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Plenary Lecture

How Do Breast Cancers Become Hormone Resistant?

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We propose that the molecular heterogeneity of estrogen receptors (ER) in breast tumor cells characterized by the presence of mutant receptor forms, generates the cellular heterogeneity evident when progesterone receptor (PR) or DNA ploidy are analyzed in cell subpopulations. Furthermore, it is likely that cellular heterogeneity leads to the lack of uniformity in response to tamoxifen that we have described. We find that heterogeneity of PR distribution and DNA ploidy reflects the existence of mixed subpopulations of breast cancer cells that are substantially remodeled under the influence of tamoxifen. It appears likely that rather than being "resistant", different subsets of cells can be inhibited or stimulated by tamoxifen and their suppression or outgrowth alters the phenotype of the tumor. PR heterogeneity in solid tumors of patients may predict for such a mixed, and potentially dangerous, response to antiestrogen treatment. Similarly, the molecular heterogeneity resulting from the presence of two normal PR isotypes can lead to inappropriate responses to progesterone antagonists in certain genes or cell types. These agonist-like responses are due to cooperative interactions between the receptors and other transcription factors. As we learn more about the heterogeneity of PR, ER and other proteins in tumors, we may be able to recognize such lethal cell subpopulations, or combinations of regulatory factors. Specifically, with respect to tamoxifen, our data suggest that its use as a chemopreventant in women at high risk of developing breast cancer [Kiang, *J. Natn. Cancer Inst.* 83, 1991, 462–463] should be viewed with caution, since in the presence of tamoxifen subpopulations of cells may arise that are stimulated, rather than inhibited, by the drug.

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INTRODUCTION

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

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ER

The molecular biology of ER has been extensively explored in recent years. Their cDNA was independently cloned and sequenced from MCF-7 breast cancer cells by Green *et al.* [1] and Greene *et al.* [2] and the ER gene was cloned and analyzed 2 years later [3]. The protein is comprised of 595 amino acids within which Kumar *et al.* [4] distinguished 6 functional domains identified by the letters A through F. The A/B domains contain regions that regulate the transcriptional function of the proteins. The C domain contains two DNA-binding zinc fingers and is the region of the protein that binds to the estrogen response element (ERE). Mutations in this portion of the protein change its affinity for DNA, resulting in suboptimal, or complete loss of DNA binding. The hormone-binding properties of the receptors map to region E by mutagenesis analysis. Since these two functions, DNA- and hormone-binding, are carried out by separate parts of the protein, they are to some extent independent. Thus, it is possible to have variant receptors that can bind to DNA with limited affinity without first binding hormone, and vice versa [4, 5]. Three additional specialized regions of steroid receptors have also been identified: a nuclear localization signal, a heat shock protein (hsp 90)-binding region, and a dimerization domain. The nuclear localization signal, located downstream of the DNA-binding domain, is a region of the protein that must be present for the receptor to remain within the nucleus in the absence of ligand [6]. It has been identified in PR and is presumed to be similar in ER. The hsp 90 appears to bind to regions in the hormone-binding domain of some steroid receptors when ligand is absent, and its binding is believed to prevent receptor dimerization and DNA-binding [7]. Ligand activation leads to hsp 90 dissociation, and monomer dimerization in solution [5]. The dimerization domain that mediates this interaction between two ER molecules has been localized to the carboxy-terminal end of the hormone-binding domain [8]. A weak dimerization domain may also be present in the second zinc finger of the DNA-binding domain [5]. Additional sites for heterologous protein–protein interactions may also be located in the hormone binding domain [9] and covalent modifications by phosphorylation [10] further enhance the complexity of these protein molecules.

MOLECULAR HETEROGENEITY: ER

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

Mutant ER in solid tumors

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

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

The weakness in all these analyses is the assumption that message variants reflect protein variants. While this may indeed be the case, until recently, given the

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

Mutant ER in breast cancer cell lines

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

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

We subsequently found that a subline, which we called $T47D_{co}$, did have ER, but they were in a permanently activated state in the nucleus. The ER were not sensitive to the action of estrogens, suggesting that the estrogen regulatory mechanism was defective at a step beyond the initial interaction of the steroid–receptor complex with DNA. The PR levels were also insensitive to estradiol or to antiestrogens but were synthesized in extraordinary amounts and were functional. Additional studies suggested that the PRs retained characteristics of inducible proteins. Thus, we suggested that persistent nuclear ER were constitutively stimulating PR, even in the absence of exogenous estradiol [29, 30]. Recently, the tools became available to test this conjecture. Two cDNA libraries were constructed from $T47TD_{co}$ cells, that yielded clones consistent with wild-type ER, plus several mutant ER cDNA clones [31]. One cDNA would encode a putative mutant protein lacking the nuclear localization signal and hormone-binding domains of ER. Another ER cDNA clone appears to be an RNA-processing intermediate or splicing error and contains ~1 kb of intron 5 linked upstream of exon 6. Three clones were found with insertions in exon 5. The inserts contain at least two blocks of direct repeats of ~130 nucleotides terminating in A residues that are 70–85% homologous to the human *alu* family. One clone has a point deletion in the hormone-binding domain just upstream of the end of exon 5. This leads to a frame-shift and a translation termination 7 codons later. This mutant cDNA would encode an ER truncated in the middle of the hormone-binding domain at aa 417, with a unique 7 aa COOH-terminal end. Such a protein could be constitutively active. Two independent clones were isolated having an identical in-frame deletion. These cDNAs would encode a mutant ER of 442 aa instead of the normal 595 aa, having a 153 aa deletion from the end of the DNA-binding domain C, through the hinge region D, to the mid-hormone-binding domain E. The deletion originates in the sequence encoding the putative nuclear localization signal (aa 256–263; R-K-D-R-R-G-G-R). However, the aa sequence encoded by the deletion mutant (R-K-D-R-N-Q-G-K) preserves 4 of the 5 basic aa residues of the wild-type sequence.

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

CONSEQUENCES OF MUTANT ER: CELLULAR HETEROGENEITY?

The consequences of this molecular diversity in ER, may reach beyond issues of hormone-dependence, to

the broader problems of tumor progression and cellular heterogeneity that also characterize advanced breast cancer. Cellular heterogeneity has usually been assumed to exist within tumors, but has been difficult to demonstrate. The concept is, however, important, since it means that in practice, the clinician must treat not just one tumor, but a variety of possibly heterogenous, subtumors. Is it possible that heterogeneity of ER among cells can lead to heterogeneity of cells among tumors? While the analyses of ER described above have led to the discovery of variant receptor forms, the methods cannot answer a fundamental question. Do all, or only some of the cells carry the variants? Moreover, wild-type ER are always present together with the variants. Are wild-type ER present alone in some cells of the tumor or are they always coexpressed with the variants in any one cell? We postulated that the genetic diversity of ER would be reflected in heterogeneity of other molecular markers and set out to develop an assay that could simultaneously measure DNA content and PR heterogeneity in subpopulations of tumor cells [32, 34]. We have used this immunologic, dual-parameter flow cytometry (FCM)-based assay to demonstrate and quantitate a remarkable heterogeneity in PR content, DNA ploidy, and mitotic indices among subpopulations of breast cancer cells [35].

Heterogeneity of PR distribution

The heterogeneity of PR distribution is illustrated by three cell lines derived from $T47D_{co}$ in which only one, called $T47D_v$, has the PR phenotype that most current receptor measurement methods assume, namely that all cells are PR-positive at a level greater than measured background. However, even these cells have PR levels that range by more than 20-fold. Two other cell lines (V22 and V26) have more than one PR-positive population despite the fact that they were derived as single cell clones from $T47D_v$.

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

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

Tumor cell "remodeling" by tamoxifen

The practical consequence of this PR heterogeneity in breast cancer cells is illustrated by an experiment in which the $T47D_v$ cell line was treated for 8 weeks with or without 1 μM tamoxifen. Tamoxifen at 1 μM generally suppresses growth and PR in estrogen target tissues [26] that carry a normal ER, and is the major endocrine therapeutic drug used in breast cancer [36]. But, what is the effect of tamoxifen in cells that carry not only normal but also variant ER? Cell growth was suppressed by 40% (not shown), and there was a marked shift in the PR pattern—mostly to the left, reflecting a complete loss or decrease in PR. However, there was an unexpected small subpopulation shifted to the right, in which PR levels have apparently been induced by tamoxifen. This subpopulation represents 5.2% of the cells in this experiment, and contains an average PR of 571.6 fluorescence intensity units (FIU), or greater than one million PR molecules/cell—levels that none of the untreated cells attain. Thus, tamoxifen, while decreasing PR levels in a majority of cells, appears paradoxically to *increase* PR levels in a selected subset of cells. The ominous consequence of tumor cell populations that may be stimulated by tamoxifen requires little comment.

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

A variety of mechanisms have been proposed for development of the acquired resistance to tamoxifen

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

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

Our data suggest that subsets of cells may actually be stimulated by tamoxifen. Little is known about the mechanisms underlying these "agonist" actions of some antiestrogens (but see progestin resistance, discussed below). It is possible that binding of tamoxifen to specific types of ER mutants, establishes a transcriptionally productive receptor complex. The agonist activities of tamoxifen are usually expressed at low doses [26], but they may also be tissue-specific [45]. While tamoxifen at high doses suppresses PR, it induces PR at low doses [26]. The tumor "flare" that occurs during initiation of tamoxifen therapy in patients [46] and the withdrawal response that occurs when the drug is stopped after tumors become resistant [47] may also be explained by this property. Additionally, we have previously shown that pretreatment of cells with an antiestrogen can sensitize them to a subsequent challenge with estrogens. In this state, cells respond more rapidly and more extensively to estrogens; for example, superinduction of PRs is observed [48]. It is possible that antiestrogen pretreatment can sensitize tumor cells to low levels of estrogens, or to weak estrogens, to which, in other settings, they would be unresponsive. The molecular mechanisms underlying the phenomenon of superinduction remain unknown.

PROGESTIN RESISTANCE

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

Progestin resistance and the two natural PR isoforms

Complementary DNAs for chicken PR were cloned by Jeltsch *et al.* [50] and Conneely *et al.* [51] and for human PR by Misrahi *et al.* [52]. The single-copy human PR gene encodes at least 9 messenger RNA species ranging in size from 2.5–11.4 kb. The 9 messages direct the synthesis of at least 2 and possibly 3, structurally related receptor proteins. The 2 major protein species, the B- and A-receptors, were originally described in the chick oviduct. Subsequent studies using breast cancer cells showed that human PR also exist as 2 isoforms; the 116 kilodalton (kDa) B-receptors and N-terminally truncated 94 kDa A-receptors. While A-receptors were originally thought to be produced by a proteolytic artifact, it is now clear that these amino-terminal truncated receptors, at least in chickens and humans, are a naturally synthesized form. In human endometrial carcinoma and breast cancer cell lines, the 2 receptor isoforms are expressed in approximately equimolar amounts. It is not known whether this quantitative relationship between the 2 isoforms is maintained in all human target tissues and tumors, and the mechanisms for their differential regulation are not known, but at least 2 of the 9 mRNA species lack the translation initiation site for B-receptors and can therefore encode only A-receptors. These messages arise by transcription from an internal promoter in the human PR gene. Five other message species can potentially encode both receptor isoforms, by alternate translation initiation from 2 in-frame AUG codons. In theory, use of the upstream codon would generate the B-receptors and use of the downstream codon generates the A-receptors, but it is not known whether initiation at the downstream site actually occurs in intact cells (reviewed in [53] and references therein).

PR are unique among steroid receptors in having 2 naturally occurring hormone-binding forms, and this structural feature may have important functional implications with respect to receptor function. Since both homo- and heterodimers can form between the A- and B-isoforms, 3 possible classes of receptor dimers (A:A, A:B, B:B) can bind DNA at a progesterone response element, each having a potentially different transcription regulatory capacity.

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

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

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

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

Each of these recent experimental models suggests that "resistance" can be a condition in which tumors respond inappropriately to hormone antagonists. We have observed this with tamoxifen and with progesterone antagonists. These studies may also explain why, in some normal target cells, antagonists have tissue-specific, agonist-like activity. We propose that these agonist-like effects occur on genes that are regulated by at least two signalling pathways. A steroid receptor antagonist complex, which is inhibitory by itself, may in the presence of other DNA-bound factors, be switched to stimulatory activity through cooperative protein–protein interactions.

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REFERENCES

1. Green S., Walter P., Kumar V., Drust A., Barnert J. M., Argos P. and Chambon P.: Human oestrogen receptor cDNA:

Sequence, expression and homology to v-*erb*-A. *Nature* **320** (1986) 134–139.
2. Greene G. L., Gilna P., Waterfield M., Baker A., Hort Y. and Shine J.: Sequence and expression of human estrogen receptor complementary DNA. *Science* **231** (1986) 1150–1154.
3. Ponglikitmongkol M., Green S. and Chambon P.: Genomic organization of the human oestrogen receptor gene. *EMBO Jl* **7** (1988) 3385–3388.
4. Kumar V., Green S., Staub A. and Chambon P.: Localisation of the oestradiol-binding and putative DNA-binding domains of the human oestrogen receptor. *EMBO Jl* **5** (1986) 2231–2236.
5. Kumar V. and Chambon P.: The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55** (1988) 145–156.
6. Guiochon-Mantel A., Loosfelt H., Ragot T., Bailly A., Atger M., Misrahi M., Perricandet M. and Milgrom E.: Receptors bound to antiprogestins form abortive complexes with hormone responsive elements. *Nature* **336** (1988) 695–698.
7. Baulieu E.-E.: Steroid hormone antagonists at the receptor level: A role for the heat-shock protein MW 90,000 (hsp 90). *J. Cell Biochem.* **35** (1987) 161–174.
8. Fawell S. E., Lees J. A., White R. and Parker M. G.: Characterization and colonization of steroid binding and dimerization activities in the mouse estrogen receptor. *Cell* **60** (1990) 953–962.
9. Adler S., Waterman M. L., He X. and Rosenfeld M. G.: Steroid-receptor mediated inhibition of rat prolactin gene expression does not require the receptor DNA-binding domain. *Cell* **52** (1988) 685–695.
10. Washburn T., Hocutt A., Brautigan D. L. and Korach K. A.: Uterine estrogen receptor *in vivo*: Phosphorylation of nuclear-specific forms on serine residues. *Molec. Endocr.* **5** (1991) 235–242.
11. Murphy L. C.: Estrogen receptor variants in human breast cancer. *Molec. Cell. Endocr.* **74** (1990) C83–C86.
12. Hill S. M., Fuqua S. A. W., Chamness G. C., Greene G. L. and McGuire W. L.: Estrogen receptor expression in human breast cancer associated with an estrogen receptor gene restriction fragment length polymorphism. *Cancer Res.* **49** (1989) 145–148.
13. Castagnoli A., Maestri I., Bernardi F. and Del Senno L.: PvuII RFLP inside the human estrogen receptor gene. *Nucleic Acids Res.* **15** (1987) 866.
14. Parl F. F., Cavener D. R. and Dupont W. D.: Genomic DNA analysis of the estrogen receptor gene in breast cancer. *Br. Cancer Res. Treat.* **14** (1989) 57–64.
15. Keaveney M., Klug J., Dawson M. T., Nestor P. V., Neilan J. G., Forde R. C. and Gannon F.: Evidence for a previously unidentified upstream exon in the human oestrogen receptor gene. *J. Molec. Endocr.* **6** (1991) 111–115.
16. Shupnik M. A., Gordon M. S. and Chin W. W.: Tissue-specific regulation of rat estrogen receptor mRNAs. *Molec. Endocr.* **3** (1989) 660–665.
17. Garcia T., Lehrer S., Bloomer W. and Schachter B.: A variant estrogen receptor messenger ribonucleic acid is associated with reduced levels of estrogen binding in human mammary tumors. *Molec. Endocr.* **2** (1988) 785–791.
18. Garcia T., Sanchez M., Cox J. L., Shaw P. A., Ross J. B., Lehrer S. and Schachter B.: Identification of a variant form of the human estrogen receptor with an amino acid replacement. *Nucleic Acids Res.* **17** (1989) 8364.
19. Lehrer S., Sanchez M., Song H. K., Dalton J., Levine E., Savoretti P., Thung S. N. and Schachter B.: Oestrogen receptor B-region polymorphism and spontaneous abortion in women with breast cancer. *Lancet* **335** (1990) 622–624.
20. Dotzlaw H., Alkhalaf M. and Murphy L. C.: Characterization of estrogen receptor variant mRNAs from human breast cancers. *Molec. Endocr.* **6** (1992) 773–785.
21. Fuqua S. A. W., Fitzgerald S. D., Chamness G. C., Tondon A. K., McDonnell D. P., Nawaz Z., O'Malley B. W. and McGuire W. L.: Variant human breast tumor estrogen receptor with constitutive transcriptional activity. *Cancer Res.* **51** (1991) 105–109.
22. Hirose T., Koga M., Matsumoto K. and Sato B.: A single nucleotide substitution in the D domain of estrogen receptor cDNA causes amino acid alteration from Glu-279 to Lys-279 in a murine transformed Leydig cell line (B-1 F). *J. Steroid Biochem. Molec. Biol.* **39** (1991) 1–4.
23. Foster B. D., Cavener D. R. and Parl F. F.: Binding analysis of the estrogen receptor to its specific DNA target site in human breast cancer. *Cancer Res.* **51** (1991) 3405–3410.
24. Scott G. K., Kushner P., Vigne J.-L. and Benz C. C.: Truncated forms of DNA-binding estrogen receptors in breast cancer. *J. Clin. Invest.* **88** (1991) 700–706.
25. Horwitz K. B., McGuire W. L., Pearson O. H. and Segaloff A.: Predicting response to endocrine therapy in human breast cancer: A hypothesis. *Science* **189** (1975) 726–727.
26. 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** (1978) 2223–2228.
27. Horwitz K. B., Zava D. T., Thiligar A. K., Jensen E. M. and McGuire W. L.: Steroid receptor analyses of nine human breast cancer cell lines. *Cancer Res.* **38** (1978) 2434–2437.
28. Keydar I., Chen L., Karbey S., Weiss F. R., Delarea J., Radu M., Chaitcik S. and Brenner M. J.: Establishment and characterization of a cell line of human breast carcinoma origin. *Eur. J. Cancer* **15** (1979) 659–670.
29. Horwitz K. B.: Is a functional estrogen receptor always required for progesterone receptor induction in breast cancer? *J. Steroid Biochem.* **15** (1981) 209–217.
30. Horwitz K. B., Mockus M. B. and Lessey B. A.: Variant T47D human breast cancer cells with high progesterone receptor levels despite estrogen and antiestrogen resistance. *Cell* **28** (1982) 633–642.
31. Graham M. L., II, Krett N. L., Miller L. A., Leslie K. K., Gordon D. F., Wood W. M., Wei L. L. and Horwitz K. B.: $T47D_{co}$ cells, genetically unstable and containing estrogen receptor mutations, are a model for the progression of breast cancers to hormone resistance. *Cancer Res.* **50** (1990) 6208–6217.
32. Graham M. L., II, Dalquist K. E. and Horwitz K. B.: Simultaneous measurement of progesterone receptors and DNA indices by flow cytometry. Analyses of breast cancer cell mixtures and genetic instability of the T47D line. *Cancer Res.* **49** (1989) 3943–3949.
33. Forman B. M., Yang C., Au M., Casanova J., Ghysdael J. and Samuels H. H.: A domain containing leucine zipper-like motifs mediate novel *in vivo* interactions between the thyroid hormone and retinoic acid receptors. *Molec. Endocr.* **3** (1989) 1610–1626.
34. Graham M. L., II, Bunn P. A. Jr, Jewett P. B., Gonzalez-Aller C. and Horwitz K. B.: Simultaneous measurement of progesterone receptors and DNA indices by flow cytometry: Characterization of an assay in breast cancer cell lines. *Cancer Res.* **49** (1989) 3934–3942.
35. Graham M. L., II, Smith J. A., Jewett P. B. and Horwitz K. B.: Heterogeneity of progesterone receptor content and remodeling by tamoxifen characterize subpopulations of cultured human breast cancer cells: Analysis by quantitative dual parameter flow cytometry. *Cancer Res.* **52** (1992) 593–602.
36. Lerner L. J. and Jordan V. C.: Development of antiestrogens and their use in breast cancer. *Cancer Res.* **50** (1990) 4177–4189.
37. Osborne C. K., Coronado E. B. and Robinson J. R.: Human breast cancer in the athymic nude mouse. Cytostatic effects of long-term antiestrogen therapy. *Eur. J. Cancer Clin. Oncol.* **23** (1987) 1189–1196.
38. Lippman M. E.: An assessment of current achievements in the systemic management of breast cancer. *Br. Cancer Res. Treat.* **4** (1984) 69–77.
39. McGuire W. L., Lippman M. E., Osborne C. K. and Thompson E. B.: Resistance to endocrine therapy. *Br. Cancer Res. Treat.* **9** (1987) 165–173.
40. Gottardis M. M., Jiang S.-Y., Jeng M.-H. and Jordan V. C.: Inhibition of tamoxifen stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res.* **49** (1989) 4090–4093.
41. Osborne C. K., Coronado E. B., Wiebe V. and DeGregorio M.: Acquired tamoxifen resistance correlates with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxy-tamoxifen. *J. Natn. Cancer Inst.* **83** (1991) 1477–1482.
42. Katzenellenbogen B. S., Kendra K. L., Normal M. J. and Berthois Y.: Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res.* **47** (1987) 4355–4360.

43. Daly R. J., King R. J. B. and Darbre P. D.: Interaction of growth factors during progression towards steroid independence in T47D human breast cancer cells. *J. Cell Biochem.* **43** (1990) 199–211.
44. Gottardis M. M. and Jordan V. C.: Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mouse after long-term tamoxifen administration. *Cancer Res.* **48** (1988) 5183–5188.
45. Turner R. T., Wakely G. K., Hannum K. S. and Bell N. H.: Tamoxifen prevents the skeletal effects of ovarian deficiency in rats. *J. Bone Min. Res.* **2** (1987) 449–456.
46. Henderson I. C., Harris, J. R., Kinne D. W. and Hellman S.: Cancer of the breast. In *Cancer: Principles and Practice of Oncology* (Edited by V. T. DeVita Jr, S. Hellman and S. A. Rosenberg). J. B. Lippincott Co., Philadelphia (1989) p. 1252.
47. Legault-Poisson S., Jolivet J., Poisson R., Beretta-Piccoli M. and Band P. R.: Tamoxifen-induced tumor stimulation and withdrawal response. *Cancer Treat. Rep.* **63** (1979) 1839–1841.
48. Horwitz K. B., Aiginger P., Kuttenn F. and McGuire W. L.: Nuclear estrogen receptor release from antiestrogen suppression: Amplified induction of progesterone receptor in MCF-7 human breast cancer cells. *Endocrinology* **108** (1981) 1703–1709.
49. Murphy L. C., Dotzlaw H., Johnson Wong M. S., Miller T. and Murphy L. J.: Mechanisms involved in the evolution of progestin resistance in human breast cancer cells. *Cancer Res.* 51 (1991) 2051.
50. Jeltsch J. M., Krozowski Z., Quirin-Stricker C., Gronomeyer H., Simpson R. J., Garnier J. M., Krust A., Jacob F. and Chambon P.: Cloning of the chicken progesterone receptor. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 5424.
51. Conneely O. M., Sullivan W. P., Toft D. O., Birnbaumer M., Cook R. G., Maxwell B. L., Zarucki-Schulz T., Green G. L., Schrader W. T. and O'Malley B. W.: Molecular cloning of the chicken progesterone receptor. *Science* **233** (1986) 767.
52. Misrahi M., Atger M., d'Auriol L., Loosfelt H., Meriel C., Fridlansky F., Guiochon-Mantel A., Galibert F. and Milgrom E.: Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. *Biochem. Biophys. Res. Commun.* **143** (1987) 740.
53. Horwitz K. B.: The molecular biology of RU486. Is there a role for antiprogestins in the treatment of breast cancer? *Endocrine Rev.* **13** (1992) 146–163.
54. Tora L., Gronemeyer H., Turcotte B., Gaub M.-P. and Chambon P.: The N-terminal region of the chicken progesterone receptor specifies target gene activation. *Nature* **333** (1988) 185.
55. Vegeto E., Allan G. F., Schrader W. T., Tsai, M.-J., McDonnell D. P. and O'Malley B. W.: The mechanisms of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell* **69** (1992) 703.
56. Sartorius C. A., Tung L., Takimoto G. S. and Horwitz K. B.: Antagonist-occupied human progesterone receptors bound to DNA are functionally switched to transcriptional agonists by cAMP. *J. Biol. Chem.* **268** (1993) 9262–9266.
57. Tung L., Mohamed M. K., Hoeffler J. P., Takimoto G. S. and Horwitz K. B.: Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements, and are dominantly inhibited by A-receptors. *Molec. Endocr.* 7 (1993) 1256–1265.
58. Meyer M. E., Pronon A., Ji J., Bocquel M.-T., Chamson P. and Gronemeyer H.: Agonistic and antagonist activities of RU486 on the functions of the human progesterone receptor. *EMBO Jl* **9** (1990) 3923.