Disruption of androgen receptor signaling by synthetic progestins may increase risk of developing breast cancer

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ABSTRACT   There is now considerable evidence that using a combination of synthetic progestins and estrogens in hormone replacement therapy (HRT) increases the risk of breast cancer compared with estrogen alone. Furthermore, the World Health Organization has recently cited combination contraceptives, which contain synthetic progestins, as potentially carcinogenic to humans, particularly for increased breast cancer risk. Given the above observations and the current trend toward progestin-only contraception, it is important that we have a comprehensive understanding of how progestins act in the millions of women worldwide who regularly take these medications. While synthetic progestins, such as medroxyprogesterone acetate (MPA), which are currently used in both HRT and oral contraceptives were designed to act exclusively through the progesterone receptor, it is clear from both clinical and experimental settings that their effects may be mediated, in part, by binding to the androgen receptor (AR). Disruption of androgen action by synthetic progestins may have serious deleterious side effects in the breast, where the balance between estrogen signaling and androgen signaling plays a critical role in breast homeostasis. Here, we review the role of androgen signaling in the normal breast and in breast cancer and present new data demonstrating that androgen receptor function can be perturbed by low doses of MPA, similar to doses achieved in serum of women taking HRT. We propose that the observed excess of breast malignancies associated with combined HRT may be explained, in part, by synthetic progestins such as MPA acting as endocrine disruptors to negate the protective effects of androgen signaling in the breast. Understanding the role of androgen signaling in the breast and how this is modulated by synthetic progestins is necessary to determine how combined HRT alters breast cancer risk, and to inform the development of optimal preventive and treatment strategies for this disease.—Birrell, S. N., Butler, L. M., Harris, J. M., Buchanan, G., and Tilley, W. D. Disruption of androgen receptor signaling by synthetic progestins may increase risk of developing breast cancer. *FASEB J.* 21, XXXX–XXXX (2007)

RECENT EVENTS HAVE necessitated evaluation of synthetic progestin use in female reproductive medicine. The World Health Organization recently cited hormonal contraceptives, of which the majority contain synthetic progestins, as contributing to increased breast cancer risk (1). This recommendation is consistent with earlier studies on HRT, including the Women's Health Initiative (WHI) (2), which reported that the use of synthetic progestins in combination HRT is associated with increased relative risk of breast cancer compared with estrogen alone (RR=1.24; 95% confidence interval 1.01–1.54). Whereas recent commentaries have highlighted a number of shortcomings in the design and analysis of the WHI trial (3–5), the weight of evidence from multiple observational studies, randomized controlled trials, and several recent meta-analyses (6–8), support the view that women taking estrogen and progestin-based HRT have an increased incidence of breast cancer. It is currently unclear whether HRT acts to increase breast cancer risk *per se*, or accelerates the development of preexisting tumors (9). Nevertheless, given that more than 25% of postmenopausal women who require HRT use a combination of estrogen and synthetic progestins, it is important to understand the actions of progestins in the breast at a molecular level. Whereas synthetic progestins are generally considered to act via progesterone receptor (PR)-mediated pathways in female reproductive tissues (10), we have demonstrated that treatment failure with the progestin medroxyprogesterone acetate (MPA) in ad-

vanced breast cancer is associated with reduced levels of androgen receptors (AR) or impaired AR function (11) and that MPA binds to the AR with an affinity comparable to the native androgenic ligand, 5α-dihydrotestosterone (DHT) (12). In addition, the observation that MPA interacts with the glucocorticoid receptor (GR) to increase levels of the metastasis suppressor gene, *nm-23*, in human breast cancer (13) provides further evidence for extensive crosstalk by progestins with non-PR signaling pathways in breast cancer cells. In this review, we will summarize the literature on the interactions between synthetic progestins, such as MPA and the AR, and discuss potential functional consequences of this interaction in breast tissue and the implications for breast cancer risk.

Estrogens, progestins, and breast cancer

Since the demonstration over 100 yr ago that removal of the ovaries could suppress the growth of breast cancer, exposure to the female sex hormone estradiol has been implicated in the development of breast cancer, potentially by modulating epithelial cell proliferation (14). Breast epithelial cells depend on a functional estrogen-signaling axis for growth and survival, and analysis of estrogen receptor knockout mice has revealed that the structural and functional development of the mammary gland is primarily controlled by one of the cellular targets for estrogens, the estrogen receptor alpha (ERα) (15). Current hormonal therapies for advanced breast cancer exploit this dependence of breast epithelial cells on estradiol for growth and survival. The most commonly used hormonal treatments either block the synthesis of estrogens or the action of estrogens at the level of ERα (16, 17). In addition to estrogens, progesterone also contributes to normal lobular-alveolar development in the breast and may influence the formation of breast cancer (17, 18). Progesterone induces the estradiol-primed endometrium into a secretory phase, and consequently synthetic progestins, which are more stable and have higher bioavailability than progesterone, have been used extensively in hormone replacement therapy (HRT) since the early 1980s to reduce the risk of uterine cancers associated with unopposed estrogen action. As synthetic progestins have diverse tissue-specific effects, which in many cases are distinct from those of the native hormone progesterone due to differences in dose, structure, specificity, and metabolism, extensive progestin-induced sequellae are well documented in women (19–22).

Synthetic progestins are classified into two main categories: *1)* the more progesterone-like pregnane derivatives (*e.g.*, medroxyprogesterone acetate, MPA) and *2)* the androstane and estrane derivatives (*e.g.*, norethisterone). In addition to binding to the two predominant PR isoforms, PR-A and PR-B, to recapitulate many of the direct effects of progesterone (17, 18), synthetic progestins bind to glucocorticoid and andro-

gen receptors (12, 20) and interact with nongenomic signaling pathways (23), to initiate a diverse range of biological effects.

The most widely prescribed progestin in the United States is medroxyprogesterone acetate (MPA). Since MPA was first formulated in 1958, it has been used in many therapeutic situations, including advanced breast cancer, as a long-acting contraceptive, and in combined HRT. Much of our understanding of MPA comes from its use in advanced breast cancer, where high doses are required to obtain a clinical response. This is in contrast to long-acting contraception and HRT, in which lower depot or oral doses are used. In the latter context, multiple studies have demonstrated that estrogen and MPA combination, but not estrogen alone, results in increased breast epithelial cell proliferation in humans and other primates and increased mammographic breast density, which is an established risk factor for breast cancer (24–27).

In Europe, MPA use is much less common than in the United States, and testosterone-like progestins (*e.g.*, levonorgestrel) or oral micronized progesterone are the preferred progestins. The testosterone-like class of progestins has, like MPA, been associated with increased breast cancer risk in observational and randomized controlled trials of HRT (28, 29). Two studies have compared the effects of the type of progestin used in combined HRT with oral estradiol and have found that systemic administration of progesterone-like and testosterone-like derivatives were both associated with increased breast cancer risk (30, 31). Notably, in France the majority of women taking combined HRT receive oral micronized progesterone rather than a synthetic progestin. In two French studies- the E3N-EPIC cohort of 54,548 women and a smaller study of 3175 women, no significant increase in breast cancer risk due to HRT use with micronized progesterone was observed compared with untreated women (32, 33). This is consistent with studies of breast epithelial cell proliferation in primates, which have shown that the combination of estrogen and progesterone has no effect on breast epithelial cell proliferation compared with estrogen alone (34, 35). These studies highlight the fact that the actions of synthetic progestins can be very different from those of the native hormone progesterone, and that classifications of progestins as progesterone- or androgen-like may not reflect the *in vivo* actions of these agents due to their interactions with PR, AR, GR and potentially other steroid receptors, especially at the doses used in HRT. For example, MPA acting through the GR may influence breast cell proliferation by modulation of the *nm-23* tumor suppressor gene expression (13). Importantly, MPA also binds with high affinity to the AR (12), and a wide body of literature indicates that the effects of MPA on androgen signaling in the breast may have important consequences for breast cancer risk and response to hormonal therapies.

Androgen signaling in the breast

There is emerging evidence that the androgen signaling pathway plays a critical protective role in breast cancer growth (36–39). The primary androgens in women are testosterone (T) synthesized in the ovaries and adrenal glands, and androstenedione and dehydroepiandrostenedione synthesized primarily in the adrenals. For the purposes of this review, we will restrict our discussion of androgens to T, and its 5α-reduced form dihydrotestostone (DHT), which has a higher affinity for binding to the AR. Androgens play key roles in regulating the functions of vital organs in women, including the reproductive tract, bone, kidneys and muscle, and can act indirectly as prohormones of estradiol or directly by binding to the androgen receptor. While the ovaries produce the majority of serum T, the bioavailability of circulating androgens is determined by the serum-binding proteins albumin and sex steroid hormone-binding globulin (SHBG), which together bind almost 99% of circulating T. There is a decline in serum T levels with increasing age, as ovarian production reduces (40), although tissue levels of androgens are potentially maintained, as both normal and cancerous breast tissues have the requisite steroid biosynthetic enzymes to produce androgens and estrogens from adrenal precursors (40–42). While little research has been performed on the role of androgen action in the normal breast, it is known that androgen excess (*e.g.*, in congenital adrenal hyperplasia or with use of anabolic steroids) suppresses breast development (reviewed in (40)). Furthermore, mice lacking a functional AR display defective mammary gland development and morphogenesis (43). In formalin-fixed, paraffin-embedded specimens of normal human mammary gland, our laboratory and others have detected AR immunoreactivity in the ductal and alveolar epithelial cells of all mammary tissues examined, with little or no expression of AR detected in the myoepithelium or stroma (44–47) (**Fig. 1**). We found AR localization to be predominantly nuclear; however, nuclear and cytoplasmic staining was observed in some tissues (*e.g.*, Fig. 1, Patient B).

Androgen signaling has a protective effect against breast cancer growth

Androgens have a predominantly inhibitory effect on the growth of breast cancer cells, both *in vitro* and *in vivo* (48–53). This inhibitory effect is mediated by the AR and is potentially due to induction of apoptosis (54, 55). Androgens (*e.g.*, fluoxymesterone) historically have been used as hormonal therapy for advanced breast cancer, demonstrating an efficacy comparable to that of tamoxifen (56, 57).

The AR is expressed in ~70–90% of primary breast tumors, a higher frequency than either estrogen (70–80%) or progesterone (50–70%) receptors, and in 75% of metastatic breast cancer deposits (44, 58–62). Expression of AR in breast tumors has been associated with increased patient survival (39, 63, 64), although a correlation between AR expression and tumor grade and stage was not found in another study (65). Levels of the androgen-regulated kallikreins (kallikreins 2, 3 (prostate-specific antigen, PSA), 6 and 10) in nipple aspirate fluid have been shown to be lower in women with breast cancer than in healthy, age-matched women (66, 67). Increased expression of PSA in breast cancer specimens and nipple aspirate fluids has been correlated with a low tumor grade, smaller tumors and a better prognosis (68). Taken together, these studies indicate that androgen signaling is protective in breast cancer and that androgen-regulated proteins such as PSA may be useful prognostic markers (66).
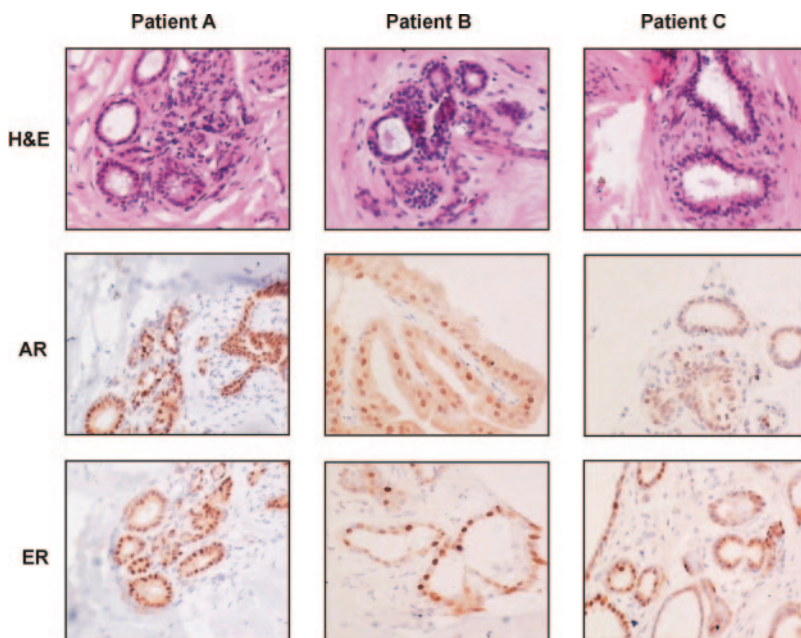

Figure 1. Expression of AR and ERα assessed by immunohistochemistry in normal breast tissue from three individuals using hematoxylin and eosin staining of the breast tissue (*A*), AR levels (*B*), ERα levels (*C*). Both AR and ERα are expressed in the ductal epithelial cells but not in the stroma. AR localization was found to be predominantly nuclear; however, nuclear and cytoplasmic staining was occasionally observed (*e.g.*, Patient B). Original magnification: ×200.

Association of AR and breast cancer risk

The length of a polymorphic glutamine repeat in the amino terminus of the AR is inversely correlated with receptor activity. As the AR is predicted to be a protective factor in breast cancer, it would be expected that less active AR alleles with longer polyglutamine repeats would predominate in women with breast cancer. However, of the numerous epidemiological studies examining the role of androgen signaling in breast cancer that measured the length of this repeat, the majority to date have shown that repeat length is not a significant modifier of breast cancer risk, age of presentation, or of tumor phenotype (69–76). Recently, the National Cancer Institute Breast and Prostate Cancer Cohort Consortium performed an extensive analysis of AR polyglutamine repeats in 5,603 breast cancer cases and 7,480 controls and found no association between repeat length and risk of breast cancer in postmenopausal women (77). Some studies have found women with AR alleles containing shorter polyglutamine repeat lengths to be at increased risk of breast cancer (78–81) and more likely to present with high-grade tumors (82) or at a younger age (83), whereas others indicated an association between longer repeat lengths and breast cancer risk (84, 85). In one study, longer polyglutamine repeat lengths in the AR were associated with increased breast cancer risk in a subset of African-American women who had a first-degree relative with breast cancer (76). Recently, increased mammographic density, a known risk factor for breast cancer, was associated with longer AR polyglutamine repeat lengths in postmenopausal women who had taken combination HRT (86). This is in contrast to the earlier Nurses' Health Study, which did not find a relationship between repeat length and mammographic density (87). However, this latter study did not stratify patients according to their prior hormonal exposure. Another study has reported that the penetrance of germline BRCA1 mutations may be influenced by AR polyglutamine repeat length (85); however, this role of the AR has been disputed by others (70, 71, 75).

While some of the variability in these population-based studies may be related to technical issues involved in polymorphism genotyping (88), a likely contributing factor to the discrepancies is nonrandom X-inactivation. Female cells heterozygous at the AR locus express only one AR allele, and it is the repeat length of this allele that will be of biological significance. Skewing of X-inactivation, involving preferential silencing of one allele, has been associated with increased risk of breast cancer (84, 89), although this has not been specifically linked to AR polyglutamine repeat length. In addition, the influence of polyglutamine repeat length on AR function, determined by *in vitro* analyses (11), is only subtle in the range observed in most of the female population and may not be a critical factor in AR function *in vivo* in some patients.

Interactions between androgen and estrogen signaling pathways in breast cancer cells

Recent studies suggest that in addition to the direct, AR-mediated genomic actions of androgens, another mechanism by which androgens inhibit the growth of breast cancer cells is to oppose the growth-stimulatory effects of estrogen-mediated activation of ERα. Androgens decrease expression of ERα mRNA and protein in breast cancer cells (90), and the AR can inhibit ERα but not ERβ activity, potentially via direct binding of AR to ERα (91). *In vivo* studies have demonstrated that administration of estradiol to ovariectomized rhesus monkeys induces breast epithelial cell proliferation, but coadministration of estradiol and testosterone prevents this increase in proliferation (34). Furthermore, coadministration of testosterone suppresses the estradiol-mediated induction of MYC, consistent with the notion that androgens suppress estrogen signaling pathways (34). Thus, the extent that androgens oppose estrogen signaling in breast tissue may play a critical role in controlling cellular proliferation and maintaining tissue homeostasis in the breast. Conventional estrogen treatment regimens, both as oral contraceptives and as HRT, may upset the normal estrogen/androgen balance and promote unopposed estrogenic stimulation of mammary epithelial cell proliferation (92). The suppression of gonadotropins by exogenous estrogen treatment results in a systemic reduction in ovarian steroidogenesis, resulting in a lower concentration of circulating T. Moreover, estrogens, particularly in oral form, stimulate the hepatic production of sex hormone-binding globulin (SHBG) (93), which binds with relatively high affinity to T, thereby reducing the bioavailability of androgens. The consequence of these dual effects is that both total and bioavailable testosterone levels are significantly reduced in women taking oral contraceptives or estrogen replacement for ovarian insufficiency (92). These findings, along with the documented inhibitory effects of androgens on breast cancer cell growth, have led to proposals for androgens to be included in combined HRT preparations in preference to synthetic progestins (94, 95).

BRCA1 modulates ERα and AR signaling in the breast

The balance between estrogen and androgen signaling pathways in breast cells may be further modulated by the tumor suppressor, BRCA1. Whereas BRCA1 inhibits both ligand-dependent and -independent transactivation activity of ERα (96, 97), we and others have reported that BRCA1 enhances the activity of the AR in prostate and breast cancer cell lines (98, 99). The potentiation of AR signaling by BRCA1 occurs predominantly via direct interaction of the two proteins (98, 99). While the *in vivo* relevance of opposing effects of BRCA1 on AR and ERα activity is not known, it is plausible that this action of BRCA1 could accentuate

the functional consequences of alterations in estrogen/androgen ratios in breast epithelial cells.

Disruption of AR signaling in the breast by synthetic progestins

Clinical studies by our laboratory and others have demonstrated that the response of breast tumors to high-dose MPA therapy is dependent on expression of the AR, but not the level of PR (100) and that the progression-free interval in response to MPA is directly proportional to the level of AR in the primary tumor (100, 101). Furthermore, we have recently identified a correlation between inactivating mutations in the AR gene in breast tumors and the failure of second-line MPA therapy (11). Our *in vitro* analyses have demonstrated that MPA has a comparably high affinity for the AR as DHT, and at high doses (100 nM), it can inhibit the proliferation of AR-positive, but not AR-negative, breast cancer cell lines (12, 102). AR antagonists can reverse the inhibitory effects of MPA on the proliferation of breast cancer cell lines (12, 102), consistent with a genomic effect of MPA-activated AR on breast cancer growth.

Crystal structure analysis of the AR has revealed that the binding of the native ligand, DHT, results in a distinct conformational arrangement of the ligand binding domain (LBD), and the formation of a conserved cleft, termed AF2, which is critical for interaction with accessory proteins that ultimately determine the program of AR-directed nuclear events (103). It is clearly evident from our recent studies that MPA, while able to act through the AR to modulate breast cancer cell growth, results in the adoption of an atypical LBD structure distinct from that mediated by DHT (**Fig. 2A-D**). In particular, the size of MPA forces the displacement of a key amino acid residue, Phe874, such that it projects abnormally into the AF2 cleft (Fig. 2A–D). It is therefore likely that DHT and MPA would have diver-

gent effects on AR-regulated gene expression, a hypothesis that has recently been corroborated experimentally (104).

Data from our and Elizabeth Wilson's laboratories suggest that at lower doses (<10 nM), MPA no longer acts as an AR agonist, but rather disrupts critical aspects of AR signaling (105). In the presence of a classical agonist, the transactivation capacity of the AR is mediated primarily by an interaction between the N-terminal transactivation domain and the AF2 cleft in the C-terminal LBD (depicted in **Fig. 3A**). This so-called "N/C interaction" is also dependent on the level of cellular cofactors that compete for the AF-2 binding surface in the LBD (106–107). Our data demonstrate that, at lower concentrations (1–10 nM), MPA is a very weak inducer of the N/C interaction in its own right (Fig. 3B), consistent with disruption of AF2 but is a potent antagonist of DHT-induced N/C interaction (Fig. 3C). This inhibition by MPA is significantly greater than that achieved by either progesterone or the specific AR antagonist hydroxyflutamide (Fig. 3C). The different molecular alterations in AR structure induced by low *vs.* high concentrations of MPA could explain both the divergent patterns of gene expression observed with the different doses, and also why MPA at high doses inhibits growth of breast cancer, whereas at low doses, such as those used in HRT, a greater effect is seen on breast density consistent with MPA treatment, resulting in an increased risk of breast cancer.

MPA–AR interaction in the breast

On the basis of current knowledge and our own data, we have generated a model for AR and ERα signaling in the normal breast and in breast cancer cells (**Fig. 4**). We hypothesize that a balance between these pathways is critical for the regulation of breast cell growth. Activation of AR results in AR binding to ERα and direct inhibition of ERα signaling in breast cells, and
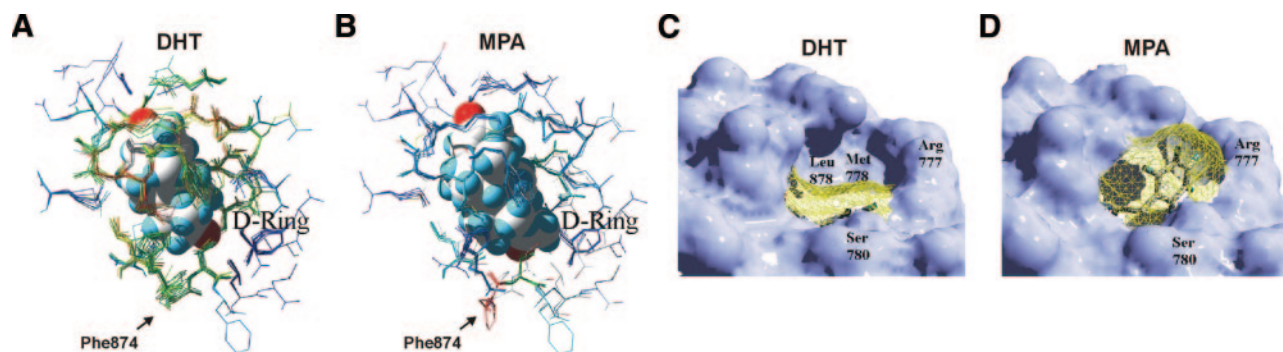

Figure 2. Divergent effects of DHT and MPA on AR structure and AR-mediated gene expression in breast cancer cells. Molecular model of the AR-LBD bound to DHT (*A*) and MPA (*B*). Shown for each is a superimposition of 10 independent molecular dynamics solutions for the AR-LBD depicting only those residues (in stick form) within 7Å of bound ligand. The shift from blue to red coloring represents minimal to maximum residue displacement, respectively, from the established crystal structure. The displacement of Phe874 in the MPA bound AR is clearly evident. Topography of the AR LBD surface in the vicinity of Phe874 when bound to DHT (*C*) and MPA (*D*) is shown. The contribution of Phe874 (shown in stick form) to the surface is represented by a gold mesh. Displacement of Phe874 by MPA disrupts the well-defined AF-2 cleft observed for the DHT-bound receptor.
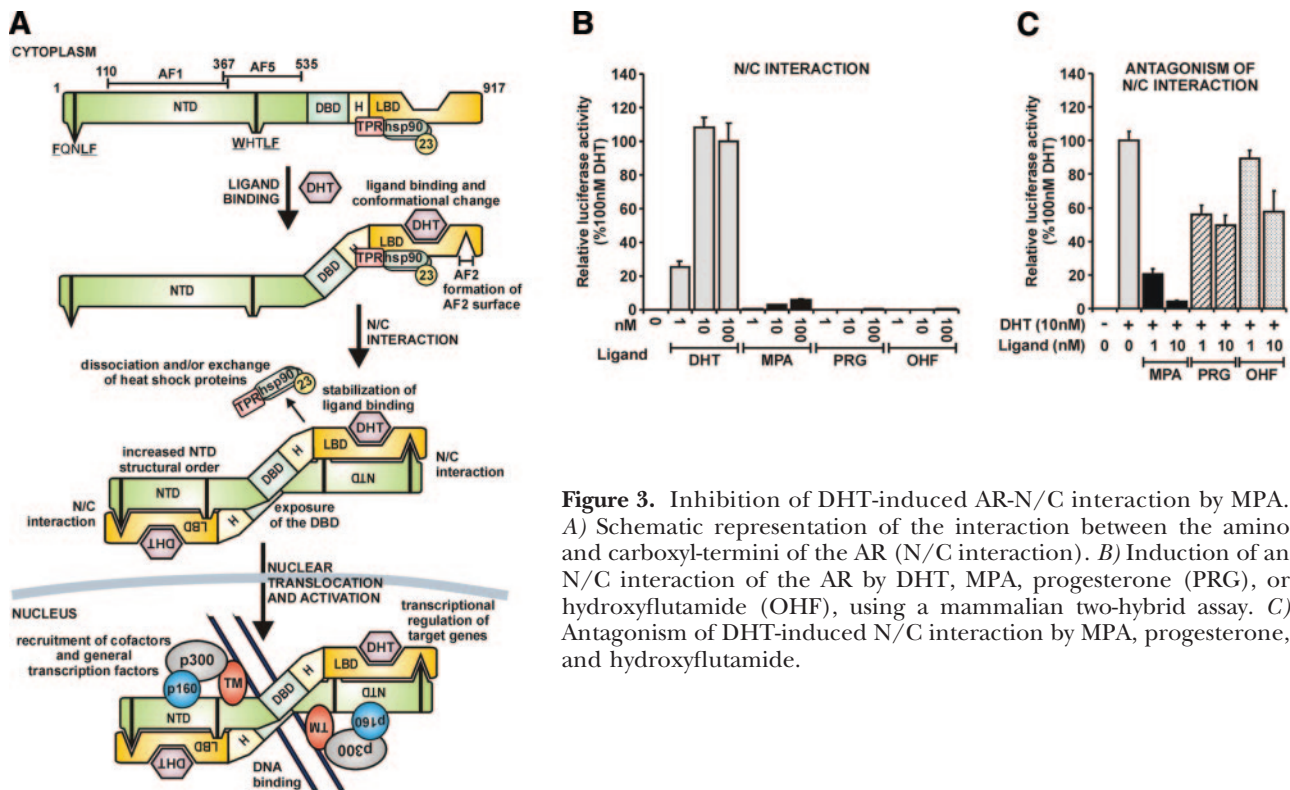
Figure 3. Inhibition of DHT-induced AR-N/C interaction by MPA. *A)* Schematic representation of the interaction between the amino and carboxyl-termini of the AR (N/C interaction). *B)* Induction of an N/C interaction of the AR by DHT, MPA, progesterone (PRG), or hydroxyflutamide (OHF), using a mammalian two-hybrid assay. *C)* Antagonism of DHT-induced N/C interaction by MPA, progesterone, and hydroxyflutamide.

this inhibition of ERα activity, in turn, suppresses growth. In addition, activation of AR may influence breast cell growth independently of ERα signaling, through direct activation of androgen-regulated genes or nongenomic mechanisms. The effects of both AR and ERα signaling may, in turn, be potentiated by the tumor suppressor protein BRCA1.

Agents such as MPA that perturb the balance between androgen and estrogen signaling in the normal breast could result in deleterious tissue-specific effects. We propose that MPA can act as an endocrine disruptor to negate the protective effects of androgen signaling in the breast (Fig. 4). In addition to its effects

Figure 4. Model of interactions between AR, ER-α, and BRCA1 in breast cells, and potential interactions of the synthetic progestin, MPA, with these pathways.

mediated by PR signaling, which may alter breast cell growth positively or negatively depending on the relative levels of A and B isoforms of the PR (18); MPA may exert direct effects on breast cell growth via AR signaling, or act indirectly through disruption of the estrogen/androgen signaling balance or by modulation of nonclassically AR-regulated genes such as BRCA1. The functional consequences of polymorphisms in the AR (*e.g.*, polyglutamine repeat length) or other enzymes involved in steroid hormone biosynthesis may ultimately fine-tune the effect of synthetic progestins on the breast at an individual level. Unfortunately, there are great deficiencies in our knowledge of the consequences of perturbating the estrogen/androgen balance in women, and it is our goal that this review will stimulate wider debate on this important aspect of a critical women's health issue. Moreover, a better understanding of androgen action in the breast is essential given the increasing use of androgens in women for sexual dysfunction and HRT, and that virtually all postmenopausal women with breast cancer will be treated for at least 5 yr with the new generation aromatase inhibitors in breast cancer adjuvant therapy. $\boxed{\text{FJ}}$
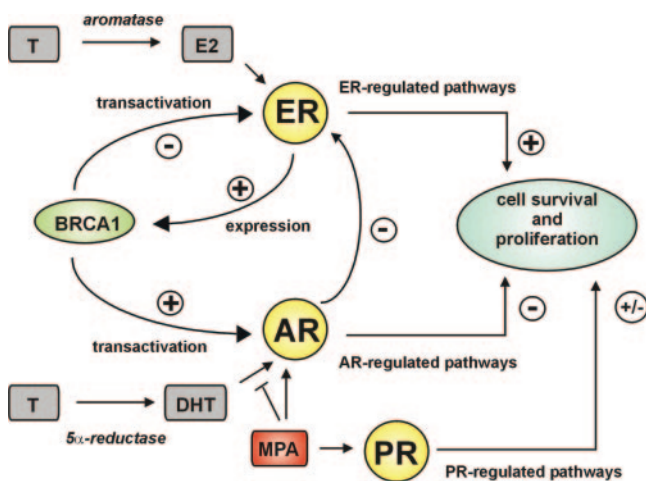
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