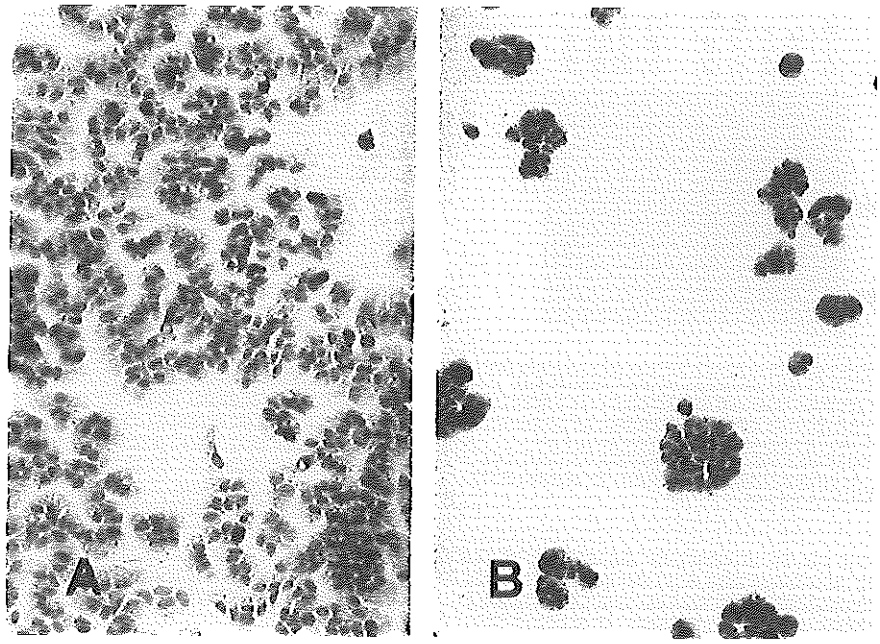


Fig. 2. Transfer of the *BCAR3* locus. Somatic cell hybrids with (A) or without (B) the *BCAR3* locus were cultured in the presence of 4-hydroxy tamoxifen. Representive examples were stained with hematoxylin–eosin at day 10. Hybrid cells with the *BCAR3* locus (A) proliferate in medium containing 4-hydroxy-tamoxifen, while hybrid cells with the non-relevant integration locus (B) are fully inhibited by 4-hydroxy-tamoxifen.

Transfer of the BCAR3 locus by somatic cell fusion

Subsequently, we wished to determine whether the alteration in the *BCAR3* locus, caused by retroviral mutagenesis, is a dominant or recessive event. Southern blotting analysis showed that one of the integration clones, XI-1-6A, carries two integrated proviruses, one in the *BCAR3* locus and an additional integration which is expected to be non-relevant for antiestrogen resistance. This cell line was chosen for generation of somatic cell hybrids between a hygromycin B-resistant subclone of the parental ZR-75-1 cells (ZH3D7) and XI-1-6A cells. The presence of the additional proviral integration in this cell line serves as an internal control. An approach was chosen in which the donor cells were lethally irradiated. Somatic cell hybrids were selected with hygromycin B and G418, resulting solely in hybrids consisting of parental ZR-75-1 cells which have acquired at least one of the NeoR-containing integration loci. In total, 11 hybrids were obtained which were characterized with an informative *Hin*dIII restriction digestion to distinguish between the two viral integrations. This analysis showed that five hybrids contained the locus with the viral integration in the *BCAR3* locus and six hybrids carried the other viral integration site. Subsequently, we tested whether the hybrids were antiestrogen resistant. The growth performance of triplicate cultures of the cell hybrids was compared with the parental ZR-75-1, ZH3D7 and XI-1-6A cells in culture medium containing 1 μM of 4-hydroxy-tamoxifen. Antiestrogen sensitivity of cell hybrids was scored on days 7 and 10. Cultures of hybrid cells showing poor, rounded cell morphology, loose attachment to the culture flask, low numbers of mitoses and little or no increase in cell numbers (measured as the percentage of covered surface, ~20–30%) comparable with ZR-75-1 and ZH3D7 cells were scored 4-hydroxy-tamoxifen sensitive (Figure 2B). Hybrids which looked healthy, with a flattened morphology, firmly attached to the culture flask with readily visible numbers of mitoses, and cell numbers occupying up to 90% of surface of the culture flask, comparable with XI-1-6A cells, were scored tamoxifen resistant (Figure 2A). This analysis showed that only the five hybrid cell lines carrying the *BCAR3* locus were resistant to 4-hydroxy-tamoxifen. These results indicate that transfer of the *BCAR3* integration locus instantly confers dominant resistance to the parental ZR-75-1 cells.

Identification of coding sequences in the BCAR3 locus

In five out of six resistant cell lines, the integration of the retrovirus occurred in a very narrow region (Figure 1). This indicates a positive selection for this region and a common mechanism of alteration of gene expression. Northern blots with RNA of the integration clones were screened with the genomic inverse PCR probe to identify possible transcribed sequences. Large transcripts were observed in the *BCAR3* clones, which vary in size in the various cell clones and were not detected in the parental ZR-75-1 cells (Figure 3). Northern blot hybridization demonstrated that these are chimeric transcripts composed of LTR, NeoR and *BCAR3* sequences (data not shown). The observed differences in mRNA sizes in individual

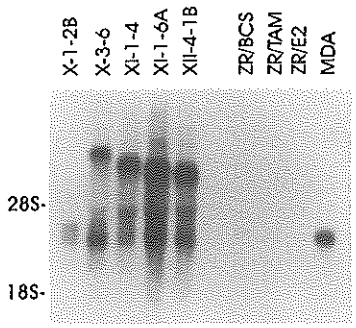


Fig. 3. mRNA expression of *BCAR3*. Northern blots containing total RNA from ZR-75-1 cells, antiestrogen-resistant cell clones and MDA-MB-231 breast cancer cells were hybridized with a *BCAR3* probe. ZR-75-1 cells were cultured in the presence (E2) or absence (BCS) of 1 nM 17β-estradiol or in medium containing 1 μM 4-hydroxy-tamoxifen (TAM). Antiestrogen-resistant cell clones were cultured in the presence of 4-hydroxy-tamoxifen. The positions of 18S and 28S rRNAs are indicated.

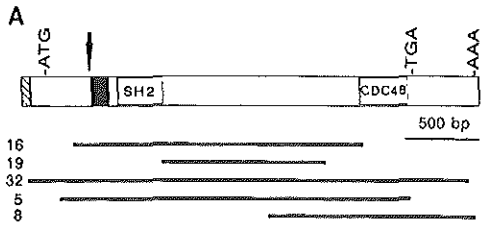
cell clones can be explained by the position of the integrated retroviruses relative to the *BCAR3* sequences and readthrough transcripts from the viral LTR (Figures 1 and 3). More importantly, screening of Northern blots with RNA of a panel of cell lines revealed a transcript of ~3.4 kb in the estrogen-independent MDA-MB-231 (Cailleau *et al.*, 1974) breast cancer cell line (Figure 3). This indicates that this genomic integration probe contains exon sequences. This *BCAR3* transcript appears also to be present in the integration clones and is barely detectable in the ZR-75-1 cells. Estrogens can modulate gene expression of target genes. However, *BCAR3* is not regulated detectably in ZR-75-1 cells. Cultivation of ZR-75-1 cells in the presence and absence of estradiol and in the presence of 4-hydroxy-tamoxifen did not result in up-regulation of *BCAR3* expression (Figure 3). Therefore, it is most likely that the observed up-regulation of the *BCAR3* RNA is induced by the integrated virus.

BCAR3 cDNA cloning and predicted amino acid sequence

To isolate the gene corresponding to the observed transcript, a human testis cDNA library was screened with the integration site-specific probe. In the first screening round, a 1.9 kb cDNA clone was isolated (#16, Figure 4A). Sequence comparison of the genomic integration probe with the cDNA showed that the genomic probe contains a 133 bp exon, identical to sequences of the cDNA clone #16 (represented by nucleotides 456–589 in Figure 4B) with consensus splice donor and acceptor sites. Northern blot analysis confirmed that this cDNA clone recognizes the *BCAR3* transcripts. In successive screening efforts to obtain the full-length cDNA, overlapping clones were isolated (Figure 4A). The assembled cDNA clones consist of 3004 bp, while the mRNA is ~3.4 kb as estimated on Northern blots. The first ATG is not preceded by an in-frame stop codon. Therefore, a 5′ RACE (rapid amplification of cDNA ends) strategy was performed to obtain the additional sequences at the 5′ end of the *BCAR3* mRNA. Total RNA of MDA-MB-231 cells, which show abundant expression of *BCAR3*, was used to obtain the full coding region of *BCAR3*. Several 5′ RACE clones were isolated and sequenced. It was shown that an in-frame stop codon (TGA) is present 72 bp upstream of the first ATG. The cDNA obtained is 3042 bp in total and contains the complete coding region. Three ATGs are present in the first 174 bp of the cDNA. Analysis of the surrounding sequences of the three putative start sites indicates that the first ATG codon at position 99 fits the Kozak consensus sequence (Kozak, 1991). The open reading frame (ORF) is flanked by an in-frame TGA translation termination codon at position 2574, followed by a 448 nucleotide 3′-untranslated region and a poly(A) tail of 19 nucleotides. *BCAR3* has a single ORF that encodes a protein of 825 amino acids with a predicted molecular mass of 92 kDa, assuming that translation starts at the first ATG (Figure 4B). Comparison of the sequence of *BCAR3* with the available protein and nucleotide databases revealed that *BCAR3* is a newly identified gene whose product has a single stretch of amino acids (codons 154–253 in Figure 4) with strong homology with Src homology 2 (SH2) domains of other proteins (Figure 4C). A profile scan utilizing the Prosite profile database detected no further putative protein domains. However, sequences homologous to part of the yeast cell division cycle protein CDC48 were observed by using the BLASTP algorithm (codons 699–812, Figure 4D).

mRNA expression of BCAR3

We next evaluated the expression of *BCAR3* mRNA in various normal tissues. For this purpose, commercially available Northern blots were hybridized with the 1.9 kb cDNA probe clone #16. *BCAR3* mRNA is widely expressed, and abundant transcripts were observed in heart, placenta, skeletal muscle, spleen, prostate, testis, ovary, small intestine, colon and fetal kidney. In skeletal muscle and heart, an additional 6 kb mRNA is present (Figure 5). The nature of this larger transcript is at present unclear but may be explained by alternative splicing. In addition, Northern analysis was performed on RNA isolated from non-malignant breast tissues. No expression of *BCAR3* was detected in these samples (n = 6). However, the amount of luminal epithelial cells in normal breast is small compared with stromal cells. Therefore, it cannot be excluded that *BCAR3* is expressed by the glandular epithelial cells. A panel of cell lines originating from breast, ovarian and endometrial cancer was screened by Northern analysis for expression of *BCAR3*. These results were compared with ER and EGF receptor protein expression (Table I). In the cell lines, only the 3.4 kb *BCAR3* mRNA was detected. In the breast cancer cell lines tested (ZR-75-1, BT-474, MCF-7, T47D, ER-positive; EVSA-T, SK-BR-3, MDA-MB-134, ER-negative), no expression of *BCAR3* was detected. In the ER-negative breast cancer cells BT-20, HS-578 and MDA-MB-231, abundant expression of *BCAR3* was observed. In two ER-negative immortalized mammary epithelial cell lines, RC-6 and HBL-100, only the latter showed expression. In ovarian cell lines (SKOV3, SKOV6, OVCAR3, 2774, 2780, HOC7 and KB3.1, all ER-negative), expression of *BCAR3* was observed except for in 2780. In the endometrial cell lines tested (SCRC, AN-3-CA, HEC1A, HEC1B, RL 95-2 and



B

BCAR3

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MAAGKFASLP RNMPVNHQFP LASSMDLLSS RSPLAEHRPD  40
AYQDVSIHGT LPRKKKGPPP IRSCDDFSHM GTLPHSKSPR  80
QNSPVTQDGI QESPWQDRHG ETFTFRDPHL LDPTVEYVKF 120
SKERHIMRT PEKLKKELEE ELLLSSEDLR SHAYYHGRIP 160
RQVSENLVQR DGDFLVRDSL SSPGNFVLTC QWKNLAQHEK 200
INRTVLRLSE AYSRVQYQFE MESFDSIPGL VRCYVGNRRP 240
ISQQSGAIIF QPINRTVPLR CLEEHYGTSP GQAREGSLTK 280
GRPDVAKRLS LTMGGVQARE QNLPRGNLLR NKEKSGSQPA 320
CLDHMQDRRA LSLKAHQSES YLPIGCKLPP QSSGVDTSPC 360
PNSPVFRTGS EPALSPAVVR RVSSDARAGE ALRGSDSQLC 400
PKPPPKPCKV PFLKVPSSPS AWLNSEANYC ELNPAPATGC 440
GRGAKLPSCA QGSHTELLTA KQNEAPGPRN SGVNYLILDD 480
DDRERFWEPA AAQMEKGQWD KGEFVTPLLE TVSSFRPNEF 520
ESKFLPPENK PLETAMLKRA KELFTNNDPK VIAQHVLSMD 560
CRVARILGVS EEMRRNMGVS SGLELITLPH GHQLRLDIIE 600
RHNTMAIGIA VDILGCTGTL EDRAATLSKI IQVAVELKDS 640
MGDLYSFSAL MKALEMPQIT RLEKTWTALR HQYTQTAILY 680
EKQLKPFSKL LHEGRESTCV PPNNVSVPLL MPLVTLMERQ 720
AVTFEGTDWN EKNDQSCEIN LNHLATAREM AEAADSYRMN 760
AERILAGFQP DEEMNEICKT EFQMRLLEGS KGAQVNQTER 800
YEKFNQILTA LSRKLEPPPV KQAEL*                 825
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C

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BCAR3 <-SSEDLRSMAMYHGRITRQVSEN-TV----GRDGDFLVRDSLSSPGNFVLTCQWKN------LAQ
SHC   <-MAGQLNGEPYFHGKLSRREAEA-LL----QLNGDFLVRESTTTPGQYVLTGLQSG------QPK
GRB2  <-NYIEMKPHYDFFPKIKPRAKAWH-MLSKQRDAETLIREGSKAGKDDSLSVKFGN------DVQ
FER   <-SEKPIAEQDLYRGAITPAIEAQE-LL----KKQGDFLVRESHGKEGEYVLSVYSDG------QRR
FES   <-VQKPLHEQLMYHGAITPAEVAE-LL----VHSQGDFLVRESQGKQ-EYVLSVLWDG------EPR
HCK   <-RVDSLETEEWFFKGISRKDAERQLLAPGNMLGSFMIRDSETTKGSYSLSVRDYDPRQGDTVK
SRC   <-PSDSIQAEEWYFGKITRRESERLLLNAENPRGTFLVRESETTKGAYCLSVSDFDNAKGLNVK
                  *                          *                        #
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BCAR3 HFKINNTVLRLISEAYSRVQTQFEMESKDSIEGLVRCYVGNRRP-ISQQSGAILFQPINRTVP->
SHC   HLLLVD-DPEGV--------VRTKDHRFESVSHLISYHMDNHLPIISAGSELCLQQTVERKL*
GRB2  HFLVLRDGAGK--------VFLVVVFGNSLNEKVDYHRSTSVSRNQQIFLRDIEQVPQQPT->
FER   QFTIHQ-YVDNN--------VRFQGTGDPSNIPQLIDHYTTKQV-ITKKSGVVLLHQIPKDK->
FES   QFIIHQ-SLDNN--------VRLQGCGFPSNIPLLIDHLLSTQQR-LTKKSGVVFLHRAVPKDK->
HCK   HYIT--NTLDNGGF------VISPRSTFSTLQELVDHYKKGNDG-LCQKLSVPCMSSKPQKP->
SRC   HYKI--NKLDSGGF------VITSRTQFNSLQQLVAYYSKHADG-LCHRLTTVCPTSKPQTQ->
       #*   #
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D

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HsBCAR3  <-VPPNNV-SVPLLMPL-VTLMERQAVTFESTDNWEKNDQSGEIMENHLATREMAEAADSYEM
ScCDC48  <-ILNAQLRKTPLEPGLELTAIAKATQGFSGADLLYIVQRAAKYAIKDSIETERQEAEKEVKV
CeCDC48a <-ILKASLRKTPLSKDLDLFLAKNTVGFSGADLTEICQRACKLAIRESIEKEIRIQKERQDPQ
CeCDC48b <-IFKASLRKTPLSADLDLNFLAKNTVGFSGADLTEICQRACKLAIRESIEREIRQKERQDRS
XlCDC48  <-ILKANLRKSPVAKDVQWDFLAKMTNGFSGADLTEICQRACKLAIRESIENEIRRERDRQTNP
MmCDC48  <-ILKANLRKSPVAKDVDLEFLAKMTNGFSGADLTEICQRACKLAIRESIESEIRREREQTNP
HsCDC48  <-ILKANLRKSPVAKDVDLEFLAKMTNGFSGADLTEICQRACKLAIRESIESEIRREREQTNP
AtCDC48  <-IFKAALRKSPIAKDVDIGALAKYTQGFSGADITEICQRACKYAIRENIEKDIEKERRSENP
SaCDC48  <-ILKVHTKNVPLAEDVSLEDIAEKLEGYTGADLEALVREATINAMR.SIYSMCDRQSRDECK
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HsBCAR3  NA-SRILAG--------FQPDENMNEICNTEFQMRLLKGSKGAQVNQTERNYEKFNQILTALSR->
ScCDC48  EGEDVEMTDEGAKAEQEPEVQPVPYITMEEFAEAMKTAKRSVSDAELRKYEAYSQQMKQSRG->
CeCDC48a ARGQELME---------DDAVDPVPEITRAHFEEAMKFARRSVTDNDIRKYEKFAQTLQQSRG->
CeCDC48b ARGQELME---------DELADPVPEITRAHFEEAMKFARRSVTDNDIRKYEKFAQTLQQSRG->
XlCDC48  SNVE-------------VEEDDPVPEIRRDHFEEAMRLARRSVSDNDIRKYEKFAQTLQQSRG->
MmCDC48  SNVE-------------VEEDDPVPEIRRDHFEEAMRFARRSVSDNDIRKYEKFAQTLQQSRG->
HsCDC48  SNVE-------------VEEDDPVPEIRRDHFEEAMRFARRSVSDNDIRKYEKFAQTLQQSRG->
AtCDC48  ENVE-------------EDGVDPVSEIKAAHFEESMKYARRSVSDADIRKYQAFAQTLQQSRG->
SaCDC48  GNMEQCY-----QKHIKECMNKTSFKVSNEDFEKALNVVKASLTQADIQKYEKFSQEFKKRAIA*
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Fig. 4. Structure of *BCAR3*. (A) Schematic representation of the *BCAR3* gene. The thick lines represent the overlapping cDNAs. The arrow indicates the position of the viral integration sites in the genome compared with the cDNA sequence. The exon detected near the integrations is indicated (black box, see also Figure 1A). The hatched box indicates the 5′ region obtained by RACE. The location of the putative SH2 domain and the region homologous to CDC48 is shown. (B) *BCAR3* deduced amino acid sequence. The amino acid sequence is given in the one-letter code. The predicted N-terminal methionine and the ATG codon (M) of the shortened product observed in the *BCAR3* cell clones and the first stop codon (*) are indicated. The region of similarity to other SH2 domain-containing proteins is underlined and the region homologous to CDC48 is double underlined. (C) Sequence alignment of the SH2 domain of the human BCAR3 and other human SH2-containing proteins. The conserved residues of the BCAR3, Shc, Grb2, Fer, Fes, Hck and Src proteins are compared by giving priority to BCAR3. Sequences used in this figure are Shc, SWISS-PROT database accession No. P29353, Grb2 P29354, Fer P16591, Fes P07332, Hck P08631 and Src G338460. Identical amino acids are represented by black boxes, whereas similar residues (A, G, S, P, T; D, E, N, Q; H, K, R; I, L, M, V; F, Y, W) are given in gray boxes. Key residues in specific interaction with phosphotyrosine (*) and substrate specificity-determining residues (#) in Src are indicated (Songyang *et al.*, 1994; Cohen *et al.*, 1995). (D) Sequence alignment of the CDC48-related domain of the human BCAR3 protein. The CDC48-related residues of the BCAR3 protein are visualized by giving priority to BCAR3. The CDC48 equivalents are derived from the following species *Saccharomyces cerevisiae* (Sc) accession No. A39977, *Caenorhabditis elegans* (Ce) P54811, *Xenopus laevis* (Xl) P23787, *Mus musculus* (Mm) Q01853, *Arabidopsis thaliana* (At) P54609, *Sulfolobus acidocaldarius* (Sa) Q07590, *Homo sapiens* (Hs) ESTs AA009995 and N72512.

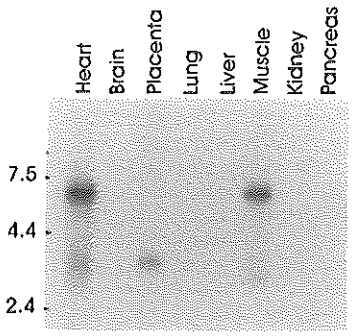

Fig. 5. The expression of *BCAR3* mRNA in various normal human tissues. Northern blots (Clontech) containing 2 μg of poly(A)$^+$ RNA from the indicated tissues were hybridized with *BCAR3* cDNA probe, clone #16. The mobility of RNA molecular size standards is indicated on the left in kilobases.

Table I. Expression of *BCAR3* mRNA was compared with expression of ER and EGF receptor protein

Cell line	BCAR3	ER	EGFR
Breast			
ZR-75-1	–	+ (100)	– (0)
MCF-7	–	+ (300)	– (20)
T-47D	–	+ (100)	+ (600)
BT-474	–	+ (23)	nd
BT-20	++	– (<10)	nd
EVSA-T	–	– (<10)	+ (67)
HS-578-T	+	– (<10)	nd
MDA-MB-134	–	– (<10)	nd
MDA-MB-231	+++	– (<10)	+ (>1600)
SK-BR-3	–	– (<10)	+ (560)
HBL-100	+	– (<10)	nd
RC-6	–	– (<10)	nd
Ovary			
2780	–	– (<10)	+ (220)
2774	++	– (<10)	+ (*)
HOC-7	+	– (<10)	+ (*)
KB3.1	+	– (<10)	nd
OVCAR-3	+	– (<10)	+ (1200)
SK-OV-3	+	– (<10)	+ (>1600)
SK-OV-6	+	– (<10)	+ (>1600)
Endometrium			
ECC-1	–	+ (82)	+ (84)
AN-3-CA	+	– (<10)	+ (1550)
HEC-1-A	++	– (<10)	+ (>1600)
HEC-1-B	+	– (<10)	+ (>1600)
KLE	–	– (<10)	+ (530)
SCRC	++	– (<10)	+ (>1600)
RL 95-2	++	– (<10)	+ (>1600)

Receptor concentrations determined with biochemical assays are expressed as fmol/mg protein. Expression of ER >10 fmol/mg and EGF receptor >50 fmol/mgof protein was scored positive (+). ND not done. (*) Expression determined by Northern analysis. Expression levels of *BCAR3* mRNA varied between –, no expression detected after 7 days exposure and +++, high expression detected after overnight exposure on film.

KLE, ER-negative and ECC1, ER-positive), high mRNA levels were observed except for in ECC1 and KLE. The integrity of the mRNA on Northern blots was examined by hybridization to a GAPDH or actin probe. Correlation of ER and EGF receptor expression with expression of

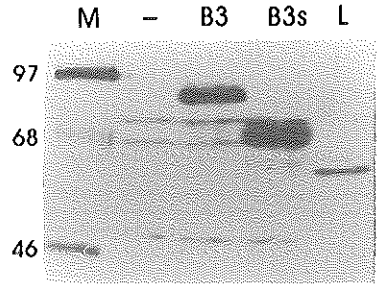

Fig. 6. *In vitro* transcription translation. *In vitro* translated full-length *BCAR3* (B3) was compared with the shortened form (B3s) of *BCAR3*, as deduced from analyses of the virus clones (nucleotides 383–2951). Control reactions with a luciferase construct (61 kDa product, 10% loaded) and reaction without plasmid DNA (–) are shown. Detection was by chemiluminescence. The mobility of molecular size standards is indicated on the left in kilodaltons.

BCAR3 showed an inverse relationship between expression of *BCAR3* and ER (McNemar test, $P = 0.025$).

In vitro transcription translation of BCAR3

Figures 1 and 4 show that all proviral integrations had occurred in an intron in the 5′ region of the *BCAR3* gene, most likely resulting in a shorter mRNA. Northern blot analysis with a specific probe for the 5′ part of the gene (represented by nucleotides 38–420) demonstrated that these sequences were not present in the shortened *BCAR3* mRNA of the integration clones, but were present in the MDA-MB-231 *BCAR3* transcripts (not shown). The shortened *BCAR3* gene in the antiestrogen-resistant clones may encode a protein of 699 amino acids with a predicted mass of 78 kDa, assuming that the ATG codon at position 477 is the initiator methionine. Both *BCAR3* and a shortened construct (represented by nucleotides 421–2989) resembling the presumed situation in the *BCAR3* clones were subcloned in a vector which allows the expressed protein to start with the authentic ATG codon. *In vitro* transcription translated BCAR3 migrated as a single band by SDS–PAGE at ~90 kDa (Figure 6). *In vitro* transcription translation analysis of the shortened construct showed a single band of ~74 kDa (Figure 6).

Ectopic expression of BCAR3 induces estrogen independence in ZR-75-1 and MCF-7 cells

Stable transfectants with the *BCAR3* gene were established to provide conclusive evidence that *BCAR3* is the gene responsible for antiestrogen resistance in this locus. For this purpose, two expression constructs were made in which the *BCAR3* cDNA #32, containing the complete coding region, was positioned under control of a cytomegalovirus (CMV) and a LTR promoter. These expression vectors were transfected into ZR-75-1 cells and stable G418-resistant clones were generated and isolated. These transfectants, designated ZR/BCAR3 cells, were expanded in the presence of 17β-estradiol to certify estrogen dependence. We next determined the proliferation capacity of the *BCAR3* transfectants in the presence of the antiestrogen 4-hydroxy-tamoxifen. In Figure 7, the increase in cell numbers over an 11 day culture period of parental ZR-75-1 cells, vector controls and eight independently derived

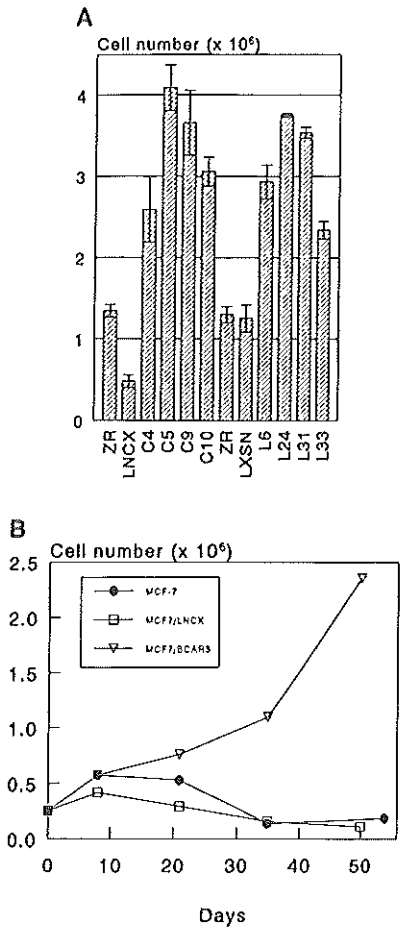

Fig. 7. *BCAR3* induces antiestrogen resistance. (A) ZR-75-1 cell lines containing the expression vector without the *BCAR3* cDNA, *BCAR3* transfectants and parental ZR-75-1 cells (0.7×10^6) were plated in culture medium with 10% BCS and 1 μM 4-hydroxy-tamoxifen in triplicate. Cell numbers were determined on day 11. Cell lines indicated are: parental ZR-75-1 cells (ZR), LNCX (average of four different cell clones) and LXSN vector controls and the *BCAR3* transfectants (C4, C5, C9 and C10 under control of a CMV promoter and L6, L24, L31 and L33 under control of the LTR promoter). (B) MCF-7: pools of MCF7/BCAR3 transfectants, MCF7/LNCX vector control transfectants and parental MCF-7 cells were plated in medium with 10% FCS with 100 nM of the pure antiestrogen ICI 182,780 in triplicate. Cells were counted at the indicated days and replated at the initial density of 0.25×10^6 cells per 25 cm^2 flask. Cumulative cell numbers are presented over a 50 day culture period.

transfectants is presented. Parental ZR-75-1 cells, which are fully dependent on estradiol for proliferation, give rise to maximally one generation in the presence of 4-hydroxy-tamoxifen (Figure 7A). As expected, similar results were obtained with the LNCX and LXSN vector controls. In contrast, the ZR/BCAR3 cells have acquired the ability to proliferate in the presence of 4-hydroxy-tamoxifen. In addition, the proliferation capacity of the transfectants is similar in the presence or absence of antiestrogens and thus not dependent on antiestrogens. Subcultivation after the 11 day culture period of ZR-75-1 cells and transfectants with the vector alone, in the presence of 4-hydroxy-tamoxifen, resulted in rapid decline of these cultures. Transfectants C5, C9, C10 with *BCAR3* under control of the CMV promoter were subcultured successfully and became expanding cultures. Cell clone L24 of the *BCAR3* transfectants with the LTR promoter only developed as a stable estrogen-independent cell line. The reason for these variations are at present not clear, but may be attributed to differences in BCAR3 protein levels. In total, 40 transfectants were tested and 25 (62.5%) showed objective growth in the presence of 4-hydroxy-tamoxifen. Similar results were obtained with the antiestrogen ICI 182,780 a pure antagonist of estrogen-stimulated proliferation. None of the vector control cell lines or the parental ZR-75-1 cells, which were tested repeatedly, were able to grow in the presence of 4-hydroxy-tamoxifen or ICI 182,780.

Additional experiments were performed to establish whether *BCAR3* can induce antiestrogen resistance in the ER-positive MCF-7 breast cancer cell line. MCF-7 cells were transfected with *BCAR3* under control of the CMV promotor (MCF7/BCAR3) or with the LNCX vector without the insert (MCF7/LNCX). Both pools of stably transfected cells and individually established clones were obtained in medium containing 10% fetal calf serum (FCS) and G418. In total, 20 MCF7/BCAR3 clones and eight MCF7/LNCX vector clones were isolated and tested for proliferation ability in the presence of the pure antiestrogen ICI 182,780. Fourteen out of 20 MCF7/BCAR3 clones (70%) showed objective growth compared with the MCF7/LNCX clones. Similar results were obtained with cultures in the presence of 4-hydroxy-tamoxifen. Growth curves of the pools of transfectants and the parental MCF-7 cells in the presence of antiestrogen are presented in Figure 7B, and demonstrate the requirement for *BCAR3* for antiestrogen-resistant proliferation.

Discussion

Treatment failure of progressive breast cancer is inevitably due to the unremitting progression of the tumor cells to a more malignant hormone-independent phenotype. Insight into the different signaling pathways of normal and breast cancer cells leading to proliferation is a prerequisite for future development of new treatment strategies. *BCAR3*, a novel gene involved in progression of breast cancer cells to estrogen independence, was identified using retroviral insertional mutagenesis *in vitro*. A panel of 80 anti-estrogen-resistant cell clones was screened for new common integration sites. In six cell clones, a proviral integration was detected in a restricted region. All viruses were integrated in an intron located in the 5′ region of the *BCAR3* gene. Somatic cell hybrids between estrogen-dependent ZR-75-1 cells and one of the estrogen-independent *BCAR3* integration clones were established. Transfer of the *BCAR3* chromosomal locus resulted in immediate induction of antiestrogen resistance of the cell hybrids. Most likely as a consequence of the proviral integrations, a truncated protein lacking the N-terminal part of the protein is expressed in these cells. It was determined by Northern analysis that the large transcripts in the *BCAR3*

integration clones consisted of *BCAR3*, LTR and NeoR sequences. In addition, shorter *BCAR3* mRNAs, lacking the first exon(s), were observed in these cells. The shorter *BCAR3* product may originate from readthrough transcripts initiated by the viral 5′ LTR (Kung *et al.*, 1991; Jonkers and Berns, 1996). These chimeric transcripts could be spliced from the splice donor site preceding the *gag* sequences (Miller and Rosman, 1989) in the retrovirus to the *BCAR3* exon sequences adjacent to the integration site. Translation of the resulting mRNAs may initiate at the ATG codon at position 477 and would result in a smaller protein. However, this truncation of *BCAR3* is not a prerequisite for the induction of the hormone-independent phenotype. Transfection of the *BCAR3* cDNA to estrogen-dependent ZR-75-1 and MCF-7 cells resulted in bypassing of hormone dependence and immediate antiestrogen resistance. These results suggest that up-regulation of *BCAR3* stimulates an alternative growth path independently of hormone, both in the presence and absence of antiestrogens. In the established cell lines studied, derived from the breast, ovary and endometrium, an inverse relationship between expression of *BCAR3* mRNA and ER protein was observed (McNemar test, $P = 0.025$). *BCAR3* expression was not detected in ER-positive cell lines. The strongest predictor for response to tamoxifen is expression of a functional ER in primary breast tumors (Foekens *et al.*, 1994; Spyratos *et al.*, 1994; Johnston *et al.*, 1995), while EGF receptor expression has been associated with lack of response to endocrine therapy (Nicholson *et al.*, 1988, 1989). In the panel of breast-derived cell lines studied so far, EGF receptor expression is not tightly linked to *BCAR3* expression.

Sequence similarity analysis showed that BCAR3 contains a copy of a putative SH2 protein domain. SH2 protein domains are found within a wide variety of cytoplasmic signaling molecules that can bind protein sequences phosphorylated at tyrosine residues (Cohen *et al.*, 1995; Pawson, 1995). The homology of BCAR3 with other SH2 domain-containing proteins is limited to this domain. The amino acid residues implicated in protein structure and in interaction with the phosphotyrosine are well conserved. Major sequence differences are observed in the amino acid sequences spacing the β-sheets and α-helices as well as residues implicated in the interaction with substrate amino acid residues adjacent to the phosphorylated tyrosine (Cohen *et al.*, 1995). The BCAR3 SH2 domain shows the strongest homology to SH2 domains of Shc, Grb2, Fer and Fes. In analogy to these related proteins, BCAR3 may act as an adaptor protein and couple activated growth factor receptors to a signaling pathway that regulates the proliferation in these breast cancer cells. Its substrate specificity may resemble that of the Grb2 protein (Songyang *et al.*, 1994), based on the conservation of the critical residues (Figure 4C, marked with #) in the βD region, and indicates a link to the Ras signaling cascade (Rozakis-Adcock *et al.*, 1992).

BCAR3 protein displays striking partial homology with a cell division cycle gene product (CDC48) from yeast. Mutation or elimination of CDC48 in yeast results in arrest of cell division in the large budded state with an undivided nucleus and unseparated spindle pole body (Fröhlich *et al.*, 1991). The characteristic feature of this protein is an internally duplicated domain for nucleotide binding, which is conserved in its mammalian homolog the valosin-containing protein (VCP). ATPase activity has been demonstrated for these proteins (Schulte *et al.*, 1994; Fröhlich *et al.*, 1995). CDC48 proteins shows significant homology to a group of NSF-like proteins which also comprise the duplicated ATPase domain and are involved in homeotypic fusion of intracellular membranes (Fröhlich *et al.*, 1991; Acharya *et al.*, 1995; Latterich *et al.*, 1995; Mellman, 1995; Rabouille *et al.*, 1995). None of the domains shared between CDC48 and the NSF-like proteins are conserved in the BCAR3 protein. BCAR3 has acquired the C-terminal part of CDC48 proteins, which has not yet been implicated in a specific function and includes ~30 amino acid residues contained within the duplicated domain. The mammalian CDC48 is strongly phosphorylated on tyrosine and serine residues in B and T cells and in v-Src transformed fibroblasts (Egerton *et al.*, 1992; Schulte *et al.*, 1994). Primarily from *in vitro* experiments with T cells it was concluded that the C-terminal Tyr805 in murine CDC48 is the predominant target for phosphorylation, but phosphorylation of this residue is non-essential for ATPase function (Egerton and Samelson, 1994). This particular tyrosine residue is not conserved in BCAR3 nor in the archaebacterial homolog of CDC48 (Figure 4D, Sa). An adjacent tyrosine residue is conserved in CDC48 proteins of all species tested and in the BCAR3 protein, and represents an attractive target for C-terminal phosphorylation and functional modulation of these proteins.

The BCAR3 protein has characteristics of a molecule involved in signal transduction. The putative SH2 domain is most likely involved in binding of tyrosine-phosphorylated proteins and may represent an essential component of cascades transporting signals from the cell membrane to the nucleus. The C-terminal domain of BCAR3 is homologous to the CDC48 protein which is responsible for cell division control in yeast and subject to tyrosine phosphorylation in mammalian cells. The combination of these features may explain its dominant role in antiestrogen-arrested breast cancer cell proliferation upon overexpression by retroviral insertion or ectopic expression. Future experiments will attempt to delineate the contribution of these components and define the signaling pathway involved. Further work will be required to establish whether BCAR3 is involved in clinical breast cancer and possibly in other endocrine-related tumors.

Materials and methods

Cell lines and culture conditions

Cell lines were obtained from the American Type Culture Collection (Rockville, MD), except for ZR-75-1 and MCF-7 which were kind gifts of R.J.B.King (ICRF, London) and R.B.Dickson (NCI, Bethesda) respectively. Cell culture was performed essentially as described (Van Agthoven *et al.*, 1992, 1994; Dorssers *et al.*, 1993; Sieuwerts *et al.*, 1997). ZR-75-1, ZH3D7, hybrid and ZR/BCAR3 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum (BCS) and 1 nM 17β-estradiol. *BCAR3* integration cell clones were maintained in RPMI 1640 medium supplemented with 15% BCS, 10% conditioned medium of CRIP cells (a mouse fibroblast cell line) and 1 μM 4-hydroxy-tamoxifen. MCF-7 and MCF7/BCAR3 cells were cultured in RPMI 1640 with 10% heat-inactivated FCS.

Cell fusion

Cell fusion experiments were performed as described (Dorssers and Veldscholte, 1997). Briefly, ZR-75-1 cells were transfected with the expression construct PGK-Hyg B^r which confers hygromycin B resist-

ance, and were used as acceptor cells (designated ZH3D7). Clone XI-1-6A, carrying two viral integrations, was selected as donor cell line. As a consequence of the retroviral insertions, these cells are both antiestrogen- and G418-resistant. Furthermore, the 4-hydroxy-tamoxifen-resistant cells were lethally irradiated to prevent outgrowth of the donor cells. ZH3D7 and XI-1-6A cells were fused with polyethylene glycol (PEG, Boehringer Mannheim, Germany) using a procedure for adherent cells (Eijdems *et al.*, 1992) and according to the manufacturer's recommended conditions. After fusion, the cells were allowed to recover for 2 days and subsequently selected with 1 mg/ml G418 (Geneticin, Gibco-BRL, Life Technologies Ltd. Paisley, UK) in the presence of 1 nM 17β-estradiol. After 10 days, the selection was continued with 500 μg/ml G418 and 25 μg/ml hygromycin B (Boehringer Mannheim) until clones developed. Clones were picked, expanded, characterized by Southern analysis and challenged for antiestrogen resistance.

Transfection of expression constructs

A near full-length *BCAR3* cDNA (#32, nucleotides 39–2989) was cloned into the pLXSN and pLNCX expression vectors (Miller and Rosman, 1989) in which transcription is driven in the former by the LTR promoter and in the latter by a CMV promoter. Expression constructs and control vectors without inserts were transfected using lipofectin reagent (Life Technologies). ZR-75-1 cells (2×10^5) were seeded in 25 cm^2 flasks (Costar, Cambridge, MA) in medium containing 17β-estradiol. After 2 days, the cells were washed twice with serum-free Optimem medium (Life Technologies). To the cells, 3 ml of Optimem medium with 30 μl of lipofectin was added. After 1 h incubation at 37°C, 2–5 μg of linearized plasmid DNA was added. Following 5 h incubation, the DNA-containing medium was replaced by RPMI medium, containing 10% BCS and 1 nM 17β-estradiol. Subsequently, after 2 days, selection with G418 was started. Individual transfectants were isolated and propagated in medium with estradiol and G418. MCF-7 cells were transfected similarly with pLNCX with and without insertion in RPMI medium containing 10% FCS.

DNA and RNA analysis

Genomic DNA was isolated using NaCl extraction procedures described by Miller *et al.* (1988). Total RNA was isolated using guanidine/cesium chloride extraction and used for Northern analyses as previously described (Van Agthoven *et al.*, 1994). Probes were random-primed labeled using [α-^{32}P]dATP (ICN Pharmaceuticals Inc. CA). Blots were exposed to X-OMAT AR film (Eastman Kodak Company, NY).

Isolation and sequencing of BCAR3 cDNA clones

A testis cDNA library (Clontech Laboratories, Inc., CA) was screened with the *BCAR3* integration-specific probe. Plaques were purified and the cDNA inserts were recloned in pGEM4Z (Promega, Madison, WI). The nucleotide sequence was determined on both strands by dideoxy sequencing reactions using T7 DNA polymerase (Pharmacia Biotech, Sweden) and [α-^{32}P]dATP (ICN Pharmaceuticals Inc.). 5' RACE experiments were performed according to the manufacturer's recommendations (Boehringer Mannheim).

In vitro translation

The near full-length *BCAR3* cDNA clone #32 was recloned in the *Eco*RI site of pGEM4Z. A shortened construct (nucleotides 421–2989) resembling the alteration in the *BCAR3* clones was obtained by subcloning the cDNA clone #32. The plasmid was transcribed from the T7 promoter and translated in rabbit reticulocyte lysate by using the TNT Coupled Reticulocyte Lysate System (Promega). After SDS–PAGE, recombinant proteins were electrotransferred to nitrocellulose membranes and proteins were detected with the Non-Radioactive Translation Detection System (Promega) and chemiluminicense (Amersham International, Bucks, UK) by exposing to X-OMAT AR film.

Growth assays

ZR-75-1 cells and transfectants cultivated in the presence of 1 nM 17β-estradiol were harvested by treatment with trypsin-EDTA. Single cells (0.7×10^5) were plated in 25 cm^2 plastic culture flasks in triplicate. The experimental medium containing 10% BCS and 1 μM 4-hydroxy-tamoxifen was changed twice a week. After 11 days, the cultures were harvested by trypsinization and counted. Subsequently, 1.5×10^5 cells were replated to determine the secondary growth rate. MCF-7 cells and transfectants routinely were cultured in medium containing 10% FCS. Cells (0.25×10^5) were plated in experimental medium with 10% FCS and 100 nM ICI 182,780, harvested at days 8 and 11, and replated at the initial density to determine the secondary growth rate.

Receptor determinations

ER and progesterone receptor were determined as described (Van Agthoven *et al.*, 1992), using commercially available enzyme immunoassay kits (ER-EIA, PR-EIA; Abbott Laboratories, Chicago, IL). EGF receptors were determined using a commercially available enzyme-linked immunosorbent assay kit (Oncogene Science, Cambridge, MA).

Nucleotide accession number

The sequence data reported here have been submitted to the DDBJ/EMBL/GenBank database under accession number U92715.

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References

Acharya,U., Jacobs,R., Peters,J.M., Watson,N., Farquhar,M.G. and Malhotra,V. (1995) The formation of Golgi stacks from vesiculated Golgi membranes requires two distinct fusion events. *Cell*, **82**, 895–904.

Cailleau,R., Young,R., Olive,M. and Reeves,W. (1974) Breast tumor cell lines from pleural effusions. *J. Natl Cancer Inst.*, **53**, 661–674.

Clarke,R. and Brünner,N. (1995) Cross-resistance and molecular mechanisms in antiestrogen resistance. *Endocr.-Related Cancer*, **2**, 59–72.

Clarke,R., Dickson,R.B. and Lippman,M.E. (1992) Hormonal aspects of breast cancer. Growth factors, drugs and stromal interactions. *Crit. Rev. Oncol. Hematol.*, **12**, 1–23.

Clarke,R., Thompson,E.W., Leonessa,F., Lippman,J., McGarvey,M., Frandsen,T.L. and Brünner,N. (1993) Hormone resistance, invasiveness, and metastatic potential in breast cancer. *Breast Cancer Res. Treat.*, **24**, 227–239.

Clarke,R., Skaar,T., Baumann,K., Leonessa,F., James,M., Lippman,J., Thompson,E.W., Freter,C. and Brunner,N. (1994) Hormonal carcinogenesis in breast cancer: cellular and molecular studies of malignant progression. *Breast Cancer Res. Treat.*, **31**, 237–248.

Cohen,G.B., Ren,R. and Baltimore,D. (1995) Modular binding domains in signal transduction proteins. *Cell*, **80**, 237–248.

Dorssers,L.C.J. and Veldscholte,J. (1997) Identification of a novel breast-cancer-anti-estrogen-resistance (BCAR2) locus by cell-fusion-mediated gene transfer in human breast-cancer cells. *Int. J. Cancer*, **72**, 700–705.

Dorssers,L.C.J., Van Agthoven,T. and Sieuwerts,A.M. (1991) Genetic mechanisms involved in progression to hormone independence of human breast cancer. In Berns,P.M.J.J., Romijn,J.C. and Schroeder,F.H. (eds), *Mechanisms of Progression to Hormone-independent Growth of Breast and Prostatic Cancer*. Parthenon Publishing Group Ltd, Carnforth, UK, pp. 169–182.

Dorssers,L.C.J., Van Agthoven,T., Dekker,A., Van Agthoven,T.L.A. and Kok,E.M. (1993) Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. *Mol. Endocrinol.*, **7**, 870–878.

Early Breast Cancer Trial Collab Group (1992) Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 Randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet*, **339**, 1–15–71–85.

Egerton,M. and Samelson,L.E. (1994) Biochemical characterization of valosin-containing protein, a protein tyrosine kinase substrate in hematopoietic cells. *J. Biol. Chem.*, **269**, 11435–11441.

Egerton,M., Ashe,O.R., Chen,D., Druker,B.J., Burgess,W.H. and Samelson,L.E. (1992) VCP, the mammalian homolog of cdc48, is tyrosine phosphorylated in response to T cell antigen receptor activation. *EMBO J.*, **11**, 3533–3540.

Eijdems,E.W.H.M., Borst,P., Jongsma,A.P.M., De Jong,S., De Vries, E.G.E., Van Groenigen,M., Versantvoort,C.H.M., Nieuwint,A.W.M. and Baas,F. (1992) Genetic transfer of non-P-glycoprotein-mediated multidrug resistance (MDR) in somatic cell fusion: dissection of

a compound MDR phenotype. *Proc. Natl Acad. Sci. USA*, **89**, 3498–3502.

Engel,L.W., Young,N.A., Tralka,T.S., Lippman,M.E., O'Brien,S.J. and Joyce,M.J (1978) Establishment and characterization of three continuous cell lines derived from human breast carcinomas. *Cancer Res.*, **38**, 3352–3364.

Foekens,J.A., Portengen,H., Look,M.P., Van Putten,W.L.J., Thirion,B., Bontenbal,M. and Klijn,J.G.M. (1994) Relationship of PS2 with response to tamoxifen therapy in patients with recurrent breast cancer. *Br. J. Cancer*, **70**, 1217–1223.

Fröhlich,K.U., Fries,H.W., Rüdiger,M., Erdmann,R., Botstein,D. and Mecke,D. (1991) Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. *J. Cell Biol.*, **114**, 443–453.

Fröhlich,K.U., Fries,H.W., Peters,J.M. and Mecke,D. (1995) The ATPase activity of purified CDC48p from *Saccharomyces cerevisiae* shows complex dependence on ATP-, ADP-, and NADH- concentrations and is completely inhibited by NEM. *Biochim. Biophys. Acta*, **1253**, 25–32.

Habets,G.G.M., Scholtes,E.H.M., Zuydgeest,D., Van der Kammen,R.A., Stam,J.C., Berns,A. and Collard,J.G. (1994) Identification of an invasion-inducing gene, *Tiam-1*, that encodes a protein with homology to GDP–GTP exchangers for Rho-like proteins. *Cell*, **77**, 537–549.

Horwitz,K.B. (1993) Mechanisms of hormone resistance in breast cancer. *Breast Cancer Res. Treat.*, **26**, 119–130.

Jensen,E.V. (1981) Hormone dependency of breast cancer. *Cancer (Philadelphia)*, **47**, 2319–2326.

Johnston,S.R.D., Dowsett,M. and Smith,I.E. (1992) Towards a molecular basis for tamoxifen resistance in breast cancer. *Ann. Oncol.*, **3**, 503–511.

Johnston,S.R.D., Saccani-Jotti,G., Smith,I.E., Salter,J., Newby,J., Coppen,M., Ebbs,S.R. and Dowsett,M. (1995) Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. *Cancer Res.*, **55**, 3331–3338.

Jonkers,J. and Berns,A. (1996) Retroviral insertional mutagenesis as a strategy to identify cancer genes. *Biochim. Biophys. Acta*, **1287**, 29–57.

Jordan,V.C. (1994) Molecular mechanisms of antiestrogen action in breast cancer. *Breast Cancer Res. Treat.*, **31**, 41–52.

Jordan,V.C. and Murphy,C.S. (1990) Endocrine pharmacology of antiestrogens as antitumor agents. *Endocr. Rev.*, **11**, 578–610.

King,R.J.B. (1990) Receptors, growth factors and steroid insensitivity of tumours. *J. Endocrinol.*, **124**, 179–181.

Klijn,J.G.M., Berns,E.M.J.J., Dorssers,L.C.J. and Foekens,J.A. (1994) Molecular markers of resistance to endocrine treatment of breast cancer. In Dickson,R.B. and Lippman,M.E. (eds), *Drug and Hormonal Resistance in Breast Cancer, Part II: Cellular and Molecular Mechanisms*. Ellis Horwood Ltd, Chichester, pp. 133–168.

Kozak,M. (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.*, **115**, 887–903.

Kung,H.J., Boerkoel,C. and Carter,T.H. (1991) Retroviral mutagenesis of cellular oncogenes: a review with insights into the mechanisms of insertional activation. *Curr. Top. Microbiol. Immunol.*, **171**, 1–25.

Latterich,M., Fröhlich,K.U. and Schekman,R. (1995) Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. *Cell*, **82**, 885–893.

Mauvais-Jarvis,P., Sitruk-Ware,R. and Kuttenn,F. (1988) The role of hormones in the pathogenesis of benign breast disease. In Grundfest-Broniatowski,S. and Esselstyn,C.B. (eds), *Controversies in Breast Disease*. Marcel Dekker, Inc., New York, pp. 43–61.

Mellman,I. (1995) Enigma variations: protein mediators of membrane fusion. *Cell*, **82**, 869–872.

Miller,A.D and Rosman,G.J. (1989) Improved retroviral vectors for gene transfer and expression. *BioTechniques*, **7**, 980–990.

Miller,S.A., Dykes,D.D. and Polesky,H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.*, **16**, 1215–1218.

Musgrove,E.A., Hamilton,J.A., Lee,C.S.L., Sweeney,K.J.E., Watts, C.K.W. and Sutherland,R.L. (1993) Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. *Mol. Cell. Biol.*, **13**, 3577–3587.

Nicholson,S., Halcrow,P., Sainsbury,J.R.C., Angus,B., Chambers,P., Farndon,J.R. and Harris,A.L. (1988) Epidermal growth factor receptor (EGFr) status associated with failure of primary endocrine therapy in elderly postmenopausal patients with breast cancer. *Br. J. Cancer*, **58**, 810–814.

Nicholson,S., Halcrow,P., Farndon,J.R., Sainsbury,J.R.C., Chambers,P. and Harris,A.L. (1989) Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. *Lancet*, **1**, 182–185.

Osborne,M.P. (1987) Breast development and anatomy. In Harris,J.R., Hellman,S., Henderson,I.C. and Kinne,W. (eds), *Breast Diseases*. Lippincott Company, Philadelphia, PA, pp. 1–14.

Osborne,C.K. and Fuqua,S.A.W. (1994) Mechanisms of tamoxifen resistance. *Breast Cancer Res. Treat.*, **32**, 49–55.

Pawson,T. (1995) Protein modules and signalling networks. *Nature*, **373**, 573–580.

Rabouille,C., Levine,T.P., Peters,J.M. and Warren,G. (1995) An NSF-like ATPase, p97, and NSF mediate cisternal regrowth from mitotic Golgi fragments. *Cell*, **82**, 905–914.

Rozakis-Adcock,M. *et al.* (1992) Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature*, **360**, 689–692.

Schulte,R.J., Campbell,M.-A., Fischer,W.H. and Sefton,B.M. (1994) Tyrosine phosphorylation of VCP, the mammalian homologue of the *Saccharomyces cerevisiae* CDC48 protein, is unusually sensitive to stimulation by sodium vanadate and hydrogen peroxide. *J. Immunol.*, **153**, 5465–5472.

Sieuwerts,A.M., Klijn,J.G.M. and Foekens,J.A. (1997) Assessment of the invasive potential of human gynecological tumor cell lines with the *in vitro* Boyden chamber assay: influences of the ability of cells to migrate through the filter membrane. *Clin. Exp. Metastasis*, **15**, 53–62.

Songyang,Z. *et al.* (1994) Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol.*, **14**, 2777–2785.

Spyratos,F., Andrieu,C., Hacène,K., Chambon,P. and Rio,M.C. (1994) pS2 and response to adjuvant hormone therapy in primary breast cancer. *Br. J. Cancer*, **69**, 394–397.

Van Agthoven,T., Van Agthoven,T.L.A., Portengen,H., Foekens,J.A. and Dorssers,L.C.J. (1992) Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res.*, **52**, 5082–5088.

Van Agthoven,T., Van Agthoven,T.L.A., Dekker,A., Foekens,J.A. and Dorssers,L.C.J. (1994) Induction of estrogen independence of ZR-75-1 human breast cancer cells by epigenetic alterations. *Mol. Endocrinol.*, **8**, 1474–1483.

Wolf,D.M. and Fuqua,S.A.W. (1996) Laboratory–clinic interface: mechanisms of action of antiestrogens. *Cancer Treat. Rev.*, **21**, 247–271.

Wolf,D.M. and Jordan,V.C. (1993) Drug resistance to tamoxifen during breast cancer therapy. *Breast Cancer Res. Treat.*, **27**, 27–40.

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Summary

Breast cancer is the most common malignancy of women in the Western world, and is one of the leading causes of cancer mortality. Breast tumor growth appears to be estrogen-dependent in early stages of the disease. The effects of estrogens are exerted through the estrogen receptor (ER) which can be inhibited with antagonists of estrogens. Adjuvant treatment of patients with primary breast cancer with antiestrogens like tamoxifen increases both the disease-free period and overall survival. Unfortunately, virtually all patients with advanced breast cancer and responsive tumors eventually experience a disease progression due to the development of hormone-independent metastases. Many mechanisms for antiestrogen resistance have been proposed including tumor heterogeneity and clonal selection of ER-negative tumor cells, functional alterations in the ER, changes in the metabolism of tamoxifen, and overexpression of various genes. Substantial evidence for any of these mechanisms in antiestrogen resistance in clinical breast cancer is still lacking.

The hypothesis, that antiestrogen resistance may be the result of (epi-) genetic alterations, resulting in changes in gene expression in the tumor cells, is the research subject of this thesis. The human ZR-75-1 breast cancer cell line was selected as a model system to study the progression to hormone independence. This cell line is fully dependent on estrogens for proliferation and growth can be inhibited with different antiestrogens. We have applied insertional mutagenesis with retroviruses in ZR-75-1 cells, to identify genes involved in development of tamoxifen resistance.

Retroviruses integrate into the DNA of the host cell as a part of their life cycle and can disturb the expression of neighboring genes. When this alteration in gene expression results in antiestrogen resistance, the integrated retrovirus can be used as a tag to isolate the involved gene. Subsequently, the candidate gene can be transfected to the parental cell line, to study its role in antiestrogen resistance *in vitro*. Next, the gene (product) should be studied in clinical breast cancer samples to establish the role in clinical hormone resistance. In this thesis, the application of retroviral insertional mutagenesis to isolate (unknown) genes involved in antiestrogen resistance is described. Several experiments were performed to evaluate the feasibility of this approach in the study of breast cancer.

Expression of ER is inversely related to expression of epidermal growth factor (EGF) receptor in breast cancer. Furthermore, the EGF receptor is implicated in poor prognosis and antiestrogen unresponsiveness of breast cancer. The EGF receptor cDNA was introduced into ZR-75-1 cells, to establish a possible role for expression of this receptor in development of antiestrogen resistance. These cells, designated ZR/HERc, showed expression of functional EGF receptor and a proliferative response to EGF even in the presence of antiestrogens. ZR/HERc cells rapidly progressed to an ER-negative phenotype in the presence of antiestrogens, suggesting a role for EGF receptor expression in progression of breast tumors to hormone independence. Interference between the ER and EGF receptor signaling cascades in these ZR/HERc cells was observed which may explain the inverse relationship of these receptors observed in breast tumors (Chapter 5.1). Furthermore, these experiments showed that alteration of the expression level of a single gene in these

cells is sufficient to bypass estrogen dependence and to allow for proliferation in th presence of antiestrogens (chapter 5.1). This notion is important, because it is prerequisite for successful retroviral insertional mutagenesis. A change of phenotyp requiring simultaneous modification of more than one gene in a single cell b retroviral insertional mutagenesis is technically not feasible.

In different experiments, it was shown that antiestrogen resistance could b induced by epigenetic changes in gene expression. ZR-75-1 cells were treated with 5 azacytidine, a DNA demethylating agent, which may result in up-regulation of gen expression. Cells were selected in the absence of estradiol or in the presence o different antiestrogens. Pools of these cells were shown to express EGF receptor an to secrete EGF-like peptides. Proliferation could be inhibited with EGF receptor blocking monoclonal antibodies, indicating the activation of an autocrine growt mechanism (Chapter 5.2). These results show that alterations in gene expression ar sufficient to induce resistance in this cell line, but this approach does not easily permi the identification of the involved genes.

As a consequence of the former studies, a double-immunohistochemical staining method was developed to study the expression of ER and EGF receptor at the singl cell level. From data of clinical studies and cell line studies, it was apparent tha expression of ER and EGF is inversely related. However, approximately 50% o primary breast tumors show co-expression of these receptors. It was not known whether ER and EGF receptor are expressed in the same individual cell or that this i mutually exclusive in a single breast (tumor) cell, because most analyses have been performed on homogenates of tumor cells. Analysis of primary breast tumor biopsies showed that expression of both receptors in the same tumor cell is a rare phenomenon. However, in non-malignant luminal epithelial cells of the breast, it was shown that simultaneous expression of ER and EGF receptor in individual cells does occur (Chapter 5.3). In Chapter 5.4 it is described that there is little difference in expression of ER and EGF receptor between primary breast tumor cells and corresponding lymph node metastases.

The results of the EGF receptor transfections and the 5-azacytidine experiments, in Chapters 5.1 and 5.2, supported the feasibility of the retroviral insertional mutagenesis approach. Large numbers of hormone-dependent breast cancer cells were infected with retroviruses and selected for proliferation in the presence of tamoxifen to establish a panel of resistant cell lines. In total, a panel of 80 antiestrogen resistant cell lines was generated. Southern blots, containing DNA of these cell clones, were screened with integration site-specific probes to identify independent cell clones with a viral integration in an identical locus. In Chapter 5.5, the first common integration site linked with antiestrogen resistance is described. In four independent cell clones, a viral integration in a locus termed breast cancer antiestrogen resistance 1 (BCAR1) was detected.

In Chapter 5.6, we describe the identification and partial characterization of the BCAR3 gene. BCAR3 is the third locus, involved in antiestrogen resistance, identified with retroviral insertional mutagenesis so far. Six independent cell clones of the panel of resistant cell clones contained an integration in the BCAR3 locus in a narrow region. Linkage of this locus with antiestrogen resistance was further documented by somatic cell hybrid experiments. Cell fusion experiments were performed with the parental ZR-75-1 cells and one of the BCAR3 clones. Somatic cell hybrids containing

the BCAR3 locus were antiestrogen resistant, in contrast to cell hybrids lacking this locus. Hybridization of Northern blots with an integration site-specific probe detected a transcript that is up-regulated in the BCAR3 clones in comparison with the parental cell line, which shows very low expression. BCAR3 cDNA was isolated from a testis cDNA library. Transfection experiments with a BCAR3 expression construct in the parental ZR-75-1 and MCF-7 cells (another estrogen-dependent breast cancer cell line) confirmed that expression of BCAR3 induces antiestrogen resistance. Characterization of the gene by sequence analysis showed little sequence homology with other known genes. Only similarity of a part of the BCAR3 protein with the SH2 domain of the adapter proteins SHC and FER was observed. In addition, some protein sequence homology was detected of a part of the protein with the yeast CDC48 gene product, which has a role in cell division and apoptosis. The presence of an SH2 domain in BCAR3 suggests a role in signal transduction.

Future experiments will be aimed at establishing the role of the different BCAR genes and the involved signaling cascades in clinical breast cancer. Most likely many, different genes are responsible for development of hormone resistance. Therefore, it is important to address the question which signaling cascades are utilized by the tumor cells progressing to a more malignant phenotype. The growing understanding of breast cancer biology will hopefully result in the development of more satisfactory treatment strategies based on biological principles.

Samenvatting

Borstkanker is de meest voorkomende maligniteit bij vrouwen in de westerse wereld en één van de belangrijkste doodsoorzaken op dit moment. In een vroeg stadium van de ziekte is de groei van borstkankercellen, naar aangenomen wordt, afhankelijk van oestrogenen. De effecten van oestrogenen worden doorgegeven via de oestrogeenreceptor, die geblokkeerd kan worden met specifieke antagonisten (anti-oestrogenen). Behandeling van patiënten met primaire borstkanker na operatie met anti-oestrogenen, zoals tamoxifen, verlengt zowel de ziektevrije periode als de totale overleving van de patiënten. Helaas blijkt bij bijna alle responderende patiënten met borstkanker in een gevorderd stadium na verloop van tijd de ziekteverschijnselen terug te keren door het ontstaan van hormoononafhankelijke metastasen. Vele mogelijke mechanismen voor resistentie tegen anti-oestrogenen zijn gepostuleerd, zoals: heterogeniteit in de tumoren en klonale uitgroei van oestrogeenreceptor-negatieve cellen; veranderingen in het functioneren van de oestrogeenreceptor; veranderingen in het metabolisme van tamoxifen en overexpressie van een verscheidenheid aan genen. Echter overtuigende bewijzen voor de verschillende gepostuleerde mechanismen van resistentie zijn nog niet geleverd.

De hypothese dat resistentie tegen tamoxifen veroorzaakt kan worden door (epi-) genetische veranderingen in de tumorcellen, resulterend in een veranderd genexpressie patroon, bepaalt het onderzoekprogramma van ons laboratorium. ZR-75-1 is een humane borstkankercellijn die is gekozen als modelsysteem voor het bestuderen van de progressie naar hormoononafhankelijkheid. Deze cellijn is voor groei volledig afhankelijk van oestrogeen. Celproliferatie kan worden geblokkeerd met verschillende soorten anti-oestrogenen. We hebben insertiemutagenese met retrovirussen toegepast op deze ZR-75-1 cellen, om genen te identificeren, die betrokken zijn bij anti-oestrogeen resistentie.

Retrovirussen integreren in het DNA van hun gastheercel als onderdeel van hun levenscyclus. Deze inserties kunnen leiden tot verandering in expressie van nabijgelegen genen, en zo (epi-) genetische effecten nabootsen. Als de integratie resulteert in resistentie tegen anti-oestrogenen, dan kan het retrovirus gebruikt worden als een label voor de isolatie van het betrokken gen. Vervolgens kan het kandidaatgen getransfecteerd worden naar de oorspronkelijke cellijn, om diens rol in de resistentie tegen anti-oestrogeen *in vitro* te bevestigen. Vervolgens moet de expressie van het gen(product) in borstkanker biopten worden geanalyseerd om de rol bij hormoonresistentie in de kliniek te kunnen bevestigen. In dit proefschrift wordt de toepassing van retrovirale insertiemutagenese beschreven, met als doel (nieuwe) genen te isoleren die betrokken zijn bij resistentie tegen anti-oestrogenen. Verschillende experimenten zijn gedaan om de haalbaarheid van deze nieuwe aanpak te onderzoeken.

De expressie van de oestrogeenreceptor is omgekeerd evenredig met de expressie van de epidermal growth factor (EGF) receptor in borstkanker. Expressie van de EGF-receptor lijkt gekoppeld te zijn aan een slechte prognose en het niet reageren op anti-oestrogenen bij de behandeling van borstkanker. Het EGF-receptor cDNA werd

in ZR-75-1 cellen ingebracht met als doel een mogelijke rol voor deze receptor vast te stellen bij de ontwikkeling van resistentie tegen anti-oestrogenen. Deze getransfecteerde cellen (ZR/HERc) brengen functionele EGF-receptor tot expressie en vertonen een groeirespons op de groeifactor EGF, ook in de aanwezigheid van anti-oestrogenen. ZR/HERc cellen veranderden snel in een oestrogeenreceptor-negatief fenotype in de aanwezigheid van tamoxifen, wat een rol suggereert voor EGF-receptor expressie bij de progressie van borstkanker naar hormoononafhankelijkheid. Er werd interferentie tussen de signaaltransductiepaden van de oestrogeenreceptor en de EGF-receptor waargenomen, die mogelijk de inverse relatie tussen deze receptoren in borstkankercellen kan verklaren (Hoofdstuk 5.1). Verder lieten deze resultaten zien dat verandering in het expressieniveau van slechts één gen voldoende is om oestrogeenafhankelijkheid te omzeilen en proliferatie in de aanwezigheid van anti-oestrogenen mogelijk te maken (Hoofdstuk 5.1). Dit gegeven is belangrijk want het is een vereiste voor het slagen van retrovirale insertiemutagenese. Een fenotypische verandering die de gelijktijdige aanpassing vereist in meer dan één gen in dezelfde cel, is technisch niet haalbaar met retrovirale insertie mutagenese.

Verder hebben we laten zien dat het mogelijk is anti-oestrogeen resistentie te induceren door epigenetische veranderingen in genexpressie teweeg te brengen. ZR-75-1 cellen werden behandeld met 5-azacytidine, een stof die demethylering van DNA tot stand brengt, wat kan leiden tot veranderingen, vooral tot verhoging van genexpressie. Cellen werden geselecteerd in de afwezigheid van oestradiol of in de aanwezigheid van verschillende anti-oestrogenen. Verzamelingen van celklonen vertoonden EGF-receptor expressie en scheidden EGF-achtige peptiden uit in het medium. Proliferatie van deze cellen kon worden geremd met EGF-receptor-blokkerende antilichamen, suggererend dat een autocrien groeimechanisme was geactiveerd (Hoofdstuk 5.2). Deze resultaten laten zien dat veranderingen in genexpressie voldoende zijn om resistentie in deze cellijn te induceren, maar met deze aanpak is het niet eenvoudig de betrokken genen te identificeren en te isoleren.

Op grond van de voorgaande studies hebben we een immunohistochemische dubbelkleuring ontwikkeld voor de gelijktijdige aankleuring van de oestrogeenreceptor en EGF-receptor, omdat immunohistochemie het mogelijk maakt de expressie van individuele cellen te bestuderen. Uit gegevens van klinische studies en studies met cellijnen was het duidelijk dat de expressie van oestrogeenreceptor en EGF-receptor omgekeerd evenredig is, maar toch leek ongeveer de helft van de primaire tumoren deze receptoren gelijktijdig tot expressie te brengen. Het was niet bekend of deze gelijktijdige expressie plaatsvond in dezelfde cel of in verschillende cellen, omdat de meeste analyses op tumorhomogenaten waren gedaan. Analyse van biopten van primaire tumoren liet zien dat gelijktijdige expressie van deze twee receptoren in één tumorcel slechts zelden voorkomt (Hoofdstuk 5.3). Echter in niet-maligne cellen van het klierepitheel in de borst komt gelijktijdige expressie wel voor. In Hoofdstuk 5.4 wordt beschreven dat er weinig verschil is in de expressie niveaus van oestrogeenreceptor en EGF-receptor in primaire tumoren en hun lymfkliermetastasen.

De resultaten van de EGF-receptor transfecties en de 5-azacytidine experimenten, beschreven in Hoofdstukken 5.1 en 5.2, steunden de haalbaarheid van de experimenten met behulp van retrovirale insertiemutagenese. Grote aantallen hormoonafhankelijke borstkankercellen werden geïnfecteerd met retrovirussen en

geselecteerd op groei in aanwezigheid van tamoxifen, met als doel een panel van resistente klonen te maken. In totaal is een panel van 80 resistente klonen gegenereerd, die elk zijn opgegroeid tot cellijnen. Southern blots met daarop DNA van deze klonen zijn gescreend met integratieplaats specifieke probes om onafhankelijke klonen te identificeren met een integratie in dezelfde locus. In Hoofdstuk 5.5 is de eerste gemeenschappelijke integratie plaats beschreven, die gekoppeld is aan resistentie tegen anti-oestrogenen. In vier onafhankelijke klonen werd een integratie in dezelfde locus gevonden. Dit locus is breast cancer antiestrogen resistance 1 (BCAR1) genoemd.

In Hoofdstuk 5.6 beschrijven we de identificatie en gedeeltelijke karakterisering van het BCAR3 gen. BCAR3 is het derde locus, dat geïdentificeerd werd door middel van retrovirale insertiemutagenese, dat betrokken is bij resistentie tegen anti-oestrogenen. Zes onafhankelijke klonen uit het panel resistente klonen bleken een integratie in een klein gebied van dit locus te bevatten. Het verband tussen dit locus en resistentie tegen anti-oestrogenen werd verder aangetoond door middel van somatische celhybriden. Celfusie experimenten werden uitgevoerd met de oudercellijn ZR-75-1 en één van de BCAR3 klonen. Hybriden die het BCAR3 integratielocus bevatten waren anti-oestrogeen resistent in tegenstelling tot de hybriden die het locus niet bevatten. Hybridisatie van Northern blots met een integratiespecifieke probe detecteerde een transcript dat verhoogd tot expressie komt in de BCAR3 klonen in vergelijking tot de oudercellijn, die slechts zeer lage expressie vertoonde. Het BCAR3 cDNA werd geïsoleerd uit een testis cDNA bank. Transfectie experimenten met een BCAR3 expressie construct in de ZR-75-1 en MCF-7, een tweede oestrogeenafhankelijke borstkanker cellijn, bevestigde dat BCAR3 resistentie tegen anti-oestrogenen induceert. Karakterisering van het gen door middel van sequentieanalyse liet weinig homologie zien met andere bekende genen. Er werd alleen een sterke overeenkomst gevonden met een gedeelte van het eiwit en het SH2 domein van de adaptergenen SHC en FER. Tevens werd er enige homologie gevonden tussen een gedeelte van het cDNA en het gistgen CDC48, dat een rol speelt bij celdeling en apoptose. De aanwezigheid van een SH2 domein duidt erop dat BCAR3 mogelijk een rol heeft in signaaltransductie.

Toekomstige experimenten zullen erop gericht zijn de rol van de verschillende BCAR genen en de betrokken signaalcascaden te bepalen bij de progressie van borstkanker. Het is zeer waarschijnlijk dat er een groot aantal genen verantwoordelijk kunnen zijn voor hormoon resistentie. Daarom is het belangrijk te onderzoeken welke signaaltransductiepaden de tumorcellen kunnen gebruiken tijdens hun progressie naar een meer kwaadaardig fenotype. De groeiende hoeveelheid kennis van de biologie van borstkanker zal dan hopelijk leiden tot de ontwikkeling van betere behandelingsmethoden, die gebaseerd zijn op biologische principes.

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Ton

CURRICULUM VITAE

Ton van Agthoven werd op 16 februari 1956 geboren te Rotterdam. Na vier jaar atheneum koos de auteur van dit proefschrift voor een meer gerichte opleiding. Hij behaalde het diploma analist in de biologische richting aan het Antoni van Leeuwenhoek Instituut te Delft in 1976. Na een onderbreking om het vaderland te dienen, werkte Ton op verschillende laboratoria. De eerste negen jaar als analist op de Afdeling Celbiologie en Genetica, Erasmus Universiteit Rotterdam bij professor D. Bootsma. In het laboratorium van Anne Hagemeijer leerde hij de fijne kneepjes van de cytogenetica en Ad Geurts van Kessel wist zijn enorme enthousiasme voor de celbiologie over te brengen. Vervolgens brachten Gerard Grosveld en Annelies de Klein hem een "no nonsens" aanpak voor de moleculaire biologie bij. Met deze vergaarde kennis werkte Ton twee en een half jaar in het Celkweeklaboratorium van Bob Löwenberg in de Daniel den Hoed kliniek te Rotterdam. Hier ontstond de drang om zelf meer inhoud aan het kankeronderzoek te geven en de behaalde resultaten te verwerken tot publicaties. Deze kans kreeg hij bij Lambert Dorssers op de Afdeling Moleculaire Biologie van de Daniel den Hoed kliniek. Na eerst enkele jaren als analist te werken kreeg hij een aanstelling als wetenschappelijk medewerker, wat het schrijven van dit proefschrift mogelijk maakte.

Bibliografie

1. Van Agthoven T, Van Agthoven TLA, Dekker A, Van der Spek PJ, Vreede L, Dorssers LCJ. Identification of BCAR3 by a random search for genes involved in antiestrogen resistance of human breast cancer cells. *EMBO J* 1998; 17:2799-2808.
2. Dorssers LCJ, Van Agthoven T. Genetic mechanisms of estrogen-indenpendence in breast cancer. *Path Res Pract* 1996; 192:743-751.
3. Dorssers LCJ, Van Agthoven T, Brinkman A, Veldscholte J, Van der Flier S. Genetische aspekten van tamoxifen resistentie van het mammacarcinoom. *IKR-bulletin* 1996; 20:55-58.
4. Dorssers LCJ, Van Agthoven T, Brinkman A. Involvement of genetic alterations in breast tumour progression to hormone independence in vitro. *Endocrine-Related Cancer* 1995; 2:123-126.
5. Timmermans M, Van Agthoven T, Foekens JA, Henzen-Logmans SC, Dorssers LCJ. Simultane detectie van oestrogeenreceptoren en de receptoren voor epidermal growth factor in mammacarcinomen. *Histotechniek/Cyto-visie* 1995; 3:11-14.
6. Van Agthoven T, Timmermans M, Dorssers LCJ, Henzen-Logmans SC. Expression of estrogen, progesterone, and epidermal growth factor receptors in primary and metastatic breast cancer. *Int J Cancer* 1995; 63:790-793.
7. Sharma AK, Horgan K, McClelland RA, Douglas-Jones AG, Van Agthoven T, Dorssers LCJ, Nicholson RI. A dual immunocytochemical assay for oestrogen and epidermal growth factor receptors in tumour cell lines. *Histochem J* 1994; 26:306-310.
8. Van Agthoven T, Timmermans M, Foekens JA, Dorssers LCJ, Henzen-Logmans SC. Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry. *Am J Pathol* 1994; 144:1238-1246.
9. Van Agthoven T, Van Agthoven TLA, Dekker A, Foekens JA, Dorssers LCJ. Induction of estrogen independence of ZR-75-1 human breast cancer cells by epigenetic alterations. *Mol Endocrinol* 1994; 8:1474-1483.
10. Van Agthoven T, Dorssers LCJ. Relatie tussen oestrogeenreceptor en receptor voor epidermal growth factor in ontwikkeling van borstkanker. *Tijdschrift Kanker* 1994; 18 no 4:24-27.
11. Dorssers LCJ, Van Agthoven T, Dekker A, Van Agthoven TLA, Kok EM. Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: Identification of bcar-1, a common integration site. *Mol Endocrinol* 1993; 7:870-878.
12. Van Agthoven T, Van Agthoven TLA, Foekens JA, Dorssers LCJ. Regulation of gene expression involved in the progression of human breast cancer to hormone independence. *Ann NY Acad Sci* 1993; 684:250-252.
13. Touw IP, Van Gurp R, Schipper P, Van Agthoven T, Lowenberg B. Introduction of the human interleukin-6 (IL-6) receptor in murine IL-3-dependent hematopoietic cells restores responsiveness to IL-6. *Blood* 1992; 79:2867-2872.
14. Van Agthoven T, Van Agthoven TLA, Portengen H, Foekens JA, Dorssers LCJ. Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res* 1992; 52:5082-5088.
15. Delwel R, Schipper P, Van Buitenen C, Van Agthoven T, Touw I, Lowenberg B. Comparative analysis of Il-1 regulated and sponteneous growth of acute myeloid leukemia in vitro. *Bone Marrow Transplant* 1990;
16. Touw IP, Pouwels K, Van Agthoven T, Van Gurp R, Budel L, Hoogerbrugge H, Delwel R, Goodwin R, Namen A, Lowenberg B. Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. *Blood* 1990; 75:2097-2104.
17. Touw IP, Pouwels K, Van Agthoven AJ, Van Gurp R, Delwel R, Hagemeijer A, Hahlen K, Lowenberg B. Role of interleukin-7 in the growth of acute lymphoblastic leukemia. *Bone Marrow Transplant* 1990;
18. Von Lindern M, Van Agthoven T, Hagemeijer A, Adriaansen H, Grosveld G. The human pim-1 gene is not directly activated by the translocation (6;9) in acute nonlymphocytic leukemia. *Oncogene* 1989; 4:75-79.

19. De Klein A, Van Agthoven T, Groffen C, Heisterkamp N, Groffen J, Grosveld G. Molecular analysis of both translocation products of a Philadelphia-positive CML patient. *Nucleic Acids Res* 1986; 14:7071-7082.
20. Grosveld G, Verwoerd T, Van Agthoven T, De Klein A, Ramachandran KL, Heisterkamp N, Stam K, Groffen J. The chronic myelocytic cell line K562 contains a breakpoint in bcr and produces a chimeric bcr/c-abl transcript. *Mol Cell Biol* 1986; 6:607-616.
21. Hagemeijer A, De Klein A, Goedde-Salz E, Turc-Carel C, Smit EME, Van Agthoven AJ, Grosveld G. Translocation of c-abl to "masked" Ph in chronic myeloid leukemia. *Cancer Genet Cytogen* 1985; 18:95-104.
22. Van Rijs J, Giguere V, Hurst J, Van Agthoven T, Geurts van Kessel AHM, Goyert S, Grosveld F. Chromosomal localization of the human Thy-1 gene. *Proc Natl Acad Sci USA* 1985; 82:5832-5835.
23. Geurts van Kessel AHM, Tetteroo P, Van Agthoven T, Paulussen R, van Dongen J, Hagemeijer A, von dem Borne A. Localization of human myeloid-associated surface antigen detected by a panel of 20 monoclonal antibodies to the q12-qter region of chromosome 11. *J Immunol* 1984; 133:1265-1269.
24. Hagemeijer A, Bartram CR, Smit EME, Van Agthoven AJ, Bootsma D. Is the chromosomal region 9q34 always involved in variants of the Ph' translocation? *Cancer Genet Cytogen* 1984; 13:1-16.
25. Bartram CR, De Klein A, Hagemeijer A, Van Agthoven T, Geurts van Kessel AHM, Bootsma D, Grosveld G, Ferguson-Smith MA, Davies T, Stone M, Heisterkamp N, Stephenson JR, Groffen J. Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* 1983; 306:277-280.
26. Geurts van Kessel AHM, Stoker K, Claas FHJ, Van Agthoven AJ, Hagemeijer A. Assignment of the leucocyte group five surface antigens to human chromosome 4. *Tissue Antigens* 1983; 21:213-218.
27. Van De Rijn M, Geurts van Kessel AHM, Kroezen V, Van Agthoven AJ, Verstijnen K, Terhorst C, Hilgers J. Localization of a gene controlling the expression of the human transferrin receptor to the region q12-qter of chromosome 3. *Cytogenetics and Cell Genetics* 1983; 36:525-531.
28. Geurts van Kessel AHM, Van Agthoven AJ, Hagemeijer A. Clonal origin of the philadelphia translocation in chronic myeloid leukemia demonstrated in somatic cell hybrids using an adenylate kinase-1 polymorphism. *Cancer Genet Cytogen* 1982; 6:55-58.
29. Geurts van Kessel AHM, Van Agthoven AJ, De Groot PG, Hagemeijer A. Characterization of a complex philadelphia translocation (1p-; 9q+; 22q-) by gene mapping. *Hum Genet* 1981; 58:162-165.
30. Geurts van Kessel AHM, Den Boer WC, Van Agthoven AJ, Hagemeijer A. Decreased tumorigenicity of rodent cells after fusion with leukocytes from normal and leukemic donors. *Somatic cell genetics* 1981; 6:645-656.