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 x 10^-3), in contrast to control cultures (<10^-4). 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 estrogendependent 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...
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). Gonzo 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, subjecting 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). ZH-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/ASA-TAM cells. Furthermore, single cell-derived subclones were generated using conditioned medium of murine C11R cells (38) and hereafter referred to as ZR/ASA 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/ASA-ICI cells). In estrogen-deprived medium, the number of estrogen-independent colonies (designated ZR/ASA-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 (≤10^-4) of spontaneous development of estrogen independence of these ZR-75-1 cells (38).

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/ASA cells had occurred and could explain antiestrogen resistance. RNase protection analyses using an antisense ER complementary DNA (cDNA) probe showed that ZR/ASA cells contained reduced ER mRNA levels in comparison with the wild type ZR-75-1 cells. In the single cell clone ZR/ASA 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/ASA 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/ASA-TAM, -ICI, and -DCC pools and ZR/ASA-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 TGFα 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).

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

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ER (fmol)</th>
<th>PR (fmol)</th>
<th>EGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR-75-1</td>
<td>115</td>
<td>625</td>
<td>ND</td>
</tr>
<tr>
<td>ZR/AZA-DCC</td>
<td>15</td>
<td>56</td>
<td>49</td>
</tr>
<tr>
<td>ZR/AZA-Tam</td>
<td>38</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>ZR/AZA-ICI</td>
<td>35</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>ZR/AZA-1</td>
<td>9</td>
<td>19</td>
<td>ND</td>
</tr>
<tr>
<td>ZR/AZA-2</td>
<td>2</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>ZR/AZA-3</td>
<td>-5</td>
<td>-1</td>
<td>28</td>
</tr>
<tr>
<td>ZR/AZA-4</td>
<td>45</td>
<td>13</td>
<td>27</td>
</tr>
</tbody>
</table>

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 (E2) for 4 days, to allow measurement of ER-induced PR expression (+E2). In addition, ER and PR of ZR/AZA-Tam, ICI, and clone 4 were determined in the absence of 17β-estradiol (−E2). Values are in femtomoles/mg protein. ND, No specific [125I]-EGF binding detected.

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 [125I]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.

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.
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 CUNA. 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, showing that 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 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 FGF 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 10⁴ 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 x 10⁴) 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 [3H]thymidine (3H-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 10⁴/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).
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 TGFβ has also been shown to be modulated by ER (54). Our results demonstrated moderately increased levels of TGFβ mRNA in estrogen-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 TGFβ 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 TGFβ, 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 TGFβ mRNA were detected in ZR-75-1 cells and ZR/AZA cells. TGFβ 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 LH 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,
cell lines (61). Association between vimentin expression documented in a subset of ER-negative breast cancer these signal pathways prohibiting rapid proliferation. ZR-75-1 cells was assessed by studying the interme-

tations, 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 anti-
estrogen 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 15% 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 (10⁶) 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 x 10⁶ cells per 75 cm² 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 fibroblasts 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 Nether-
lands) 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 bro-

mide (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 (30, 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 (H-Thy 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 10⁴ cells in RPMI medium containing 10% 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 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-32P (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 BamHI/EcoRI 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 preincubation in the absence of estradiol. EGF receptor expression was determined by Scatchard analyses of 125I EGF binding and using hydroxyapatite to separate bound and free ligand as previously described (39, 77).

**Immunofluorescence**

Immunological detection of keratin was performed on cytokeratin-18 stain and 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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