Chapter 8: Estrogen Receptor-Mediated Processes in Normal and Cancer Cells

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The role of estrogens in breast and other cancers has been extensively investigated for many years, and historically most of these studies have focused on the hormonal regulation of cell proliferation. The most recent work in this area has focused on the expression of genes likely to mediate proliferation (e.g., growth factors, proto-oncogenes, etc.) and their regulation by the classic nuclear estrogen receptor, ER-α. In this chapter, we present a synopsis of several new developments in this area of ER-regulated gene expression. These developments include the following: 1) the selective activation of ER domains by partial estrogen antagonists, such as tamoxifen and other ligands; 2) the effects of ER-α overexpression and gene knockout on the development of breast and uterine cancers in experimental animal models; 3) mechanisms by which steroid hormones regulate programmed cell death, cell cycle progression, cell-substratum interactions, and genomic instability in cancer cells; 4) identification of nuclear proteins that interact with the ER in the presence of agonists and antagonists, the effect of ligand binding on the receptor structure, and the interactions of liganded and nonliganded receptors with coactivators, corepressors, and other regulatory proteins; and 5) the biochemical properties, cellular distribution, and potential biologic roles for the newly discovered ER-β. Although there is an increasing interest in understanding the role of estrogens as endogenous carcinogens, it remains clear that ER-mediated regulation of gene expression plays many significant roles in normal and cancer cells, and increased knowledge of the mechanisms involved will improve our overall understanding of hormonal carcinogenesis. [J Natl Cancer Inst Monogr 2000;27:135–45]

Background and Rationale for Session

The primary focus of this meeting was "Estrogens as Endogenous Carcinogens in the Breast and Prostate," and most speakers thus discussed the actions of estrogens that are potentially related to initiation events (Chapters 3–5). This focus is somewhat different from most historic studies on estrogens and cancer, which focused largely on the role of estrogens in the process of proliferation. The view was that estrogens increased proliferation of target cells in the breast and other tissues, and this proliferation contributed to breast cancer by one of two major mechanisms. First, an increase in cell proliferation would be expected to cause an increase in spontaneous errors associated with DNA replication. Second, after mutations were introduced into a target cell by this or other mechanisms, estrogens would enhance the replication of clones of cells carrying such genetic errors. Much of the focus on estrogens in cancer to date has thus been on the mechanisms by which estrogens increase cell proliferation.

The general view has been that estrogens regulate proliferation of target cells by transcriptional mechanisms involving the classic estrogen receptor (ER), initially discovered in the laboratories of Elwood Jensen at the University of Chicago, IL, and Jack Gorski at the University of Illinois (Champaign-Urbana). Work from their laboratories, along with metabolic inhibitor studies of Gerald Mueller, then at the University of Wisconsin (Madison), led to the concept that estrogens increased proliferation by stimulating RNA synthesis in target cells. This concept led to a search for target genes for which transcription was regulated by estrogenic hormones, and many laboratories identified a number of growth factors, proto-oncogenes, and other regulatory molecules that were likely candidates for such genes.

In recent years the emphasis of these studies has progressed to investigating the transcriptional regulation of such target genes by the ER, with special emphasis on identifying the regulatory factors involved and their molecular mechanism of action, differences in the activity of various ER ligands, the identification of new ER subtypes, and the types of gene families regulated by estrogens during hormonally induced increases in proliferation. It was thus felt important to have a session on ER-mediated processes in normal and target cells. Because it was impossible to present a comprehensive review of this body of work in a single session, the goal was to invite a group of speakers who would address some of the most rapidly emerging paradigms of estrogen action that the Organizing Committee felt were particularly relevant to understanding the actions of estrogens in cancer cells.

Readers interested in additional information on this aspect of estrogen action are referred to a number of recent reviews and articles and references therein, in addition to the references provided throughout the body of this article. These references include information on the structure and function of the nuclear ERs *(1–9),* the roles of nuclear receptor coactivators and corepressors in steroid hormone action *(10–12),* recent advances in the development of selective ER modulator(s) (SERM) *(13–17),* and the various phenotypes observed in ER knockout mice, which indicate biologic actions mediated by these nuclear receptors *(18–21).*

Overview of Speakers and Topics

Until quite recently, the stimulation of transcription by estrogens was viewed in terms of a relatively straightforward set of interactions initiated by estrogen binding to a single type of ER that was thought to be identical in all target tissues. Ligand

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See "Note" following "References."

binding was viewed as a "trigger" that activated the receptor from an "off" state to an "on" state, and this activation enabled the receptor to activate or repress transcription of target genes. The activated receptor was then thought to interact with an estrogen response element (ERE) in the 5′-flanking region of responsive genes. It was thought that EREs of most endogenous hormone-regulated genes would have sequences similar to the palindromic sequence, GGTCAnnnTGACC, originally identified in the vitellogenin gene and generally referred to as the consensus ERE. This scheme of estrogen action is illustrated in Fig. 1, and, although highly schematized and oversimplified, it represents, to a good approximation, the state of our basic knowledge of estrogen-regulated transcription about 5 years ago.

In terms of cancer, it has been known for many years that breast cancer cells contain steroid receptors, and the content of ERs and progesterone receptors (PRs) in individual tumors is a valuable predictor of whether an individual patient will respond to endocrine therapy. However, the correlations between receptor content and responses to endocrine therapy are far from perfect, and many tumors progress to states of hormone independence. Antihormones, such as tamoxifen, were known to compete with estrogenic agonists for receptor binding, but little else was known about the specific biochemical mechanisms by which these important drugs produced their actions in experimental or therapeutic settings. In addition, their use is complicated because most breast tumors eventually become refractory to antiestrogen treatment. Paradoxically, drugs such as tamoxifen also display strong agonist activity in the endometrium, which is highly problematic for their therapeutic use. Such observations were difficult to reconcile with a view of the ER as a simple "on/off" switch that interacted with the same regulatory sequence in all target genes.

Within recent years, significant advances in our understanding of ER-mediated events have occurred at the conceptual level, and major new experimental approaches to the study of hormone action have become available. Many of these approaches will be discussed in this session, and several key points are enumerated below.

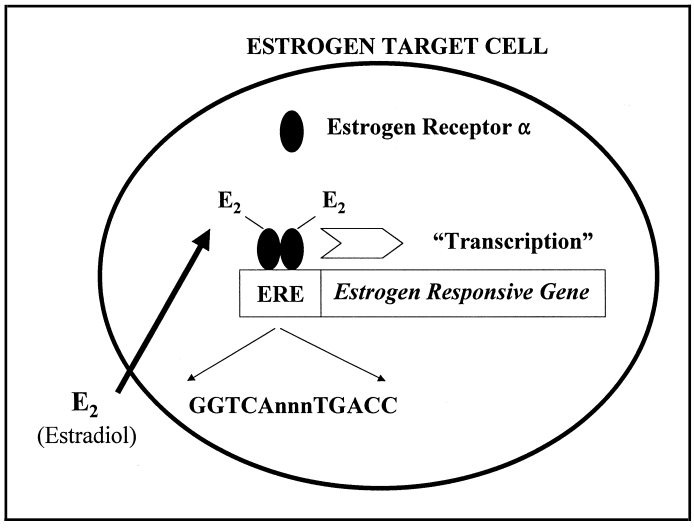

Fig. 1. Model of estrogen action circa 1990. Estrogens such as estradiol (E_2) enter target cells by diffusion and bind the classic estrogen receptor-α (filled ovals). The receptor-hormone complex stimulates transcription of target genes via interactions with an estrogen response element similar to the sequence 5′-GGTCAnnnTGACC-3′ identified in the vitellogenin A2 gene.

1) It is now known that the ER contains several "domains" that are involved in transcriptional regulation and that different ligands may selectively activate these functions. This knowledge raises the exciting prospect of developing estrogens that can be used to selectively produce desired therapeutic actions while minimizing untoward side effects; several such agents have already been discovered. Such functional studies on the actions of different estrogens and antiestrogens are being accompanied by structural studies of the molecular interactions between ligands and the receptor, and this combination will almost certainly lead to the discovery of even more selective agents. A related question of special importance to understand breast cancer etiology is whether structurally diverse estrogens differentially stimulate proliferation of breast cancer cells. Dr. McDonnell discusses the role of the ER transcriptional activation functions (AFs) in ligand selective responses.
2) A major experimental advance has been the production of experimental animals that overexpress the ER or knockout animals that do *not* express the receptor. These experimental animals provide heretofore unavailable approaches to define unequivocally the role of ER in estrogen-mediated events, to identify redundant signaling pathways that compensate for changes in ER levels, and to identify previously unrecognized actions of estrogens. Dr. Couse describes the generation and phenotypes of ER-α knockout (ERαKO) and overexpressing mice as well as the effect that receptor levels have on the development of breast and uterine cancers in experimental models.
3) In the past, a major focus of study has been the regulation of growth factor and proto-oncogene expression. More recently, attention has increased to other ways by which estrogens might affect breast cancer. This attention includes the study of mechanisms that regulate cell death, the factors that control cell–cycle progression, and the mechanisms that contribute to genomic instability of cancer cells. In addition, interest has increased in processes, such as angiogenesis and cell–substratum interactions, that can affect tumor growth and metastases, and understanding these processes may also improve our understanding of the etiology of breast cancer and potential therapeutic targets. Dr. Dickson addresses several mechanisms regulating these pathways in mammary cancer cells.
4) It has been known for some time that cross talk exists between ER-mediated events and other signaling pathways (e.g., those regulated by peptide growth factors and their second messenger systems), and the ER itself undergoes phosphorylation/dephosphorylation events that could alter its activity. More recent studies have also identified a number of other nuclear factors, including coactivators, corepressors, and integrator proteins that play important roles in ER-mediated transcriptional events. A key observation is that these factors can alter the magnitude of cellular responses to estrogens and other steroids. Identification of these factors and the mechanisms by which they operate are likely to provide additional indices that can be used in conjunction with ER/PR levels to classify breast tumors and predict the efficacy of current hormonal therapies and to develop new therapeutic targets. A related advance has been the recognition that substantial diversity is found in the location and sequence of EREs in endogenous hormone responsive genes,

and these differences may also increase our understanding of mechanisms by which ER-mediated processes affect breast cancer. Dr. Greene's talk discusses the interaction of several factors with the ER and illustrates that different ligands produce different structural states of the receptor that could interact differentially with other regulatory molecules, such as coactivators and corepressors.

5) In addition to the classic ER, now referred to as ER-α, a second receptor termed ER-β has been identified in humans and in animals. The two receptors show different tissue distributions, and, although they have generally similar ligand binding patterns, at least several differences appear to exist. An exciting era of endocrine research will be to define further the properties, distribution, and regulation of these receptors and to identify the biologic responses that they mediate. This new receptor is discussed by Dr. Gustafsson, whose laboratory has been the leader in the identification and characterization of ER-β.

Cellular Components That Distinguish Between Agonist- and Antagonist-Activated Steroid Receptors

Research in Dr. McDonnell's laboratory has been driven in large part by two key issues in estrogen and antiestrogen pharmacology. First is the issue of how to obtain tissue selectivity with estrogens used for hormone replacement therapy. It is clearly established that estrogens diminish vasomotor instability ("hot flashes"), preserve bone mass, and have beneficial effects on cardiovascular health. An emerging view is also based on epidemiologic evidence that they may also benefit cognitive function and delay the onset of Alzheimer's disease. However, it is highly problematic that estrogens used for these desirable purposes produce proliferative effects on the breast and endometrium. It is the fear of breast cancer, in particular, that greatly limits the use of estrogen replacement therapy by many women. The second pharmacologic issue is the use of tamoxifen as an adjuvant treatment of breast cancer. The drug has established efficacy in the treatment of the disease, but tamoxifen treatment generally fails after a period of time, and use for prolonged periods (e.g., 10 years) may actually be less beneficial than use for shorter times (e.g., 5 years). These observations were very difficult to reconcile with a simple mechanism of estrogen action in which "all estrogens are alike" in that they simply activate the receptor, and antiestrogens simply act by "freezing" the ER in an inactive state akin to that of an unliganded receptor protein.

Roughly 5 years ago, a number of studies began appearing that were inconsistent with this simple view of ER activation. One study was a clinical paper published by Love et al. *(22)* in 1992. These workers examined the effect of tamoxifen on bone mineral density of the lumbar spine in women receiving the drug for the treatment of breast cancer, and their data indicated that tamoxifen increased bone mass. In other words, the drug acts as an estrogen agonist in bone, in contrast to its antiestrogen action in the breast. This study was one of the first well-documented clinical studies of a SERM and clearly indicated that an ER ligand could have opposite effects in different target tissues.

Shortly thereafter, studies in Dr. McDonnell's own laboratory demonstrated that the binding of different ligands caused the ER to assume different conformations *(23).* In these studies, he used protease digestion to probe subtle differences in ER conformation. When trypsin was incubated with the unliganded ER, the 66-kd molecular weight native receptor was degraded to very low-molecular-weight fragments. When estradiol was bound to the receptor, however, the receptor assumed a conformation less susceptible to protease digestion because a relatively large receptor fragment (32 kd) remained after prolonged digestion. When tamoxifen was bound to the receptor, the protein was not degraded to very small fragments, indicating that tamoxifen did not simply hold the receptor in an inactive conformation similar to that of the unliganded protein. Rather, tamoxifen binding produced a conformational change that protected a relatively large protein fragment (28 kd) from trypsin digestion. This finding indicated that tamoxifen actually put the ER in a conformation that was distinct from either that produced by the endogenous hormone (estradiol) or that of the unliganded receptor, which was previously presumed to represent its inactive conformation. This study provided physical evidence that different ligands caused the receptor to assume different conformations.

These laboratory investigations suggested a molecular explanation for clinical findings such as those reported by Love et al. *(22)* in different tissues (i.e., bone versus breast cancer cells). Because tamoxifen and estradiol put the receptor into different conformations, this investigation suggested that the different tissues had ways to functionally "distinguish" structural difference in the receptor (i.e., the conformation of the ER–tamoxifen complex could function as an agonist in bone but not in breast cancer cells). It was known at this time that the ER had a modular structure and that two different regions of the protein could function to activate transcription. One such region, termed transcription-activating function 1 (referred to as either TAF-1 or AF-1 in the literature) was present in the N-terminal region of the receptor, and a second (AF-2) was present in its carboxyl-terminal region. This knowledge raised the possibility that different ligands (e.g., estradiol versus tamoxifen) might put the receptor into conformations in which the two AFs were differentially active. To test this hypothesis, Dr. McDonnell's group performed a series of co-transfection studies with the use of wild-type ERs that contained both AFs and ER mutants in which only one of the AFs was active *(24).*

A series of such studies indicated that most cultured cells (approximately 90% of those tested) required both AF-1 and AF-2 functions for transcriptional activity when stimulated by estradiol, but the hormone could stimulate transcription in some cells via receptors with only an active AF-1 or an active AF-2 function. Tamoxifen failed to activate transcription in all cases in which both the AF-1 and AF-2 functions were required and in cases in which the AF-2 function alone could mediate estradiol-induced transcription. In these cell types, tamoxifen functioned as a pure estrogen antagonist to block the action of estradiol. In contrast, in those cells in which estradiol could activate transcription from receptors with only a functional AF-1, tamoxifen could act as a partial agonist with substantial estrogen-like activity. These interactions are illustrated schematically in Fig. 2. These studies were also important because they established that tamoxifen could function both as a partial estrogen agonist and as a pure estrogen antagonist via the same receptor system. This finding ruled out the possibility that the antagonist and partial agonist activities of antiestrogens were mediated by different receptor systems.

In the early 1990s, the concept was also emerging that the role of the receptor AF was to serve as "contact" points for the interaction with other cellular proteins involved in transcription control, the so-called coactivators and corepressors. The idea

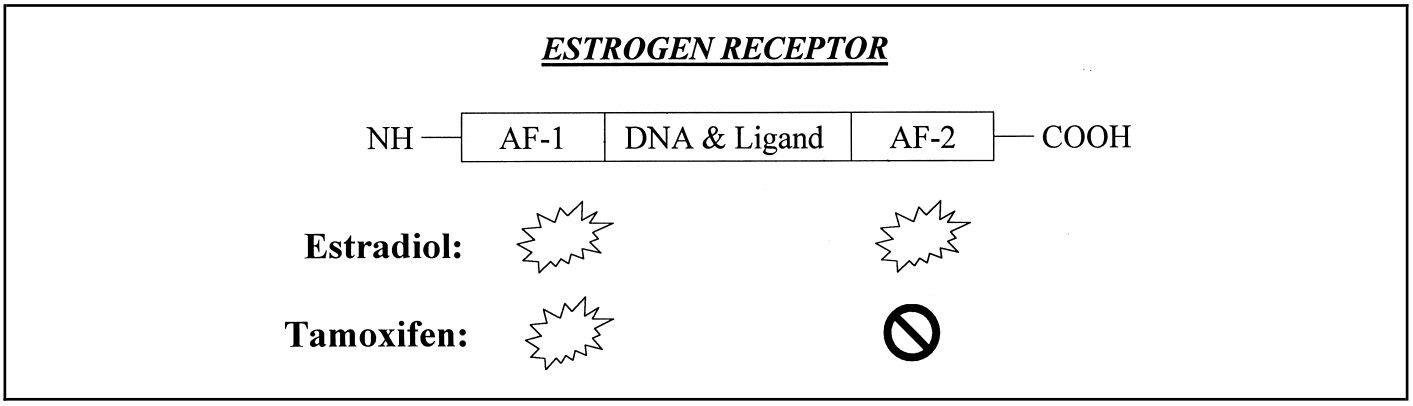

Fig. 2. Agonists and antagonists have differential effects on transcription-activating functions (AF) of the estrogen receptor (ER). The ER has a modular structure involving a N-terminal transcription activation function, termed AF-1, a DNA-binding domain, a ligand-binding domain, and a more C-terminal activation function, termed AF-2. Both estradiol and tamoxifen bind to a similar site in the ligand-binding domain of the receptor. Estradiol is able to activate both AFs. In contrast, tamoxifen prevents activation of AF-2, although the drug can activate the AF-1 function of the receptor.

was that the steroid receptor would bind to EREs in the regulatory regions of target genes and that AF functions (activated by bound hormone) would then recruit these factors, which would alter transcription. This idea raised the possibility that different cells required different AFs in the ER for transcription activation because they contained different complements of coactivators, corepressors, or other regulator proteins. Such differences could be qualitative (i.e., different cells would express different types of proteins) or quantitative (different cells would contain different levels of coactivators/corepressors), or both.

To investigate this possibility, Dr. McDonnell's laboratory performed a series of studies in which they co-transfected one such coactivator (termed GRIP) into cells along with either the wild-type or mutated ER *(25).* In the cell line used, estradiol produced a full response (100%) of transfected reporter genes with the wild-type receptor, but only a 50% response was produced with receptors in which only AF-1 was active and only 20% in receptors in which only AF-2 was active. However, when vectors expressing GRIP were used to raise cellular levels of this protein, receptors with only AF-1 or AF-2 function produced the same transcriptional response as wild-type receptors. This finding established the principle that coactivators present in some cells are sufficient to enable the ER to activate transcription when only one of its two AFs is activated by ligand binding.

Collectively, studies such as these indicated that tamoxifen could function as a partial agonist if only the AF-1 function of the receptor was required, but it always functioned as an antagonist if the AF-2 function was required, either alone or in combination with the AF-1 function. A related question that Dr. McDonnell also considered was whether all antiestrogens would display this same type of behavior, and he thus began a series of studies to investigate the ability of different antiestrogens to activate AF-1 activity but block AF-2 activity. These studies led to the recent discovery of an antiestrogen, GW-5638, with an activity profile different from that of tamoxifen.

GW-5638 is a triphenylethylene antiestrogen that appears totally devoid of either the AF-1 or AF-2 type of activity *(26).* This lack of AF activity is not due to poor entry into target cells, because the drug can block the transcriptional effects of estradiol and tamoxifen in cultured cell systems. This drug is thus a pure antiestrogen in the breast, but it retains the ability to maintain bone mass without producing any uterotrophic action in rats. This ability indicates that ER ligands *without* AF-1 or AF-2 activity can function as estrogen agonists in bone. This function suggests that ER-mediated actions in target cells are even more complex than previously recognized and that other factors besides AF-1 and AF-2 functions are likely to be involved in estrogen actions in some cell types.

Role of ER-α in Carcinogenesis With the Use of Transgenic Mouse Models

The estrogen signaling system has long been implicated as a possible factor in the induction and/or promotion of carcinogenesis, especially in the tissues of the female reproductive tract and of the breast. The proliferative effects of the natural ligand, 17β-estradiol, as well as the synthetic estrogen diethylstilbestrol (DES) in the uterus, vagina, and mammary gland have been well studied. The majority of the cellular effects of estrogens are thought to be mediated by the ER, now known to exist in two types, the well-characterized ER-α and the newly discovered ER-β. Although it has been established that the ER must be present for most estrogen-induced mechanisms, the relationship between the levels of ER and the extent to which a tissue is estrogen responsive is less understood. Furthermore, the influence of varied ER levels in carcinogenesis is even less well known. Efforts to understand further the role of the ER-α in carcinogenesis have led to the generation and characterization of a series of transgenic mouse models that possess altered levels of ER-α expression. The MT-mER mice possess a transgene that results in overexpression of the ER-α protein, whereas the ERαKO mice are homozygous for a targeted disruption of the ER-α gene and, therefore, possess no functional levels of ER-α *(27).* By using these models, studies have been conducted to elucidate further the role of ER-α in the induction and promotion of hormonally (DES)-induced tumors of the reproductive tract *(28)* and of oncogene-induced tumors of the mammary gland *(29).*

In utero exposure to DES, a potent synthetic estrogen, has been linked to a significantly higher risk of a rare form of vaginal cancer, as well as other reproductive abnormalities in humans. The effects of neonatal DES exposure in the female mouse include structural abnormalities in the uterus, oviduct, and bone; uterine tumors; and vaginal adenosis and adenocarcinoma, whereas, in the male, increased incidence of retained testes and hypoplasia of the accessory sex organs have been reported. However, the exact mechanisms by which develop-

mental exposure to DES leads to such abnormalities remain unknown. DES is able to bind ER-α and to mimic the proliferative effects of the natural hormone 17β-estradiol in the uterus and the vagina. However, DES and its metabolites are also able to directly bind DNA and tubulin, reportedly increasing the incidence of aneuploidy and of nondisjunction in dividing cells. Therefore, it is possible that the developmental and carcinogenic effects of DES may be a direct result of its ER-α-mediated activity, its nonreceptor-mediated genotoxic effects, or both.

The role of ER-α in the induction and promotion of DES-induced tumors was first investigated by using the transgenic MT-mER mice. The uteri of adult MT-mER mice possessed approximately 25% more ER-α than their wild-type littermates *(29).* It was hypothesized that, because of this abnormal expression of ER-α, the reproductive tract tissues of the MT-mER mice may be more susceptible to tumors after neonatal exposure to DES. Wild-type and MT-mER littermates were treated with DES on days 1–5 at 2 mg/pup per day and then killed at 4, 8, 12, and 18 months of age. At 8 months of age, DES-treated MT-mER mice demonstrated a significantly higher incidence of uterine adenocarcinoma at 73% compared with 46% in the DES-treated wild-type mice (Table 1). These tumors were also preceded at 4 months by a significantly higher incidence of the preneoplastic lesion, atypical hyperplasia, in the MT-mER mice at 26% compared with 0% in the wild-type mice. These data indicate that the level of ER-α present in a tissue may be a determining factor in the progression of estrogen-responsive tumors.

Further studies *(30,31)* designed to possibly segregate the estrogenic and genotoxic effects of DES have utilized the ERαKO mice, which possess no functional levels of the ER-α protein. Wild-type, heterozygous, and ERαKO littermates were treated as described above in the MT-mER study and also killed at 4, 8, 12, and 18 months. At all time points, uterine weight was significantly reduced in DES-treated wild-type and heterozygous females, whereas no difference was observed in the ERαKO females. Furthermore, the persistent cornification and hyperplasia of the vaginal epithelium as well as the progressive proliferative lesions of the oviduct that are characteristic of neonatal exposure to DES were observed in the wild-type and heterozygous mice but absent in the ERαKO females. At 4 months of age, squamous metaplasia was occasionally observed in the uteri of DES-treated wild-type females but not in the DES-treated ERαKO mice. In the males, significant atrophy of the seminal vesicle was observed at all time points in both DES-treated wild-type and heterozygous mice, whereas no difference was observed between control and DES-treated ERαKO males. The incidence of tumors, as well as possible altered gene expression in reproductive tract tissues of the different genotype/treatment groups, is currently being assessed. These results thus far indicate that certain developmental effects of DES are, indeed, ER-α mediated.

Finally, the group of Couse and Korach has utilized the ERαKO to study the influence of ER-α in mammary tumors induced by the ectopic expression of the Wnt-1 oncogene. Mice possessing the MMTV-LTR-driven Wnt-1 transgenic construct are known to develop hyperplastic ductal and alveolar epithelium and eventually mammary adenocarcinoma during adulthood *(32).* Therefore, they have crossed Wnt-1 transgenic mice with the ERαKO mice to generate mice that possess the Wnt-1 transgene on a background of altered ER-α levels *(33).* The adult female ERαKO mammary gland is completely undeveloped, exhibiting only a rudimentary ductal structure and lacking any terminal end or alveolar buds. However, ectopic expression of the Wnt-1 gene in the ERαKO mammary gland did result in hyperplasia of the existing ductal structure, but it did not lead to further ductal branching or the development of terminal end buds as exhibited by the Wnt-1/wild-type ER-α females. In addition, the average time of tumor onset in the Wnt-1/ERαKO females was much delayed (50% at 48 weeks) compared with the Wnt-1/wild-type ER-α littermates (50% at 24 weeks), even though the serum levels of estradiol in the ERαKO females are approximately 10-fold higher than normal. Postpubertal ovariectomy, as well as pregnancy, had no effect on the growth rate of the mammary tumors in the Wnt-1/wild-type ER-α females. However, prepubertal ovariectomy did result in a delayed average time to tumor onset in the Wnt-1/wild-type ER-α as well as the Wnt-1/ERαKO females compared with that of their respective intact study groups. The results of these studies indicated that Wnt-1-induced mammary tumors can arise in the absence of functional ER-α, as well as ovarian hormones. However, their results have demonstrated that estrogen actions, as mediated by the ER-α, do act to promote the growth of Wnt-1-induced tumors.

Table 1. Effect of increased ER-α on the progression of DES-induced tumors in the mouse reproductive tract

		No. of affected mice/total No. of mice			
		Control		DES	
Observation	Age, mo	Wild type	MT-mER	Wild type	MT-mER
Squamous metaplasia	4	0/15	0/14	2/19 (11%)	12/19 (63%)*
	8	0/11	0/10	6/24 (25%)	1/26 (4%)
Atypical hyperplasia	4	0/15	0/14	0/19 (0%)	5/19 (26%)*
	8	0/11	0/10	3/24 (12%)	1/26 (4%)
Adenocarcinoma	4	0/15	0/14	0/19 (0%)	0/19 (0%)
	8	0/11	0/10	11/24 (46%)	19/26 (73%)*
	12	0/15	0/15	11/15 (73%)	13/15 (87%)
	18	0/19	0/19	13/14 (93%)	12/13 (92%)

*$P<.05$ as calculated by the Fisher exact probability test, comparing DES-treated MT-mER to DES-treated wild type. Adapted from *(28).* DES = diethylstilbestrol; ER = estrogen receptor.

Regulation of Cell Cycle and Cell Death in Mammary Cancer

Physiologic levels of estrogens and progestins are well known to promote both onset and malignant progression of breast cancer. A number of investigators in the field believe that an imbalance of mammary epithelial proliferation and death (apoptosis) contributes to tumor formation, genomic instability, and metastasis. They have hypothesized that imbalanced expression of steroid-regulated genes triggers this diverse cascade of processes *(34,35).* Both estrogenic and progestational steroids are known to regulate expression of genes encoding several polypeptide growth factors, growth factor-binding proteins, and growth factor receptors. In the case of the epidermal growth factor (EGF) family of ligands and receptors (including transforming growth factor-α [TGF-α], amphiregulin, EGF receptor, and c-erbB_2), their pathologic overexpression and functional relevance for breast cancer has received experimental support *in vitro, in vivo,* and in ongoing clinical studies. Conversely,

growth factors have been shown to regulate expression and function of steroid receptors *(34,35).* Sex steroids and growth factors appear to exert their principal influences on the cell cycle and cell survival through regulation of cyclin D_1 and Bcl-2/$BclX_L$, respectively *(35–37).* The c-myc gene and the bcl-2 gene family have been shown to be important downstream mediators, both of the actions of steroids and of the EGF ligand/receptor family on cellular proliferation and survival; of particular interest, the c-myc gene is amplified in 20%–30% of breast cancer cases and aberrantly expressed in a much higher proportion of cases *(38,39).* A recent meta-analysis of published clinical pathologic studies *(39)* has demonstrated that amplification of the c-myc gene is associated with increased lymph node metastases and poorer survival, irrespective of expression of the ER. Myc appears to exert its principal effect on the cell cycle through activation of CDK-2; however, its overexpression sensitizes cells to apoptosis coincident with induction of the proapoptotic p53 and bax genes *(38).*

As a model system to examine the consequences *in vivo* of disregulated expression of two important, but functionally quite distinct, mediators of the action of estrogen, Dr. Dickson's group has carried out a cross of transgenic Myc- and TGF-α-overexpressing mouse strains. Bitransgenic progeny exhibited a remarkable synergy between the two genes for mammary tumorigenesis, independent of sex, parity, and reproductive hormonal status, indicating that disregulated expression of these two estrogen-inducible genes can entirely supplant an etiologic role for estrogen in malignancy *(40).* They observed that the mechanism of interaction of Myc and TGFα involved a coordinated stimulation of the cell cycle and suppression of apoptosis (Fig. 3) *(41).* First, the two gene products interacted such that TGF-α-induced $BclX_L$ allowed cellular survival in the presence of Myc-induced p53 and Bax *(36).* The EGF receptor-mediated effect on survival appears to depend on signal transduction, both through the Erk1/Erk2 and the PI3K pathways *(42).* An independent survival effect is also mediated in these cells by collagen IV acting through a β_1 integrin-PI3K mechanism *(43).* Second, through their concordant activation of CDK-4 (by induction of cyclin D_1) and CDK-2 (by destruction of the CDK inhibitor p27), the two gene products markedly stimulated aberrant cell cycles and promoted the appearance of multiple chromosomal aberrations *(44,45).* The results of decreased modulation of p27 by c-myc appear to be sufficiently potent to allow abrogation of the anchorage-independent G_1/S cell cycle checkpoint *(46).* The appearance of dicentric chromosomes and of a wide array of other chromosomal abnormalities was suggestive of a bridge–break–fusion cycle mechanism at work. The p53 gene was observed not to be mutated in Myc-expressing mammary tumors; it played no obvious role in surveillance of the chromosomal defects observed *(47,48).* Future studies must further address these mechanisms of cell cycle disregulation, apoptosis suppression, genomic instability, their relevance to sex steroid action, and their roles in human breast cancer.

ER Structure, Modulators, and Targets in Hormone-Responsive Tissues and Cancers

Dr. Greene emphasized that the ER does not function in a vacuum but that it interacts with many other proteins. For example, it is well established that the ER and other steroid receptors interact with the heat-shock proteins *(49)* during the initial synthesis of the receptor to ensure its proper folding and trafficking, and, in turn, dissociation of heat-shock proteins seems to be required for the ligand-occupied receptor to activate transcription. He also emphasized that one of the major functions of ligand binding is to change the nature of protein–protein interactions between steroid receptors and other proteins and, conversely, that other proteins can alter the state of the ER independent of ligand binding (e.g., by receptor phosphorylation). In

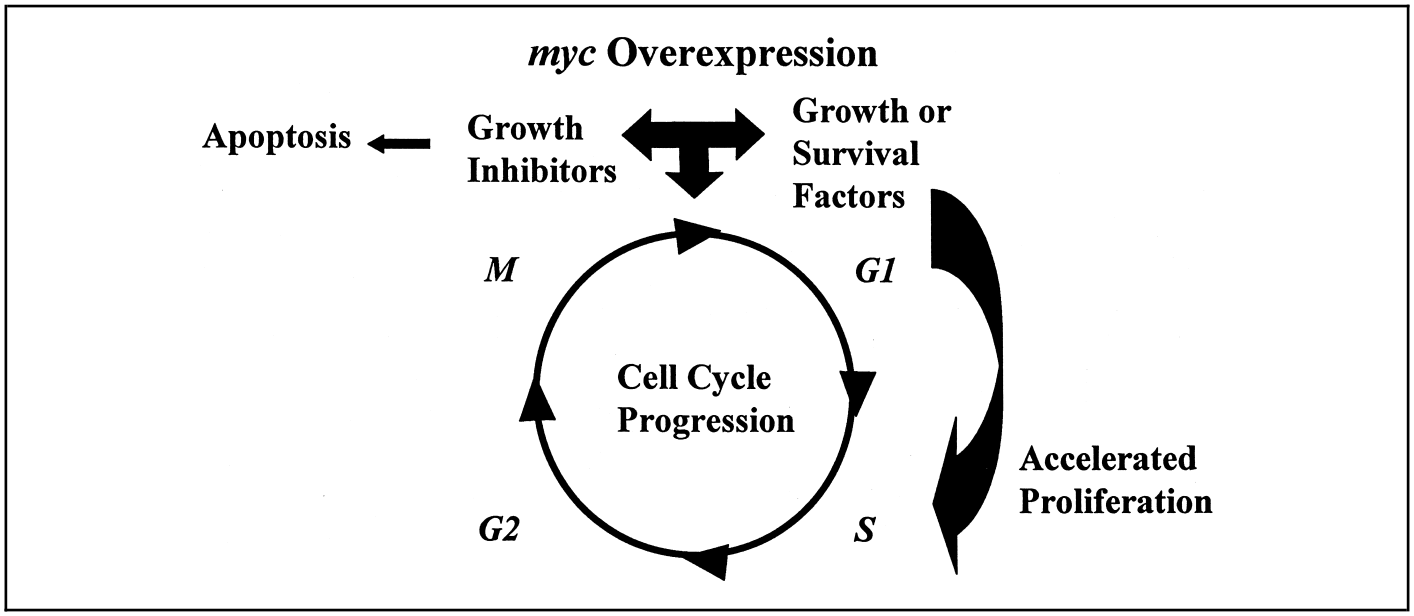

Fig. 3. Model for the dual action of c-myc overexpression in mammary epithelial cells. Deregulated c-myc expression promotes cell–cycle progression through a mechanism that is currently under investigation. The end result of such inappropriate cell–cycle stimulation depends on a number of factors, such as cell genotype and environment. For example, in the presence of certain growth or survival factors (such as activators of the epidermal growth factor receptor or integrin-mediated adhesion), c-myc expression is proposed to accelerate cell proliferation and promote cell survival. In the absence of such factors or in the presence of certain growth inhibitors (such as transforming growth factor-β), constitutive expression of c-myc is more likely to induce apoptosis [adapted from *(38)*].

his talk, Dr. Greene presented data from his laboratory on the following three related topics: 1) the identification of gene targets for the ER-ligand complex, 2) the factors that serve to modulate the actions of the ER in target cells, and 3) the structural changes produced in the ER by the binding of different estrogenic and antiestrogenic ligands.

In the first series of studies, Dr. Greene used the technique of RNA differential display to identify transcripts in breast cancer cells that are regulated by estrogens and antiestrogens. This technique identified a number of transcripts with expression altered by ER ligands. Sequence and northern blot analysis of one clone that was decreasingly regulated by estradiol revealed its identity as monocyte chemoattractant protein-1 (MCP-1). The basal expression of MCP-1 is low in MCF-7 breast cancer cells, but it is stimulated by TNF-α, and estradiol blocks induction of the MCP-1 message by the cytokine.

TNF-α is known to regulate MCP-1 expression via the NF-κB pathway. The MCP-1 gene is known to contain an NF-κB regulatory element, and reporters containing this element are induced by TNF-α in MCF-7 cells. Despite the fact that reporters containing the MCP-1 promoter do *not* contain any sequences resembling the classic ERE, estradiol blocks induction of such reporters following transfection into breast cancer cells. Extracts from estrogen-treated MCF-7 cells also decrease the binding of NF-κB to its regulatory element in gel shift studies, and the ER and NF-κB can be co-immunoprecipitated. Collectively, these studies suggest that the ER blocks TNF-α induction of MCP-1 by directly or indirectly decreasing the binding of NF-κB to its regulatory site in the 5′-regulatory region of the MCP-1 gene. This mechanism is illustrated schematically in Fig. 4.

TNF-α is known to act via a membrane receptor to stimulate a protein kinase cascade that leads to the phosphorylation of IκB (Fig. 4). Before phosphorylation, this protein forms a dimeric complex with NF-κB in the cytoplasm to prevent its movement to the nucleus. On phosphorylation, the IκB inhibitor dissociates from the complex and is degraded by an ubiquitin-mediated pathway. This process allows the NF-κB to translocate to the nucleus, where it binds to NF-κB sites in target genes and activates their transcription. The ER appears to decrease transcription by preventing the binding of NF-κB to its regulatory site in the 5′-flanking region of the gene, most likely by a direct interaction of the two proteins, as illustrated in Fig. 4. This finding emphasizes that estrogens and antiestrogens can regulate expression of target genes that do not contain hormone response elements, and such regulation is thus likely to occur via protein–protein interactions. Another recent similar example of regulation by protein–protein interactions occurs via binding of the ER to AP-1 components *(50,51)*.

A second series of studies was aimed at identifying cellular factors that modulate the activity of the ER. To identify such factors, Dr. Greene and his colleagues utilized the ligand-binding domain B *(52)* of the ER to "capture" proteins from breast cancer cells that interact with the receptor *(53)*. In this approach, a fusion protein between glutathione *S*-transferase and the ligand-binding domain of the ER is used as an affinity matrix to bind proteins in cell extracts that bind to this domain of the receptor in the presence or absence of estrogens and/or antiestrogens. At present, this approach has already identified at least five to six proteins from MCF-7 cell extracts that bind to the ER.

One such "modulator" protein, which has been identified, is a kinase that binds to the ER in the presence of estrogenic ligands and dissociates from the receptor when it is liganded with antiestrogens, such as hydroxy-tamoxifen. This action enabled the kinase to be purified by first binding proteins in cell extracts to glutathione *S*-transferase-ER in the presence of estradiol, followed by washing to remove unwanted proteins, and then eluting in the presence of antiestrogens. Similar purification

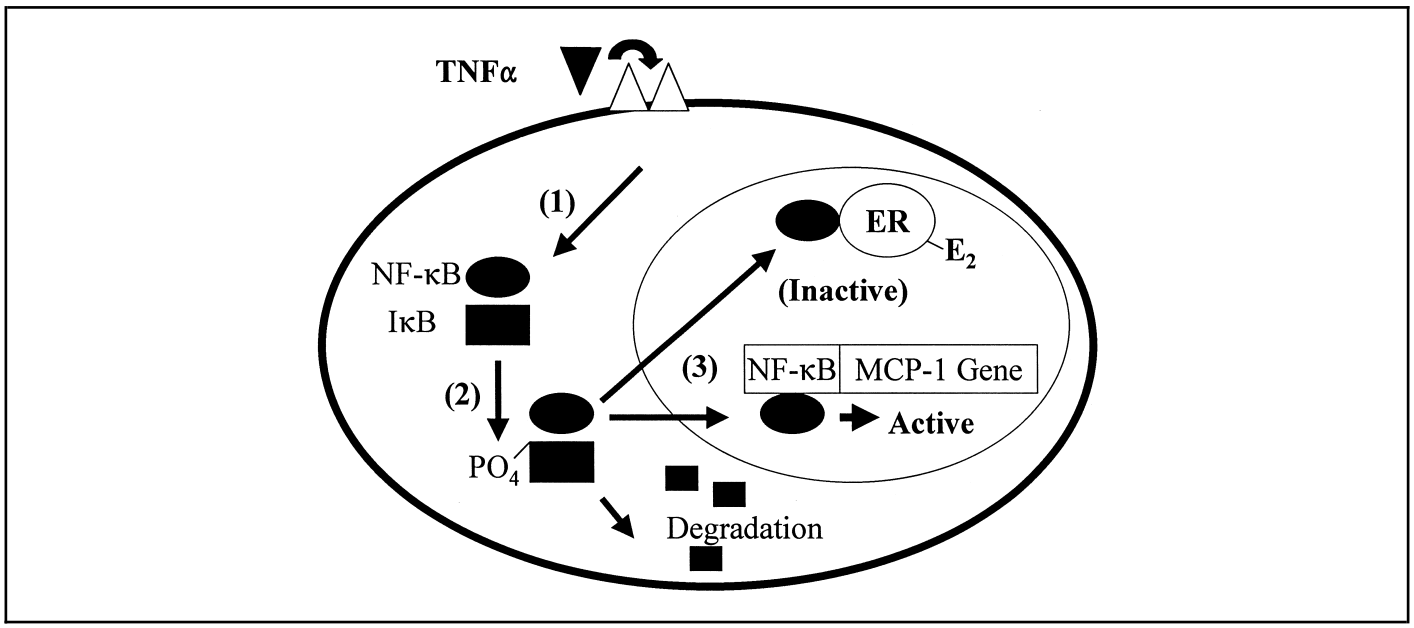

Fig. 4. Proposed model for regulation of monocyte chemoattractant protein-1 (MCP-1) in human breast cancer cells. Tumor necrosis factor-α binds to a membrane receptor and initiates a kinase cascade (step 1) that phosphorylates the IκB protein shown as a filled rectangle (step 2). Before phosphorylation, IκB forms a dimer with the transcription factor NF-κB (filled oval) in the cytoplasm to prevent its entry to the nucleus. Following phosphorylation, IκB is degraded, and the free NF-κB can translocate to the nucleus (step 3). In the absence of estrogens, NF-κB is "active" as a transcription factor and drives transcription of the MCP-1 gene via a regulatory element in the 5′-flanking region of the gene. When occupied by estradiol, the estrogen receptor (open oval) forms a nuclear complex with NF-κB so that it is inactive as a transcription factor.

steps were performed by displacing the ER-bound kinase with a peptide with sequences similar to the motifs in coactivators that bind to steroid receptors. This kinase phosphorylates the ER on a serine residue, although the functional consequences of phosphorylation at this site are unknown at present. This series of experiments also emphasizes that protein–protein interactions are increasingly being recognized as playing potential roles in estrogen action.

In a third major series of studies, Dr. Greene and a number of colleagues examined the effects of ligand binding on the structure of the ER. In this work, they solved the crystal structures of the human ER ligand-binding domain (amino acids 301–553) complexed with either estradiol or the mixed antagonist raloxifene *(54).* This study provided definitive evidence that different ligands produce distinct structural alterations in the receptor. The crystal structures revealed that both ligands bind to the same site within the core of the ligand-binding domain of the receptor, but the two ligands induce major conformational differences in the positioning of the most c-terminal a helix in the receptor (helix-12). This evidence is of major significance because helix-12 is located in the transactivation domain of the ER and appears to be a major site for contact with coactivators and corepressors. Hence, the different structures observed following binding of the two ligands are expected to interact quite differently with these accessory proteins, which drive the transcriptional responses to steroid hormones.

Structure and Function of ER-β

A novel form of the ER, termed ER-β, was originally cloned from the rat prostate *(55)* and has also been identified in the mouse *(56)* and in humans *(57).* The rat ER-β complimentary DNA encodes a protein of 485 amino acids with a predicted molecular weight of 54 200 that is highly homologous to the ER-α, particularly in the DNA-binding domain (97% amino acid homology) and the c-terminal ligand-binding domain (59% homology). The amino acid homologies between the three ER-βs identified to date and the human ER-α are illustrated in Table 2 *(58),* and it is clearly seen that there is nearly perfect homology in the DNA-binding domains of the α and β receptors, substantial homology in the ligand-binding domains, but far less homology in the N-terminal regions. The genes for the two receptors are on separate chromosomes in the human—ER-β on chromosome 14 and ER-α on chromosome 6—removing all doubts that the two receptors are totally distinct species. The major difference in the structures of the two receptors is in the N-terminal region, which is considerably shortened in the β-receptor *(58).* In addition, another form of the ER-β is present in the rat that contains an in-frame insertion of 54 nucleotides coding for an insertion of 18 amino acids with the ligand-binding domain. A major difference in the genes for the two ERs is that the α-gene is much larger (approximately threefold) than the β-gene, which has led to the speculation that the latter might be preferentially expressed at certain times in development when shorter genes are more rapidly transcribed, but this speculation remains to be established.

Table 2. Homologies among various ERs, given as percentage of amino acid identity to the human ER-β*

	Domain					
Receptor	NH-term	DBD	Hinge	LBD	F	Overall
Human β	100.0	100.0	100.0	100.0	100.0	100.0
Human α	17.5	97.0	30.0	59.0	17.9	47.0
Mouse β	80.6	98.5	84.4	91.9	78.6	88.0
Rat β	79.6	98.5	85.6	93.4	78.6	89.0

*ER = estrogen receptor; NH-term = amino terminal; DBD = DNA-binding domain; hinge = Hinge region; LBD = ligand-binding domain; F = F domain.

Despite considerable differences in sequence in the ligand-binding domain, both ER-α and ER-β bind the endogenous hormone 17β-estradiol with about the same affinity. Of interest, however, the β-receptor seems to bind some androgens (e.g., 5-androstenedione) with reasonable affinity, leading to the speculation that this receptor might be activated by androgenic steroids in some situations. In contrast to binding of estradiol, the two receptors show differences in the binding of phytoestrogens, such as genistein and coumestrol, with ER-β having substantially better affinity for these compounds than its α counterpart *(59).*

It is also clear that both receptors can stimulate transcription from the consensus ERE and that phytoestrogens, as well as estradiol, can stimulate the transcriptional activity of both receptors *(59).* In addition, ER-α and ER-β are able to form heterodimers that have transcriptional activity when assayed with the traditional ERE. It now appears that both receptors may also stimulate transcription by other non-ERE mechanisms, such as protein–protein interactions with AP-1 components. Of interest, classic ER-α agonists, such as estradiol and DES, function as antagonists in situations in which ER-β stimulates transcription via such AP-1-dependent mechanisms, whereas classic antagonists, such as tamoxifen, act as agonists *(51).*

In the prostate, ER-β appears to be under androgenic regulation, because its levels decrease with castration and can be restored by testosterone administration. Its expression in the adult prostate is highest in the epithelium and very low in the stroma. This pattern is developmentally regulated, however, because ER-β is present at high levels in both the epithelial and mesenchymal layers of the tissue at birth but is then lost from the stroma in the adult. ER-β does not appear to be regulated by ER-α, because levels of the β receptor are similar in wild-type and ERαKO mice. Although estrogens do not seem to directly regulate ER-β expression, neonatal estrogen treatment appears to decrease the expression of this receptor in certain regions of the adult prostate *(60).*

Dr. Gustafsson and his colleagues have also investigated the expression of ER-β in models of vascular injury because estrogens appear to offer protection against atherosclerotic disease. By using a model of aortic lesions in mice, it is established that estrogens promote healing and that this effect occurs equally in ERαKO mice and wild-type animals *(61).* This finding suggests that ER-β may have an important role in the vascular response to estrogens. Of interest, ER-β expression (but not ER-α) is dramatically increased in both the endothelial cells and smooth muscle cells following vascular injury, again suggesting a potential protective role for estrogens acting via ER-β.

In the female reproductive system, ER-β may play a prominent role in the ovary. During follicular development, the granulosa cells express high amounts of this receptor, and its level seems to associate with mitotic activity, whereas little receptor is seen in the thecal cells. During the second half of the cycle, ER-β levels then decline. The β-receptor is also widespread through the urogenital tract, leading to speculation that it may

mediate estrogen action on tissues such as the bladder. In this regard, there are many anecdotal reports that estrogen replacement has beneficial effects on urogenital atrophy and micturition in postmenopausal women even though the bladder does not appear to contain significant levels of ER-α, thus suggesting that the newly discovered ER-β may mediate these actions. Another major site of ER-β expression in female animals is the mammary epithelium of pregnant animals. This finding is particularly significant because these cells have previously been reported *not* to express ER-α, and it was thus thought that estrogen effects on these epithelial cells were mediated by stromal ERs. This recent finding now suggests that ER-β may directly mediate hormonal actions on the mammary epithelium.

Because of increasing interest in the possible actions of estrogens on cognitive function and Alzheimer's disease, Dr. Gustafsson and his colleagues have compared the expression patterns of ER-α and ER-β in the central nervous system of developing and mature rodents. Expression of both receptors is widespread in the central nervous system, but differences are seen in the relative expression in different brain regions *(62),* suggesting that the two receptors may mediate different functions in the brain. One speculation is that the α-receptor may play a more prominent role in reproductive behaviors and the β-receptor might play an important role in certain aspects of cognitive function, but these roles remain to be established.

Other sites in which the ER-β shows substantial levels of expression include the bone, kidney, lung, adrenal cortex, intestinal mucosa, lymph nodes, testis, sperm, thymus, spleen, and peripheral leukocytes. This expression raises the possibility that ER-β-selective agonists and antagonists might be able to produce selective effects in such tissues. These selective effects might offer some distinct advantages (e.g., for hormone replacement therapy, when one wishes to minimize the hyperproliferative actions of estrogens on the endometrium and breast). The possibility for producing selective estrogenic actions via this newly discovered receptor has thus prompted the search for such receptor selective agents.

Summary and Potential Implications for Hormonal Carcinogenesis

A number of key points emerged from this session that are likely to have particular relevance for understanding the transcriptional actions of estrogens and antiestrogens in breast, prostate, and other cancers.

1) Studies with transgenic animals overexpressing the ER-α have clearly shown that the level of expression of this protein can affect the rate of progression of several cancers. In addition, it is now clear that the ER interacts with a large number of other proteins to regulate transcription. These proteins include the so-called coactivators and corepressors as well a variety of other regulatory proteins [for recent reviews, *see (63–65)*]. Thus, in addition to the levels of the classic ER-α and the newly discovered ER-β, the levels and activities of these proteins may affect the etiology of hormone-dependent cancers, their growth responses to estrogenic substances, and their response to hormonal therapies.
2) It is now clear that different estrogens and antiestrogens can have differential effects on the multiple activation functions of ERs and that these activation functions provide the surfaces that interact with coactivators and corepressors to regulate target gene expression. This finding has radically changed our thinking about the pharmacology of estrogens, and it now appears theoretically feasible to design highly selective estrogens with minimal growth-promoting effects on breast and other tumors *(66,67).* Conversely, this finding raises the possibility that certain estrogens might play a greater role in breast and prostate cancers than in others.
3) At the cellular level, it is now clear that we must consider hormone and antihormone effects on cell death, as well as cell proliferation. We must also understand how estrogens affect the interaction of cells with their environment (e.g., substratum, vascular system, etc.) as well as mechanisms by which estrogens regulate internal production of factors that regulate cell function at intracellular sites and understand the basis for the genomic instability commonly seen in cancer cells.
4) One area briefly mentioned by Dr. Greene was the possibility that antiestrogens may play a more "active" role in the treatment of breast cancer than the simple competitive blockade of estrogen actions at the receptor site. Thus, several reports are available that antiestrogens, acting through the ER or other mechanisms, may induce the synthesis of factors normally suppressed by estrogens, or at least not expressed in the absence of antiestrogens. One area of great potential significance when considering estrogens as endogenous carcinogens are the reports that antiestrogens may induce expression of quinone reductase *(68,69).* This action would certainly be expected to decrease effects of reactive estrogenic metabolites, such as catechol estrogen quinones, or other genotoxic chemical species generated by redox cycling.

Finally, it should be emphasized that, although there is increasing interest in understanding the role of estrogens as endogenous carcinogens (Chapters 3–5) that may have actions independent of the classic ER, it is clear that ER-mediated processes play significant roles in normal and cancer cells. An increased knowledge of both types of processes is thus certain to enhance our understanding of the causes, treatments, and mechanisms to prevent many of the most prevalent human cancers.

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NOTE

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