

# Androgen Receptor in Prostate Cancer

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The normal development and maintenance of the prostate is dependent on androgen acting through the androgen receptor (AR). AR remains important in the development and progression of prostate cancer. AR expression is maintained throughout prostate cancer progression, and the majority of androgen-independent or hormone refractory prostate cancers express AR. Mutation of AR, especially mutations that result in a relaxation of AR ligand specificity, may contribute to the progression of prostate cancer and the failure of endocrine therapy by allowing AR transcriptional activation in response to antiandrogens or other endogenous hormones. Similarly, alterations in the relative expression of AR coregu-

lators have been found to occur with prostate cancer progression and may contribute to differences in AR ligand specificity or transcriptional activity. Prostate cancer progression is also associated with increased growth factor production and an altered response to growth factors by prostate cancer cells. The kinase signal transduction cascades initiated by mitogenic growth factors modulate the transcriptional activity of AR and the interaction between AR and AR coactivators. The inhibition of AR activity through mechanisms in addition to androgen ablation, such as modulation of signal transduction pathways, may delay prostate cancer progression. (*Endocrine Reviews* 25: 276–308, 2004)

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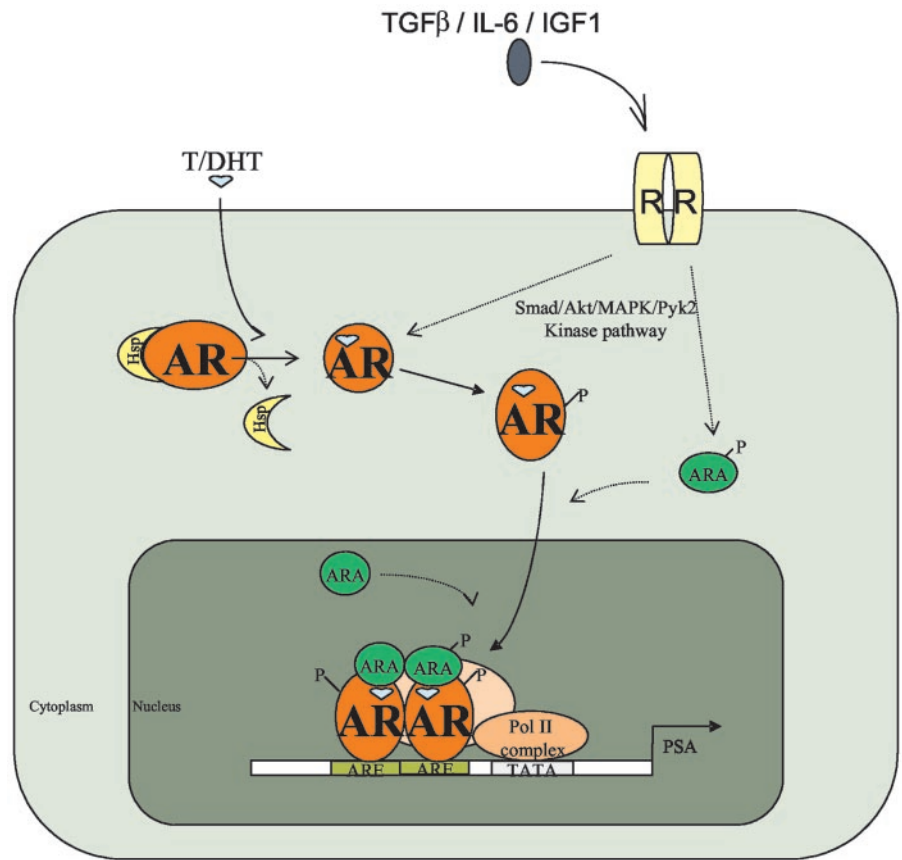
## I. Introduction

ANDROGENS, ACTING THROUGH the androgen receptor (AR), are required for prostate development and normal prostate function (1). Androgen action can be considered to function through an axis involving the testicular synthesis of testosterone, its transport to target tissues, and the conversion by 5 $\alpha$ -reductase to the more active metabolite 5 $\alpha$ -dihydrotestosterone (DHT). Testosterone and DHT exert their biological effects through binding to AR and inducing AR transcriptional activity (Fig. 1). The androgen-induced transcriptional activation of AR is modulated by the interaction of AR with coregulators and by phosphorylation of AR and AR coregulators in response to growth factors (1–4). AR and the modulators of AR activity remain important in prostate cancer. Approximately 80–90% of prostate cancers are dependent on androgen at initial diagnosis, and endocrine therapy of prostate cancer is directed toward the reduction of serum androgens and inhibition of AR (5). However, androgen ablation therapy ultimately fails, and prostate cancer progresses to a hormone refractory state. AR is expressed throughout prostate cancer progression and persists in the majority of patients with hormone refractory disease (6–10). Also, most identified AR mutations from hormone refractory prostate cancer are capable of transcriptional activity (Table 1). These observations suggest that loss of AR function is not a major cause of androgen ablation failure and that AR-negative prostate cancer cells do not have a significant growth or survival advantage. Instead, the avail-

Abbreviations: AIB1, Amplified in breast cancer-1; AR, androgen receptor; ARE, androgen response element; bFGF, basic FGF; DES, diethylstilbestrol; DHEA, dehydroepiandrosterone; DHT, 5 $\alpha$ -dihydrotestosterone; DIM, 3,3'-diindolymethane; EGF, epidermal growth factor; EGFR, EGF receptor; ESE2, epithelium-specific Ets factor 2; Ets, E twenty-six; FGF, fibroblast growth factor; HF, hydroxyflutamide; IGF-IR, IGF-I receptor; IGFBP, IGF binding protein; LBD, ligand binding domain; nmt55, nuclear matrix protein, 55 kDa; PDEF, prostate-derived Ets factor; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog; PYK2, proline-rich tyrosine kinase 2; Rb, retinoblastoma susceptibility gene; SH2, Src homology 2; SMRT, silencing mediator of retinoid and thyroid hormone receptor; SRC, steroid receptor coactivator; STAT, signal transducer and activator of transcription; Tfm, testicular feminized; TIF, transcriptional intermediary factor.

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FIG. 1. Androgen-AR action in the prostate. Testosterone (T) and DHT bind to AR and promote the association of AR coregulators (ARAs). AR then translocates to the nucleus and binds to AREs in the promoter regions of target genes to induce cell proliferation and apoptosis. Other signal transduction pathways, such as those involving TGF $\beta$ , IL-6, and IGF-I, can also enhance AR activity via phosphorylation of AR and/or ARAs. Hsp, Heat shock protein; R, membrane receptor; P, protein phosphorylation.



able clinical and experimental evidence suggests that prostate cancer progression occurs through alteration of the normal androgen axis by dysregulation of AR activity through signal transduction cascades, alteration in the expression of AR coregulators, and mutations of AR that enable it to become transcriptionally active in response to ligands in addition to testosterone and DHT.

## II. AR in the Normal Prostate

### A. Androgens and AR in normal prostate development

The prenatal development of the prostate is dependent on androgen, particularly on DHT. Although the fetal testis produces testosterone, the intracellular reduction of testosterone by 5 $\alpha$ -reductase is necessary for complete prostate morphogenesis. At about 10 wk gestation in the human, the ductal structure of the prostate arises from epithelial outgrowths of the urogenital sinus and moves into the surrounding mesenchyme immediately below the developing bladder. The 5 $\alpha$ -reductase enzyme is present in the urogenital sinus before and during prostate development (11, 12). In individuals lacking a functional 5 $\alpha$ -reductase gene, the prostate is small or undetectable. In rats, inhibition of 5 $\alpha$ -reductase during fetal development results in partial prostate development (13). The partial prostate formation that occurs with 5 $\alpha$ -reductase deficiency suggests that the initiation of prostate development can occur in response to extremely low levels of DHT or in response to testosterone alone, but also that a threshold level of DHT is necessary for

complete prostate morphogenesis (14). The initiation of prostate development is dependent on a functional AR. The prostate is absent in AR knockout mice (15), testicular feminized (Tfm) mice, and individuals with complete androgen insensitivity due to an inactivating mutation of AR (4, 16, 17). The formation of prostatic buds results from epithelia-stroma interaction that requires the presence of a functional AR in the urogenital mesenchyme but not in the epithelia, suggesting that DHT-regulated growth factors are secreted by the mesenchyme and act upon the developing prostate epithelium (4, 11). In tissue recombinants, the urogenital epithelia of fetal Tfm mice can form prostatic buds when placed in contact with wild-type fetal stroma and grafted into intact male mice. However, wild-type urogenital epithelia is unable to form prostatic buds when combined with Tfm urogenital stroma (18, 19). Consistent with these experiments, AR is expressed in the fetal mouse urogenital mesenchyme but not in the prostate epithelial buds. Prostate epithelium expresses AR in late fetal or early neonatal development when AR function has been implicated in the final morphogenesis of the prostate and the initiation of prostate secretory protein expression (20–22).

The hypothesis that a receptor might be needed to mediate the biological effects of androgens developed in the 1960s. At first, numerous efforts were made to isolate/purify the AR without success. Autoimmune anti-AR antibodies from human serum were found to be able to precipitate an [ $^3$ H]-R1881-AR complex (22a), yet attempts to use those autoimmune anti-AR antibodies to purify AR or isolate AR cDNA were still unsuccessful, due to their lack of specificity. Even-

TABLE 1. Mutations associated with prostate cancer

Exon	Position	Change codon/ amino acid	Characteristics	Ref.
TIS	+2 bp	CAG→CAT	Germline mutation from a prostate cancer patient with no known family history of prostate cancer. Mutation abolishes the initiation CAG of the transcription initiation site II (TISII).	404
5' UTR	+214 bp	GCC→GAC	Germline mutation from a patient with a familial history of prostate cancer.	404
1			Contraction of the polyglutamine repeat from 20 to 18	151
1			Contraction of the polyglutamine repeat from 24 to 18. Detected in a radical prostatectomy sample prior to hormonal therapy.	405
1	57	CTG→CAG L→Q	Isolated from a TURP sample prior to hormonal therapy.	330
1	111	CAG→CAT Q→H	Isolated from a TURP sample prior to hormonal therapy.	330
1	167	GGC→AGC G→S	Identified in a TURP sample from a hormone refractory tumor after combined treatment of orchiectomy and bicalutamide.	406
1	179	AAA→AGA K→R	Isolated from a TURP sample prior to hormonal therapy.	330
1	198	GAA→GGA E→G	Isolated from a bone marrow metastasis from a patient with hormone refractory cancer after treatment with bicalutamide.	407
1	269	CCA→TCA P→S	Isolated from a TURP sample prior to hormonal therapy.	330
1	330	TCC→CCC S→P	Isolated from a bone marrow metastasis from a patient with hormone refractory cancer after treatment with flutamide	407
1	527	GAT→GGT D→G	Isolated from a TURP sample prior to hormonal therapy.	330
2	546	TTG→TTC L→F	Deletion resulting in a frameshift mutation expected to result in 12 missense amino acids prior to a stop codon. Detected in an archival latent prostatic tumor sample from a Japanese man.	327
2	553	CCA→CCC P→P	Deletion causing a frameshift expected to result in 5 missense amino acids prior to a stop codon. Detected in two archival latent prostatic tumor samples from Japanese men.	327
2	574	ACA→GCA T→A	Isolated from a pelvic lymph node metastasis. This mutant can be weakly activated by DHEA <i>in vitro</i> .	152, 328
2	579	AAG→AGG K→R	Isolated from a pelvic lymph node metastasis. This mutant exhibits weak constitutive activity and can be transcriptionally activated by DHT, DHEA, flutamide, bicalutamide, hydrocortisone, estradiol, and progesterone.	152, 328
2	585	GCC→GTC A→V	Isolated from a pelvic lymph node metastasis sample. This mutant is transcriptionally inactive <i>in vitro</i> .	152, 328
2	586	GTC→TCT A→S	Isolated from a pelvic lymph node metastasis. This mutant can be activated by DHEA <i>in vitro</i> .	152, 328
3	618	TGT→TAT C→Y	Identified in a pelvic lymph node metastasis. This mutation prevents AR DNA binding, resulting in loss of transcriptional activity.	328, 408
4	670	ATC→ACC I→T	Isolated from a TURP sample prior to hormonal therapy. This mutant can be weakly activated by DHEA <i>in vitro</i> .	152, 330
4	683	GGT→GCT G→A	Isolated from two separate individuals with hormone refractory tumors carrying an amplification of AR. Conflicting results regarding the <i>in vitro</i> activity of this mutation have been reported, with one group finding that the transcriptional activity does not differ from the wild-type receptor and another group observing that the response of the mutant receptor to DHT is significantly reduced compared to wild type.	150–152
4	700	CTC→CAC L→H	Isolated from a prostatic autopsy sample from a patient treated with castration and chlormadinone acetate whose cancer had become hormone resistant. Cancerous tissues from metastatic sites from the same patient showed a separate AR mutation (T876A). This mutant can be transcriptionally activated by DHT, DHEA, estradiol, hydrocortisone, progesterone, bicalutamide, and flutamide.	152, 329
4	714	GTG→ATG V→M	Isolated from a fine-needle biopsy sample from a patient with metastatic prostate cancer. Prior to biopsy, the patient had been treated by castration followed by flutamide and estracyte. At the time of biopsy, the cancer was hormone refractory. There is no significant difference in the relative binding affinity between this mutant and the wild-type AR. However, this mutant demonstrates an enhanced transcriptional activation in response to HF, progesterone, DHEA, estradiol, androstene, androstanediol and androstenedione.	152, 340, 341, 409
4	719	AAG→GAG K→E	Isolated from a bone metastasis. Transcriptional activation of the mutant receptor <i>in vitro</i> does not differ significantly from the wild-type receptor in response to R1881 or DHT.	152, 410

TABLE 1. Continued

Exon	Position	Change codon/ amino acid	Characteristics	Ref.
4	720	GCC→ACC A→T	Isolated from a hormone refractory bone marrow metastasis from a patient treated with flutamide and an LHRH agonist. Does not become transcriptionally active in response to estradiol or progesterone. Shows moderate transcriptional activation in response to 1 μM nilutamide, but not HF or bicalutamide. Demonstrates a reduced response to DHT <i>in vitro</i> compared to the wild-type receptor.	152, 336, 341
5	725	CGC→CTC R→L	Germline mutation observed in 2% of Finnish sporadic prostate cancer patients. The AR CAG repeat length is 26 in 85% of mutation carriers. This mutation does not alter the ability of the mutant AR to bind mibolerone, DHT, estradiol, or progesterone. However, this mutation enhances AR transcription in response to estradiol compared to the wild-type receptor in CV-1 cells. This mutation also can be activated by DHEA <i>in vitro</i> .	152, 342, 411
5	729	GTG→ATG V→M	Isolated from a patient with organ-confined prostate cancer who had not received hormonal therapy. This mutant shows enhanced transactivation in response to HF, androsterone, and androstenediol. However, the relative binding affinity of the mutant receptor for R1881, androstenediol, androsterone, and androsterone is the same as the wild-type receptor.	409, 412
5	740	TGG→TAG W→Stop	Detected in an archival latent prostatic tumor sample from a Japanese man.	327
5	740	TGG→TGT W→C	Identified from a TURP sample from a hormone refractory tumor treated with a combination of orchiectomy and bicalutamide. The same amino acid substitution has also been isolated from a bone marrow metastasis from a patient with hormone refractory cancer after treatment with bicalutamide.	406, 407
5	742	GGG→GGC G→G Stop	Deletion causing a frameshift mutation resulting in a stop codon after 1 amino acid. Detected in an archival latent prostatic tumor sample from a Japanese man. A separate latent tumor foci in the same individual contained the L743F mutation.	327
5	743	CTC→TTC L→F	Detected in an archival latent prostatic tumor sample from a Japanese man. A separate latent tumor foci from the same individual contained the Δ742 mutation.	327
5	747	GGC→GTC A→V	Detected in an archival latent prostatic tumor sample from a Japanese man. This mutation results in a decreased transcriptional response to DHT compared to the wild-type receptor <i>in vitro</i> . Can also be transcriptionally activated by DHEA <i>in vitro</i> .	152, 327
5	748	ATG→ATA M→I	Detected in an archival latent prostatic tumor sample from a Japanese man. This mutant does not become transcriptionally active <i>in vitro</i> in response to DHT, DHEA, progesterone, estradiol, hydrocortisone, flutamide, or bicalutamide.	152, 327
5	748	ATG→ATA M→I	Isolated from a TURP sample from a hormone refractory tumor after treatment by orchiectomy and bicalutamide.	406
5	749	GGC→AGC G→S	Detected in an archival latent prostatic tumor sample from a Japanese man. This mutant can be transcriptionally activated <i>in vitro</i> by DHEA.	152, 327
5	750	TGG→TAG W→Stop	Detected in two archival latent prostatic tumor samples from Japanese men.	327
5	754	ACC→GCC T→A	Detected in an archival latent prostatic tumor sample from a Japanese man.	327
5	756	GTC→GCC V→A	Isolated from a pelvic lymph node metastasis. The mutant receptor can be transcriptionally activated by DHEA <i>in vitro</i> .	152, 328
5	758	TCC→CCC S→P	Detected in an archival latent prostatic tumor sample from a Japanese man. This mutant shows a reduced transcriptional activity in response to DHT <i>in vitro</i> compared to the wild-type receptor.	152, 327
5	762	TAC→TGC Y→C	Detected in an archival latent prostatic tumor sample from a Japanese man. This mutant become transcriptionally active <i>in vitro</i> in response to DHT but not estradiol, DHEA, progesterone, hydrocortisone, flutamide, or bicalutamide.	152, 327
6	781	AGC→AAC S→N	Isolated from a TURP sample prior to hormonal therapy. The mutant can be transcriptionally activated by DHT and DHEA <i>in vitro</i> .	152, 330
6	795	TGG→TGA W→STOP	Isolated from a TURP sample prior to hormonal therapy.	330
7	845	AGA→GGA R→G	Isolated from a pelvic lymph node metastasis. This mutation can show transcriptional activation <i>in vitro</i> in response to DHT and DHEA.	152, 328
8	865	GTG→ATG V→M	Detected in an archival latent prostatic tumor sample from a Japanese man. This mutation fails to be activated by DHT, DHEA, estradiol, progesterone, hydrocortisone, flutamide, or bicalutamide <i>in vitro</i> .	152, 327

Continued on next page

TABLE 1. Continued

Exon	Position	Change codon/ amino acid	Characteristics	Ref.
8	873	CAT→TAT H→Y	Isolated from a hormone refractory bone marrow metastasis from a patient treated with flutamide and an LHRH agonist. The tumor was initially treated with local radiotherapy. Shows enhanced <i>in vitro</i> transcriptional activity in response to DHEA, estradiol, and progesterone compared to the wild-type receptor. Transcription can also be induced by the antiandrogens nilutamide and HF, but not bicalutamide.	152, 336, 341
8	876	ACT→AGT T→S	Isolated from a hormone refractory bone marrow metastasis from a patient treated with flutamide and an LHRH agonist. The tumor was initially treated with local radiotherapy. Also identified in a patient whose tumor contained both the T876S and T876A mutations. In addition to testosterone and DHT, this mutant can be induced by HF, casodex, and cyptoteron acetate.	335, 336, 341, 343
8	876	ACT→GCT T→A	In one study, this mutation was found in 31% of patients examined with hormone refractory disease after surgical or chemical castration plus flutamide treatment. A separate study found this mutation in three metastatic cancerous loci in 1 of 8 (12.5%) of patients with hormone-resistant prostate cancer that had been treated with chlormadinone acetate. This patient had a different AR mutation (L700H) in cancerous foci in the prostate. A separate study found this mutation in archival TURP sections from 6 of 24 (25%) prostate cancer patients. This mutation is present in the AR expressed in LNCaP cells.	329, 334, 335, 337
8	878	GAC→GCC D→G	Isolated from a bone marrow metastasis from a patient with hormone refractory cancer after treatment with bicalutamide. Shows enhanced <i>in vitro</i> transcriptional activity in response to estradiol and progesterone compared to the wild-type receptor. In addition, the antagonists nilutamide and HF, but not bicalutamide, activate transcription of this mutant.	407
8	889	GAC→AAC D→N	Isolated from a bone marrow metastasis from a patient treated with LHRH agonist therapy with hormone refractory disease. Mutation is associated with an increase in transcription in response to DHEA <i>in vitro</i> .	152, 335
8	901	CAA→CGA Q→R	Isolated from a hormone refractory bone marrow metastasis from a patient treated with flutamide and an LHRH agonist. The tumor was initially treated by radical prostatectomy. In transfection assays, shows approximately 37% of wild-type AR activity at 1 nM DHT. Demonstrates marginal transcriptional activity in response to 0.01–10 nM androstenedione and no activity in response to up to 1 μM estradiol or progesterone.	336, 341
8	908	GGG→GAG G→E	Detected in an archival latent prostatic tumor sample from a Japanese man.	327

Because AR contains two trinucleotide repeat regions that are polymorphic in length, amino acid positions may vary between publications. In this table, amino acid positions are as numbered in Ref. 99. Silent mutations have not been included. TURP, Transurethral resection of the prostate; UTR, untranslated region.

tually, use of a DNA oligonucleotide probe that was homologous to other steroid receptors allowed Chang *et al.* (22b) and Lubahn *et al.* (22c) to isolate full-length human AR cDNAs, from which *in vitro* transcribed/translated protein was generated, and found to bind to [<sup>3</sup>H]-R1881 with a  $K_d$  of 0.3 nM (22b). Structural analysis of AR revealed that it contains four functional domains, similar to other members of the steroid receptor superfamily: a conserved DNA binding domain (DBD), a hinge region, a ligand-binding domain (LBD), and a less conserved amino-terminal domain (22b, 22c). Further analysis of AR structure revealed two transcriptional activation function domains, including the N-terminal ligand-independent AF-1 domain and the C-terminal ligand-dependent AF-2 domain.

### B. Androgens and AR in the maintenance of prostate epithelia

After the development of the prostate, androgens continue to function in promoting the survival of the secretory epi-

thelia, the primary cell type thought to be transformed in prostate adenocarcinoma (23). In the normal prostate, the rate of cell death is 1–2% per day, which is balanced by a 1–2% rate of proliferation (24, 25). The reduction of serum and prostatic DHT levels by castration results in a loss of 70% of the prostate secretory epithelial cells due to apoptosis in adult male rats, but the basal epithelia and stromal cell populations are relatively unaffected (26). In the intact rat prostate, the secretory epithelial cells show strong AR immunoreactivity, whereas the majority of basal epithelial cells are AR negative (27), suggesting an explanation for their different sensitivity to androgen. However, AR is also expressed in the prostatic stroma, although castration results in the loss of stromal AR expression (27, 28). The prostatic stroma therefore has the capacity to respond to androgen, but androgen is not required for its survival. Physiological testosterone levels prevent secretory rat prostate epithelial apoptosis. However, normal epithelial function is dependent on prostatic DHT levels (29). Superphysiological levels of serum

androgen in dogs and in human habitual anabolic steroid users result in an increase in cellular proliferation in the prostate (30, 31). In humans, the proliferation occurs predominantly in the transitional zone of the prostate, the region that is primarily affected in benign prostatic hypertrophy but is seldom the initial site of prostate carcinoma formation (30, 32). Although individual cases of prostate cancer have been reported in anabolic steroid users (33), epidemiological studies have failed to establish a link between elevated serum testosterone, DHT, or adrenal androgens and prostate cancer risk (reviewed in Ref. 34), suggesting that elevated testicular and adrenal androgens alone do not significantly promote prostate carcinogenesis.

In addition to apoptosis of secretory epithelial cells, castration also results in apoptosis and degeneration of prostatic capillaries and constriction of larger blood vessels, which precedes the appearance of epithelial apoptosis (35, 36). These observations suggest that the reduction of blood flow to the prostate may contribute to epithelial apoptosis. However, castration does not induce necrosis or apoptosis in all prostatic cell types, suggesting that if secretory epithelial cell loss is influenced by the alteration in blood flow, these cells are more sensitive to this change than other prostate cell types. Administration of testosterone to castrated rats results in vascular regrowth followed by reconstitution of the secretory epithelia (37). However, the vascular endothelial cells of the rat prostate do not express AR (27). In the normal prostate, cellular homeostasis is modulated in part by paracrine growth factor regulation between epithelial and stromal cells (38). A subset of these growth factors, including basic fibroblast growth factor (bFGF) and vascular endothelial growth factor, can be regulated by androgens and can influence vascular survival (38–41). It is possible that castration initially alters prostatic growth factor production in the stroma, which contributes to a decrease in vascular function. The resulting reduction in blood flow, combined with an altered growth factor environment and decreased expression of other androgen regulated proteins, may contribute to apoptosis of the secretory epithelia.

### III. AR Expression and Prostate Carcinogenesis

Although serum androgens alone may not promote prostate carcinogenesis, androgen action and the functional status of AR are important mediators of prostate cancer progression. Low serum testosterone levels in men with newly diagnosed and untreated prostate cancer have been found to correlate with higher AR expression, increased capillary vessel density within the tumor, and higher Gleason score (42). Recent analysis of clinical prostate cancer specimens also collected from patients without preoperative treatment demonstrated that high AR expression correlated with lower recurrence-free survival and disease progression (43). The endocrinological treatment of prostate cancer primarily involves the modulation of AR activity through the deprivation of circulating testicular androgens by surgical castration or chemical castration with LHRH agonists. The activity of AR may also be blocked by the administration of antiandro-

gens, either alone or in combination with surgical or chemical castration (referred to as combined androgen blockade). Over 80% of patients show a positive response to androgen ablation. However, patients with metastatic prostate cancer eventually experience disease progression in a median of 12 to 18 months after androgen deprivation therapy. The tumors of these patients are considered to be hormone refractory. Although these tumors are refractory in the sense that they have progressed despite a reduction in serum androgen and/or treatment with antiandrogens, the majority of these tumors are unlikely to be completely resistant to androgen. In 97% of patients with hormone refractory metastatic prostate cancer, exogenous androgen treatment results in disease flare and unfavorable response (reviewed in Ref. 44). Secondary therapy for patients with hormone refractory prostate cancer is also predominantly targeted at androgen production and AR function and includes administration of a secondary antiandrogen, inhibition of adrenal androgen production, and further LH inhibition with progesterone or estrogenic agents (45). Although secondary hormonal therapy also eventually fails, the ability of therapies directed toward AR to provide positive therapeutic benefit suggests that AR activity is an important mediator of prostate cancer growth and survival.

#### A. AR expression in prostate cancer

AR expression is observed in primary prostate cancer and can be detected throughout progression in both hormone-sensitive and hormone refractory cancers (8, 9, 46). Immunohistochemical studies have shown that AR expression is heterogeneous in prostate cancer and that the degree of heterogeneity does not generally correlate with response to androgen deprivation therapy (8, 46). However, we and others have observed that a higher degree of AR positivity correlates with a greater degree of differentiation or lower Gleason score (9, 47, 48), although this is not a universal observation (10, 46). Although animal models of prostate cancer have suggested that elevation of AR expression can initiate prostate cancer development (49) or is associated with recurrent growth in the presence of low androgen (50), the persistent heterogeneity of human prostate cancer suggests that increased AR expression is not generally associated with prostate cancer initiation, and that hormone refractory prostate cancers are not clonally selected from AR-negative foci. The cause of the loss of AR expression in some cells of tumor foci is unclear. X chromosome losses, including loss of the AR gene, are extremely rare in prostate cancer (51–53). Epigenetic silencing of AR expression by methylation may occur and has been observed in 8% of primary prostate cancers (54). Another possibility for the loss of AR expression in some tumor cells is a decrease in AR protein stability that reduces the AR protein level to one difficult to detect immunohistologically. AR is degraded by ubiquitin targeting to the proteasome (55). Ubiquitination of AR is promoted by Akt kinase-mediated phosphorylation of the receptor, suggesting that cells with increased Akt activation may have a reduced AR protein level (55).

### B. Androgen availability in the prostate after androgen ablation

Androgen ablation by surgical castration or treatment with LHRH agonists results in a 90–95% decrease in serum testosterone levels. However, intraprostatic DHT levels only decline by approximately 50% (5, 56, 57). Although this reduction is able to cause the death of over 70% of normal prostate secretory epithelial cells as discussed above, prostate cancer cells surviving this treatment would be exposed to a relative abundance of DHT. In contrast, GnRH agonist treatment has been reported to reduce intraprostatic DHT by 90% (58). Combined treatment of castration and flutamide has been found to reduce prostate DHT levels to approximately 20% of pretreatment levels (56, 59). Flutamide is a nonsteroidal antiandrogen, and the mechanism of its effect on prostatic DHT levels has not yet been determined. In contrast to castration alone, the presence of flutamide in combined androgen ablation would be expected to substantially block the ability of the residual prostatic DHT to activate AR transcription (57). Although the differences in remaining DHT and accessibility of the DHT to AR might be expected to correlate with a difference in prostate cancer prognosis after different androgen ablation regimes, a recent meta-analysis of studies comparing patient survival with combined androgen ablation or with either castration or long-term LHRH agonist treatment alone found no statistically significant difference between the two groups (60). Although monotherapy and combined androgen blockade may not differ in overall prostate cancer survival, it is possible that these treatment regimes may differ in the molecular mechanism used by the tumors to become androgen insensitive.

### C. Androgen deprivation and prostate cancer proliferation and apoptosis

Although androgen deprivation results in a dramatic reduction of a population of prostate secretory cells through apoptosis, there is some evidence to suggest that prostate cancer cells acquire a relative resistance to androgen ablation-induced apoptosis early in transformation and that androgen primarily regulates the proliferation of prostate cancer cells *in vivo*. In analysis of castration-induced involution of the normal rat prostate, the majority of epithelial cell loss occurs within 7 d (25, 26). Several studies of human prostate cancer samples obtained several months after the initiation of androgen deprivation have detected the presence of prostate cancer foci that appear to be morphologically altered by androgen deprivation but have not undergone necrosis or apoptosis (61–63). Histochemical analysis of human prostate tumors 7 d after orchiectomy showed that 88% of tumors demonstrated a decrease in proliferation as determined by Ki-67 immunopositivity (64). However, in 60% of tumors, castration either had no effect or reduced the rate of apoptosis (64). In a separate study, androgen deprivation was found to result in only a 3.4% apoptotic index (65). Similar results have been obtained in the androgen-sensitive Dunning R3327 rat prostate tumor model. Castration induced involution of the normal prostate epithelia and reduced the mitotic index of tumor cells, but did not significantly influ-

ence tumor cell number up to 2 wk after castration (66, 67). In CWR22 xenograft tumors, castration initially induced growth arrest in tumor cells. However, foci of Ki-67 immunopositive cells were detected by 120 d after castration (50). The clinical and animal prostate cancer data suggest that a significant proportion of prostate tumors are resistant to androgen ablation-induced apoptosis at the time of treatment and that the observed therapeutic benefit may be the result of a decrease in the proliferation rate of the tumor cells. The mechanism of the resistance to apoptosis in prostate tumors remains to be determined, however apoptosis resistance may be at least partially due to an increase in the expression of apoptosis suppressor genes. Elevation of the antiapoptosis proteins bcl-2, bcl-x, and mcl-1 has been found in prostatic intraepithelial neoplasia (68, 69), suggesting that resistance to apoptosis may be an early event in prostate cancer. The expression of these antiapoptotic proteins is found to further increase with prostate cancer progression (68, 69). Overexpression of bcl-2 in the androgen-dependent prostate cancer cell line LNCaP enables cell growth in androgen-depleted media and enhances tumor formation in castrated male mice (70). The suppression of bcl-2 expression through AR-mediated androgen action has been proposed as a mechanism for enhanced bcl-2 expression upon androgen deprivation (71). These observations suggest that elevated expression of antiapoptotic proteins, particularly bcl-2, in prostate tumors may contribute to the resistance of some tumors to androgen deprivation-induced apoptosis. The stimulation of antiapoptotic genes may represent a secondary event in response to modulation by other factors, such as growth factors. For example, fibroblast growth factor 2 (FGF2) has been found to promote growth and survival of prostate cancer cells through induction of bcl-2 expression (72).

On the basis of cell line models, it has been suggested that prostate cancer cells surviving androgen deprivation therapy may be sensitive to apoptosis induced by androgen (73). The growth of LNCaP cells is normally dependent on androgen. However, LNCaP cell growth is inhibited at high concentrations (300 nM) of DHT (74). The growth of several LNCaP sublines selected for growth in reduced androgen-containing media can be inhibited by physiological levels of DHT (1–10 nM) (75–77). Additionally, DHT, acting through AR, has been shown to potentiate the apoptotic effect of 12-O-tetradecanoylphorbolacetate, a protein kinase C activator, via interruption of nuclear factor  $\kappa$ B signaling and activation of the c-Jun NH<sub>2</sub>-terminal kinase pathway in LNCaP cells (S. Altuwaijri, and C. Chang, unpublished observations). Similarly, stable transfection of AR into the AR-negative prostate cancer cell line PC-3 has been reported to generate sublines that undergo growth arrest or apoptosis in the presence of physiological levels of androgen (78, 79). However, it is unclear to what extent these cell lines represent prostate cancer *in vivo*. Treatment of prostate cancer patients with hormone refractory metastases with either physiological or superphysiological doses of androgen results in a negative response in 97% of patients (44), suggesting that androgen-induced growth arrest in tissue culture cell lines does not represent a common physiological response *in vivo*.

Although continuous androgenic therapy may not be ther-

apeutically beneficial for the majority of prostate cancer patients, intermittent androgen ablation has been proposed as a therapy to delay the development of tumors that cannot respond to androgen deprivation (80, 81). It has been proposed that androgen-dependent cells surviving androgen ablation adapt to a low androgen environment and eventually become androgen insensitive (81, 81a). According to this model, periodic exposure to androgen would prevent cells that can grow independent of androgen from becoming predominant. Consistent with this model, the development of androgen-independent LNCaP xenografts in castrated nude mice is delayed by intermittent treatment with testosterone (82). Phase II clinical trials have suggested that intermittent androgen deprivation may improve quality of life and sexual function compared with continuous androgen deprivation, although it remains unclear whether intermittent therapy provides a survival benefit (reviewed in Refs. 80 and 81).

#### D. Androgen regulation of prostate-specific antigen (PSA)

PSA is generally considered to be the most sensitive biochemical marker available for monitoring the presence of prostatic disease, particularly prostate cancer, and response to therapy. PSA is a glycoprotein and a member of the kallikrein family of serine proteases (83). In the normal prostate, PSA is secreted into the glandular ducts where it functions to degrade high molecular weight proteins produced in the seminal vesicles to prevent coagulation of the semen (84). PSA levels in the normal prostate are approximately 1 million-fold higher than in the serum. PSA normally enters the serum only through leakage into the prostatic extracellular fluid. During prostate cancer progression, serum PSA levels become progressively elevated due to aberration of the normal prostate ductal structure by the neoplastic epithelial cells. The increasingly abnormal ductal structure allows PSA to be actively secreted into the extracellular space and enter the circulation (85, 86). In addition to the prostate, a low level of PSA expression is found in amniotic fluid, in the lactating breast, and in a subset of breast and ovarian tumors (87).

The primary regulator of PSA expression is AR, which induces PSA expression through three androgen response element-containing enhancer elements located in the proximal 6 kb of the PSA promoter (88, 89). The androgen-independent prostate cancer cell line PC-3, which does not express either AR or PSA, was induced to produce PSA after transfection of AR and treatment with androgen. This result highlights the importance of AR activity for PSA expression in this cell line (90). In addition to androgens, PSA expression has been reported to be induced by glucocorticoids in T47D breast cancer cells (91) and LNCaP cells transfected with the glucocorticoid receptor (92). Progestins are also able to stimulate PSA expression at low concentrations ( $10^{-11}$  to  $10^{-10}$  M) in breast cancer cell lines (91, 93), and oral contraceptives containing progestin can induce PSA expression in breast tissue (94). However, these cell lines and tissues are all known to express a functional AR, and the cell culture assays were done in media that is expected to contain residual androgen. Therefore, it remains possible that the glucocorticoid receptor or progesterone receptor may cooperate with AR to promote PSA expression. Recently, a novel transcrip-

tion factor, GAGATA binding protein, has been identified and found to affect androgen-mediated expression of PSA through binding to an alternative enhancer site (GAGATA) in the PSA promoter (95). Two E twenty-six (Ets) family transcription factors, epithelium-specific Ets factor 2 (ESE2) and prostate-derived Ets factor (PDEF), have also been found to induce transcription of a PSA reporter gene in the AR-negative cell line CV-1 (96, 97). PDEF is highly expressed in the prostate and weakly expressed in the ovary (97). Although PDEF is capable of inducing PSA expression in the absence of AR, PDEF can heterodimerize with AR to enhance AR-induced transcription (97). ESE2 is weakly expressed in the normal prostate, and it is not yet known whether this transcription factor directly interacts with AR (96). The ability of Ets transcription factors to regulate PSA expression in prostate cancer remains to be determined. A small percentage of cells in local prostate tumors have been found to express PSA but lack detectable AR expression by immunohistochemistry (46). It is possible that PSA expression in these cells is regulated by an Ets transcription factor. As discussed above, the majority of prostate tumors express AR, and therefore the significance of AR-independent PSA expression is unclear.

## IV. Prostate Cancer Progression and the Modulation of AR Transcriptional Activity

### A. AR trinucleotide CAG and GGN repeats: effect on prostate cancer development and progression

The NH<sub>2</sub>-terminal transactivation domain of AR contains two trinucleotide repeat regions, both of which are polymorphic in length. The CAG repeat, encoding a polyglutamine region, is located within a region of the NH<sub>2</sub>-terminal that is required for full ligand-inducible transcription (98–101). Charged, glutamine-rich regions have been identified in other transcription factors, including cAMP response element-binding protein (CREB), amplified in breast cancer-1 (AIB1), and specificity protein 1 (Sp1), where they mediate protein-protein interactions with coregulators or members of the basal transcriptional machinery (102–104). The second trinucleotide repeat is the GGN or polyglycine repeat that lies 3' of the CAG repeat. The two repeat regions are separated by 248 amino acids of nonpolymorphic sequence. Polymorphic variation in the trinucleotide repeat lengths of the NH<sub>2</sub>-terminal of AR is associated with altered AR transcriptional activity *in vitro* (105–107), as well as variations in prostate growth upon testosterone substitution in hypogonadal men (108), and may therefore contribute to prostate cancer risk or progression (109, 110). The occurrence of prostate cancer demonstrates familial aggregation, with a 2- to 4-fold increased risk among men reporting prostate cancer in a father or brother after adjustment for age and dietary factors (111–113). Recently, several hereditary prostate cancer loci have been identified. The cancer-associated alleles of these loci are rare, autosomal dominant or X-linked, and show high penetrance (114–117). In contrast, epidemiological studies suggest that AR trinucleotide repeat polymorphisms associated with prostate cancer are common alleles of relatively low penetrance (118).



The AR CAG repeat normally varies between eight and 30 contiguous repeats in length (119). However, the modal CAG repeat number varies between ethnic groups, with 18 repeats being the most abundant allele in African-Americans and 21 and 22 repeat alleles most abundant in non-Hispanic whites and Asians, respectively (102, 120, 121). Expansion of the CAG repeat to over 40 causes the rare neuromuscular disorder spinal and bulbar muscular atrophy, which is also often associated with reduced virilization (122). Ethnic differences in prostate cancer incidence are inversely correlated to the predominant AR CAG repeat length in each group, with Asians having the lowest prostate cancer incidence and the longest AR CAG repeats, whereas African-Americans have the highest incidence and shortest CAG repeat length. Longer CAG repeat lengths have been correlated with decreased AR transcriptional activity *in vitro*. AR molecules carrying more than 40 CAG repeats show reduced transcriptional activity compared with AR molecules with 25, 20, or no CAG repeats (106, 107). However, analysis of the transcriptional effect of CAG repeat lengths within the normal repeat range suggests that the correlation between short CAG repeat lengths and increased transactivation is cell-type dependent. In the fibroblastic COS-1 cell line, a 25% reduction in AR transcription is seen between receptors having 12 CAG repeats and those having 20 (107). Similarly, a 40% progressive decrease in the level of AR transcription occurs between a CAG repeat length of 15 and that of 31 in COS-1 cells (123). In contrast, one study found no significant difference in AR transcription, in the epithelial prostate cancer cell line PC-3, between AR molecules with 15, 24, or 31 CAG repeats (123). In a separate study using PC-3 cells, a 7% decrease in AR transcription was observed between receptors with nine and 21 CAG repeats, and a 13% decrease was shown between receptors with nine and 29 CAG repeats (124).

Although differences in AR transcription with CAG repeat lengths in the normal range may be difficult to observe *in vitro*, it is possible that small differences in AR transactivation may cumulatively contribute to lifetime prostate cancer risk or age of diagnosis. In healthy men without prostate cancer, a short AR CAG repeat length correlates to a modestly higher, but statistically significant, serum PSA level (125), suggesting that the CAG repeat number influences AR transactivation *in vivo*. Short CAG repeat lengths (CAG repeat length  $\leq 17$  to  $\leq 23$ , depending on the study) have been found to correlate with an increased prostate cancer risk (120, 126, 127). This association has been shown in both American non-Hispanic white men (126, 127) and in a population-based study in China (120). The association of short AR CAG repeat length with prostate cancer risk in both a moderate risk non-Hispanic white population and a low-risk Chinese population suggests that this may represent a genuine prostate cancer predictor. Several studies have also reported an association between a short AR CAG repeat length and an earlier age of diagnosis (128–130) or more advanced cancer grade and stage at diagnosis (127, 131).

However, a number of studies have failed to link AR CAG repeat number to sporadic or familial prostate cancer (132–135). In men without known prostate disease, CAG repeat length was not found to be related to the volume of the

central zone of the prostate (136), considered to be the most hormonally sensitive prostatic region (30). The reason for the inconsistent association between the AR CAG repeat number and prostate cancer or *in vivo* parameters of androgen action is unclear. Differences in study design and reference CAG lengths may contribute to the divergent results in the epidemiological studies. It has been proposed that the polymorphic CAG repeats function as low penetrance prostate cancer alleles that may require additional genetic or environmental factors to result in increased cancer risk (118, 137).

The CAG repeat region is located in an AR domain that is known to interact with some AR coregulators (2). It is possible that variation in the prostatic coregulator milieu contributes to the association between CAG repeat length and prostatic disease. Transfection assays have demonstrated that the interaction between AR and the coactivator ARA24 decreases with increasing AR CAG repeat length, resulting in decreased AR transactivation (138). Similarly, longer AR CAG repeat lengths result in a decrease in the ability of AR to be coactivated by members of the steroid receptor coactivator (SRC) family of coregulators [SRC-1, transcriptional intermediary factor 2 (TIF-2), and SRC-3] (124). The expression of SRC-1 and TIF-2 has been found to be elevated in some prostate tumor specimens (139). It is possible that individuals who normally have an increased expression of an SRC coregulator in the prostate and carry an AR allele with a short CAG repeat length may have a greater risk of prostate cancer. Alternatively, polymorphisms in the promoters of AR target genes in combination with short CAG AR alleles may contribute to prostate cancer susceptibility. The PSA gene promoter contains a polymorphic androgen response element (ARE), referred to as the A and G alleles. Individuals carrying an AR allele with less than 20 CAG repeats and homozygous for the PSA G allele have been reported to have a 5-fold increased risk of prostate cancer (125). The protease activity of PSA has been hypothesized to contribute to prostate carcinogenesis through cleavage of extracellular matrix proteins or through modulation of the availability of IGF-I by cleavage of IGF binding protein-3 (IGFBP-3) (140–142). It is possible that AR binds to the PSA G allele with greater affinity and that in combination with the increased transcriptional activity of short CAG repeat alleles of AR, contributes to prostate carcinogenesis (143, 143a).

The second polymorphic AR trinucleotide repeat, the GGN or polyglycine repeat, is less well studied than the CAG repeat. Deletion of the GGN repeat results in a 30% reduction in AR transcriptional activation in transfection experiments (105), but it has not yet been determined whether this trinucleotide repeat functions as a protein interaction domain. Because a comparison of AR transcriptional activation with varying GGN repeat lengths has not been performed, it remains to be determined whether the reduction in AR activity with the deletion of the GGN region reflects a reduction in AR transactivation with decreasing repeat length. Although the AR GGN repeat shows a lesser degree of polymorphism than the CAG repeat (120, 121, 132), several studies have examined the GGN repeat length and prostate cancer susceptibility. Short GGN repeat lengths (GGN  $\leq 14$  or GGN  $\leq 16$ , depending on the study) have been found to be associated with increased prostate cancer risk (126, 132). If there is a

direct relationship between GGN repeat length and AR transactivation, then this result is unexpected. However, one study found that long GGN repeat lengths (GGN  $\geq$  16) were associated with an increased risk of prostate cancer recurrence and increased risk of death (135). Two separate studies failed to find a link between GGN repeat number and prostate cancer risk (133, 135). Additional molecular and epidemiological studies will be required to more firmly establish the role of the AR GGN repeat in AR transcriptional activity and prostate disease.

### B. AR amplification

The amplification of the AR gene has been suggested as a mechanism that enables prostate cancer cells to become sensitive to the reduced level of androgens present after androgen ablation therapy. AR amplification occurs rarely in untreated primary prostate cancers, with an observed frequency between 0 and 5% (144–147). However, amplification of AR is found in 20–30% of hormone refractory prostate cancers (145–149). In the prostate cancers analyzed, the AR amplification predominantly involves the wild-type sequence (150, 151). In two separate cases, the amplified AR gene contained a point mutation at codon 683 resulting in a glycine to alanine substitution (150, 151). This mutation, however, does not alter the functional properties of AR in transfection assays (150) and does not allow AR to become activated by other steroids or antiandrogens (152). The association between AR amplification and hormone refractory prostate cancer has led some authors to suggest that selection for increased AR gene copy number may occur under conditions of androgen deprivation because an elevated level of AR gene expression could contribute to the ability of cancer cells to proliferate in a reduced androgen environment (150, 153, 154).

It remains unclear whether amplification of the AR gene in hormone refractory tumors results in an increase in AR protein levels. Using *in situ* hybridization, one study found that hormone refractory prostate tumors carrying an amplified AR expressed a higher level of AR mRNA compared with untreated primary tumors with a single copy of AR per cell (150). However, using the more quantitative technique of real time RT-PCR, hormone refractory tumors carrying an amplification of AR were not found to express a higher level of AR mRNA than hormone refractory tumors with a normal AR copy number (148). Divergent results have been obtained for the influence of AR amplification on PSA expression. Although one study found AR amplification positively correlated with an increase in tumor PSA (149), two subsequent studies failed to correlate the presence of AR amplification in hormone refractory carcinomas with either tumor or serum PSA levels (148, 155). Therefore, the significance of AR amplification in prostate cancer is currently unclear.

Genome instability, including microsatellite instability, amplification of cellular oncogenes, and gain or loss of chromosomal regions, is associated with the progression of multiple tumor types, including prostate cancer (156, 157). Instability of microsatellites, particularly those located on chromosomes 8 and 16, has been found to be associated with higher Gleason scores in prostate cancer patients (52, 158),

although generalized microsatellite instability may represent an early event in prostate carcinogenesis (159, 160). Similarly, chromosomal aberrations are found to increase with prostate cancer progression, both in patients initially treated with hormonal therapy and in patients treated by radical prostatectomy without hormonal intervention (51, 53). Therefore, genomic instability in general does not appear to be related to primary therapy. Because AR amplification has not been found to consistently result in an increase in the expression of AR target genes, the amplification event may reflect an increased level of genome instability with prostate cancer progression. However, it is possible that the primary therapy may influence the prevalence of particular genetic changes. In addition to amplification of AR, hormone refractory tumors of patients have a higher frequency of loss of chromosomal markers on chromosomes 15, 19, and 22, compared with recurrent tumors of patients treated by radical prostatectomy without hormonal therapy (51, 53). The cause and functional consequences of these differences remain to be determined.

### C. AR coregulator overexpression

Because coactivators enhance the transcriptional activity of steroid receptors and enable steroid receptors to become transcriptionally active at lower ligand concentrations, it has been suggested that overexpression of select coactivators may contribute to carcinogenesis in steroid-responsive cancers such as those of the breast and prostate. Support for this model originally came from the observation that SRC-3 is overexpressed in 64% of primary breast cancers (161). Subsequently, the SRCs peroxisome proliferator-activated receptor- $\gamma$  binding protein (PBP)/thyroid hormone receptor-associated protein 220 (TRAP220)/vitamin D receptor-interacting protein 205 (DRIP205) and TRAP100 have been found to be amplified and overexpressed in breast cancers (162, 163). In prostate cancer, several SRCs that are capable of enhancing AR transcription have also been found to be overexpressed. The expression of the three members of the SRC, or p160, family of coactivators, SRC-1, TIF-2, and SRC-3 (2, 164), is elevated in prostate cancer. SRC-1, but not TIF-2, is overexpressed in 50% of androgen-dependent prostate cancers, compared with normal prostate tissue and benign prostatic hyperplasia specimens (139). In hormone refractory prostate cancers, both SRC-1 and TIF-2 are overexpressed in 63% of samples (139). In a separate study, an increase in SRC-3 expression was found to correlate with increased prostate cancer grade and stage and decreased disease-free survival (165). A recent study also demonstrated that enhanced expression of ACTR/AIB1/SRC-3 resulted in higher PSA levels, with or without androgen stimulation. More specifically, it was determined that ACTR/AIB1/SRC-3 facilitates RNA polymerase II recruitment to a distant enhancer element of the PSA gene, thereby producing the observed enhancement of PSA expression (166). In addition to the SRC family of coactivators, the AR coactivator ARA70 (167) is also found to be overexpressed in prostate cancer specimens (S. Yeh, and C. Chang, unpublished observations) and in the CWR22 xenograft tumors that have become hormone refractory after castration (168). The cdk-activating phosphatase,

cdc25B, has recently been identified as an AR coactivator and found to be overexpressed in human prostate cancer, with the highest expression in late-stage tumors of high Gleason score (169). Similarly, the AR coactivator Tat interactive protein, 60 kDa (Tip60), has been shown to increase in expression as well as in nuclear localization upon androgen withdrawal in both the CWR22 prostate xenograft and LNCaP prostate cancer cell line (170). Finally, through differential display gene expression analysis, the putative AR coactivator, nuclear matrix protein, 55 kDa (nmt55) was identified as up-regulated in human prostate cancer tissue. The nmt55 expression was positively correlated with that of AR, and expression of the PSA promoter was enhanced by transfection of nmt55 (171).

These studies suggest that prostate cancer is associated with an increase in the expression of multiple AR coactivators and that the number of coactivators that are overexpressed may increase with prostate cancer progression. It remains to be determined which coactivators are most frequently overexpressed and contribute most significantly to prostate carcinogenesis. However, in transfection experiments, coactivators are able to enhance AR transcription at reduced concentrations of agonistic ligands (172–175). Therefore, an increase in the abundance of a subset of AR coactivators may contribute to the sensitization of AR to low levels of androgen after androgen deprivation therapy (176).

It has been suggested by some authors that interruption of AR-coactivator interaction using gene therapy is a possible future therapeutic modality for the treatment of prostate cancer (177, 178). Because prostate cancer cells appear to be capable of overexpressing more than one coactivator simultaneously (139), this method would presumably be effective only if the interacting peptide were able to block AR interaction with multiple coactivators. Different AR coactivators interact predominantly with different AR domains (reviewed in Ref. 2). For example, ARA70 interacts primarily with the AR LBD (167), although N-terminal interaction has been reported (179). In contrast, members of the SRC family interact primarily with the AR N terminal (180, 181). It may therefore prove difficult to design interacting peptides or proteins that can effectively block the multiple coactivator binding sites of AR.

#### D. AR and tumor suppressor genes

**1. Retinoblastoma susceptibility gene (Rb).** Several tumor suppressor gene products have been found to interact with AR to influence AR transcriptional activity. Rb is able to enhance AR transcription, and inhibition of the Rb-AR interaction results in a decrease in AR activity (182, 183). Rb also functions to negatively regulate cell cycle progression through G<sub>1</sub>, and loss of the normal Rb cell cycle control is associated with multiple cancer types (184). In the case of prostate cancer, Rb expression is found to decrease with increasing tumor grade and stage (185–187). The decrease in Rb expression in prostate cancer has been reported to be associated with mutation of the Rb gene (187). The paradoxical observation that Rb enhances AR activity but is lost with prostate cancer progression suggests that the role of Rb in cell cycle control may be dominant to its role as an AR coregulator. As

discussed above, the increase in certain AR coactivators with prostate cancer progression may compensate for the loss of Rb and allow a continuation of a high level of AR transcriptional activity. Rb phosphorylation modulates its control of the cell cycle. Rb blocks cell cycle progression when it is not phosphorylated, however, phosphorylation of Rb by cyclin-dependent kinases relieves this block (188). It is possible that phospho-Rb functions as an AR coactivator, whereas unphosphorylated Rb does not enhance AR transcription. In the subset of prostate cancers that retain Rb expression, phosphorylation of Rb may contribute to cellular proliferation through both regulation of cell cycle progression and AR transcriptional activity. In the normal prostate, the Rb may function to maintain normal cellular turnover.

**2. BRCA1/BRCA2.** AR transcriptional activity has also been found to be enhanced through interaction with the breast and ovarian cancer susceptibility gene BRCA1 (189). BRCA1 may function as an AR coregulator by promoting androgen-AR mediated apoptosis (189). Although an initial report found an increase in prostate cancer risk in men carrying mutant alleles of BRCA1 (190), several subsequent studies have failed to find a significant relationship between BRCA1 mutations and prostate cancer (191–193). Although BRCA1 is implicated in the regulation of cell cycle progression and DNA repair (194), normal BRCA1 function is apparently not critical in the development or progression of prostate cancer. Additionally, the tumor suppressor gene BRCA2 has recently been shown to increase AR transcriptional activation through a synergistic effect involving GRIP1/TIF-2, a member of the p160 family of nuclear receptor coactivators (195).

**3. Phosphatase and tensin homolog (PTEN).** In contrast to Rb, BRCA1, and BRCA2, the tumor suppressor PTEN inhibits AR function by promoting the degradation of AR (H. K. Lin, and C. Chang, unpublished observations). As discussed below, PTEN is a phosphatase that negatively regulates the activity of phosphatidylinositol 3-kinase (PI3K) and Akt kinase (Fig. 2 and Ref. 196). Loss of PTEN results in an increase in PI3K and Akt activity resulting in an increase in cellular proliferation and a decrease in apoptosis (196). In prostate cancer, loss of PTEN expression correlates with increasing tumor Gleason score and clinical stage, with approximately 20% of Gleason 7–9 tumors completely negative for PTEN expression (197). In the absence of PTEN, the normal AR protein turnover may be impaired, contributing to an increase in AR transcriptional activity promoting prostate cancer progression through regulation of both AR and PI3K/Akt signaling.

#### E. Growth factor modulation of AR activity

Prostate cancer progression is often associated with alteration of growth factor or growth factor receptor expression by the tumor (38, 198). Unlike steroid hormones, growth factors and cytokines regulate cellular responses through binding to membrane receptors. Growth factor or cytokine binding initiates a phosphorylation cascade that ultimately results in phosphorylation of transcription factors or transcription factor-interacting proteins. In the prostate, AR is among the transcription factors whose activity is influenced by signal transduction cascades, and disruption of the nor-

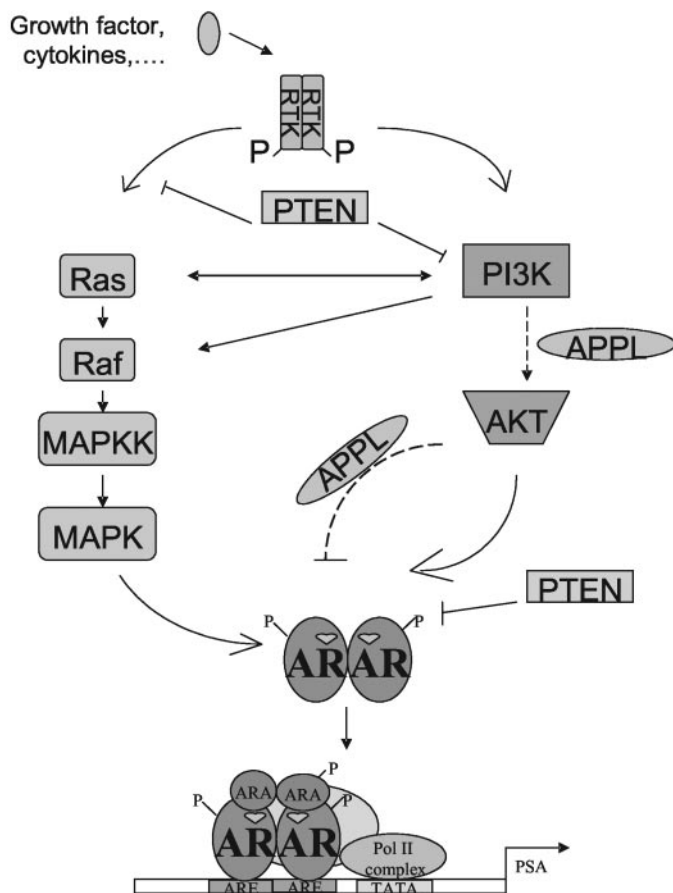


FIG. 2. Crosstalk of MAPK and PI3K/Akt pathways with A/AR. Both MAPK and PI3K/Akt may influence the phosphorylation of AR and AR coregulators, resulting in modulation of AR activity. The tumor suppressor PTEN can modulate AR activity via PI3K/Akt pathways or by interacting directly with AR. MAPKK, MAPK kinase; A/AR, androgen/androgen receptor; RTK, receptor tyrosine kinase; APPL, adaptor protein containing PH domain, PTB domain, and leucine zipper motif; P, protein phosphorylation.

mal interaction between signal transduction and AR transactivation may contribute to the progression of prostate cancer. Not all growth factors that influence prostate cancer do so through modulation of AR activity. For example, bFGF or FGF-2 is elevated in the tumors and serum of some prostate cancer patients (199) but does not influence AR transcriptional activity (200) and can function as a mitogen for AR-negative prostate cancer cell lines (201). Instead, bFGF may promote angiogenesis of prostate cancers (38). However, an increasing number of signal transduction cascades have been found to influence AR transcriptional activity either through direct phosphorylation of AR or through phosphorylation of AR coregulators. Growth factor modulation can result in either stimulation or inhibition of AR transcriptional activity. In prostate cancers carrying a growth factor response that enhances AR activity, AR may promote proliferation at low levels of androgen. In tumors with a growth factor response that is suppressive of AR activity, clonal populations may be selected that are able to proliferate in an AR-independent manner.

1. *Her2/Neu/ErbB2*. The epidermal growth factor (EGF) family is composed of four structurally related membrane tyrosine kinases: the prototypic EGF receptor (EGFR, also called Her1 or ErbB1), Her2 (ErbB2/neu), Her3 (ErbB3), and Her4 (ErbB4). The amplification and/or overexpression of the Her family members, particularly Her2, have been observed clinically in a number of cancer types, including malignancies of the brain, urinary tract, and male and female reproductive systems (202). Overexpression of Her2 is found in 10–40% of breast tumors and is associated with poor prognosis in patients with nodal metastases (203, 204). However, whether Her2 is overexpressed in prostate cancer is more controversial, possibly due to methodological differences in tissue preparation and the antibodies used. Although initial studies were unable to detect Her2 protein or mRNA in prostate cancer samples (205, 206), more recent studies have found Her2 protein to be elevated (207–210). It is possible that increased expression of Her2 in prostate carcinomas is related to the development of hormone resistance. Signoretti *et al.* (210) have observed an increase in the proportion of Her2-positive prostate tumors in patients receiving combined androgen ablation therapy before prostatectomy compared with patients treated by prostatectomy alone. A further increase in Her2-positive cases was seen in patients with metastatic, hormone refractory prostate cancer (210). The response to androgen withdrawal was independent of tumor stage and grade. These observations are consistent with previous studies which found that elevated Her2 in tumor cells compared with normal adjacent epithelia but that the level of Her2 expression or percentage of Her2-positive tumors did not correlate with tumor stage (208, 211). The Her2-positive tumors from patients treated with combined androgen blockade and from patients with hormone refractory disease also expressed AR and PSA (210). Overexpression of Her2 in the normally androgen-sensitive LNCaP cells allows androgen-independent cell proliferation and decreases the tumor latency of xenografts in castrated mice (212, 213). Her2 overexpression was also found to induce androgen-independent expression from the PSA promoter that could not be completely blocked by antiandrogens (212, 213). The elevation of Her2 expression may protect prostate cancer cells from the growth inhibitory effect of combined androgen ablation in part through allowing AR transcription under conditions of extremely low levels of circulating androgen. This may contribute to the development of hormone refractory tumors.

Her2 stimulation of AR has been reported through MAPK and PI3K. As shown in Fig. 2, the MAPK and PI3K pathways are not completely distinct, and activation of one pathway may either stimulate (214–216) or inhibit (217, 218) the other, possibly in a cell-type or signal-specific manner. Overexpression of Her2 in prostate cancer cells has been demonstrated to enhance AR transcription in the presence of DHT (213), raising the possibility that elevated Her2 may permit the proliferation of malignant prostate cells before therapeutic intervention. Overexpression of Her2 increased proliferation of xenografts in intact mice, consistent with this hypothesis (212). Yeh *et al.* (213) have shown that the effect of Her2 on AR transcriptional activity in the presence of androgen occurs at least partly through the MAPK pathway.

Treatment with the MAPK kinase-1 inhibitor PB98059 partially reduces Her2 stimulated enhancement of AR transcription in DU145 cells (213). Her2 stimulation of AR transactivation enhances the interaction between AR and the AR coregulator ARA70 (213), although it has not yet been determined whether this increase is due solely to MAPK phosphorylation of AR or whether phosphorylation of ARA70 also contributes to this effect. Phosphorylation of estrogen receptor  $\beta$  and steroidogenic factor 1 by MAPK kinase has been found to enhance the ability of those receptors to recruit coactivators (219, 220), suggesting that this represents a general regulatory mechanism of nuclear receptors. MAPK also phosphorylates SRC family coactivators, enhancing their ability to form a coactivator complex to facilitate transcription (221–224). Therefore, it is possible that both mechanisms contribute to the enhanced interaction between AR and ARA70 found with Her2 overexpression. It is unclear whether Her2 activates MAPK through homodimerization induced by a high concentration of Her2 at the cell surface or through heterodimerization with other Her receptors conferring sensitization to endogenous EGF-related ligands secreted by the cells (225, 226). Clinically, elevation of the EGFR ligands EGF and TNF $\alpha$  has been detected in prostate cancer cells compared with benign tissue, although alteration of EGFR in prostate cancer specimens is contradictory between different studies (38). EGF has been found to enhance AR transcription in the presence of androgen (227, 228). However, EGF has been found to suppress AR transcription in LNCaP cells (229, 230). It therefore remains unclear to what extent the effect of Her2 on AR transcription in prostate cancer is mediated by Her2 alone or through heterodimerization with other EGF receptor family members.

In LNCaP cells, expression of constitutively active Her2 enhances AR transcription at the very low level of androgen found in charcoal-stripped serum-supplemented media. Addition of androgen further increases AR transcription in the presence of constitutively active Her2 (231). The Her2-mediated AR transactivation is reduced by transfection of a dominant negative mutant of Akt, a PI3K target (231). Akt is able to bind directly to AR and phosphorylates AR at S213 in the N terminal and at S791 in the AR LBD (231). These observations suggest that Akt phosphorylation of AR can enhance AR transcription at a low level of androgen. Akt activity is increased in androgen-independent xenograft tumors (232). Therefore, stimuli that increase Akt activity, including Her2, may contribute to the progression of prostate cancer. The activity of PI3K, and thus Akt, is regulated by the phosphatase PTEN (196, 233, 234). PTEN functions as a tumor suppressor, and loss of PTEN function is observed in a number of human cancers, including prostate cancer (235–237). LNCaP and PC-3 cells lack endogenous PTEN (238). Exogenous PTEN expression in these prostate cancer lines results in growth inhibition and repression of AR transcription (Refs. 239 and 240; and H. K. Lin and C. Chang, unpublished observations), consistent with a stimulatory effect of Akt on AR transcription. However, PTEN is also able to inhibit AR transcription directly. PTEN interacts with androgen-bound AR and decreases AR protein stability (H. K. Lin and C. Chang, unpublished observations). Although Her2 apparently enhances AR activity through PI3K and Akt

(231), the direct binding of PI3K to Her2 has not been reported (241). LNCaP cells have been characterized as expressing a high level of endogenous Her3, known to bind PI3K (226). The AR activation with Her2 overexpression in LNCaP cells was observed in the absence of exogenous Her3 ligands (231), possibly indicating that elevated Her2 sensitizes Her2-Her3 heterodimers to low levels of Her3 ligands present in cell culture serum or that Her2 homodimers have a previously unreported ability to activate PI3K/Akt signaling. However, elevated expression of Her3 and the Her3 ligand neuregulin have been detected in some human prostate cancers (242), suggesting that autocrine stimulation of Her2-Her3 heterodimers can occur and allow AR transcription in patients treated with combined androgen ablation therapy. Another possibility is that PI3K is activated by Her2 through crosstalk between the MAPK and PI3K pathways (Fig. 2). PI3K-dependent activation of Ras (214) and Ras-dependent activation of PI3K (215, 216, 243) have both been reported in response to EGF stimulation. It is possible that Her2 enables communication between these two pathways in LNCaP cells.

2. *TGF $\beta$* . TGF $\beta$  is the prototypic member of a family of polypeptide growth factors that also includes bone morphogenic protein and Mullerian-inhibiting substance. In the normal prostate, TGF $\beta$  functions as a growth inhibitor of prostatic epithelia and possibly functions as a differentiation factor for prostatic stroma (244). The mediators of TGF $\beta$  signaling are the Smad proteins that function as phosphorylation-regulated transcription factors.

In the normal prostate, TGF $\beta$  is predominantly produced by prostatic stromal cells (245, 246), functions as a paracrine inhibitor of normal prostate epithelial cell proliferation (244, 247, 248), and is thought to be a mediator of castration-induced epithelial apoptosis (244, 247, 249). *In vitro*, TGF $\beta$  inhibits the growth of primary human prostate epithelial cells (248) and of the human prostate cell lines PC-3 and DU145 (250). The prostate cancer cell line TSU-Pr1 has alternately been reported to proliferate (251) or be growth inhibited (252) in response to TGF $\beta$  treatment. These divergent results may be due to differences in cell culture conditions, which are known to influence the TGF $\beta$  responsiveness of some cell lines (253). Several observations suggest that a decreased sensitivity to the inhibitory effect of TGF $\beta$  contributes to prostate cancer cell proliferation and cancer progression.

The clinical observation that an elevation of serum TGF $\beta$  is associated with elevated serum PSA (254) suggests that interaction between the TGF $\beta$  pathway and AR transcriptional activity may exist. AR has been reported to interact with Smad3 (255, 256). Transfection of AR into the AR-negative prostate cells DU145 and PC-3 and cotreatment with DHT and TGF $\beta$  results in an increase in AR transcription. Cotransfection of Smad3 results in a further increase in AR transactivation (256). However, the ability of Smad3 to enhance AR transactivation is reversed in the presence of Smad4. Smad4 is also able to directly interact with AR, and this interaction decreases the interaction between AR and Smad3 (257). It has not yet been determined whether Smad4 levels are altered in prostate cancer, but it is possible that loss

of Smad4 may enable Smad3 to enhance AR transcription and facilitate prostate cancer progression. In these cases, autocrine production of TGF $\beta$  may phosphorylate Smad3, which enhances AR transcription in the absence, or with very low levels, of Smad4. Smad4 is not directly responsive to TGF $\beta$  (258), but it is possible that the AR response to TGF $\beta$  is modulated by Smad4 levels.

Alternatively, Hayes *et al.* (255) observed that AR transcriptional activity was reduced by exogenous transfected Smad3 in cells treated with TGF $\beta$  and DHT. This is consistent with a model in which TGF $\beta$  plays a modulatory role for the proliferative effect of DHT-bound AR in normal prostate epithelial cells. In prostate cancer cells, the decreased sensitivity to TGF $\beta$  due to reduced TGF $\beta$  receptor levels results in decreased phosphorylated Smad3. In prostate cancer cells with this phenotype, the decreased phosphorylated Smad3 removes an inhibitory mechanism for AR transcription, allowing cellular proliferation and PSA expression even at the low levels of androgen present after androgen ablation therapy. It is therefore possible that two distinct mechanisms of TGF $\beta$  responsiveness operate in prostate cancer cells depending on the expression level of different proteins in the TGF $\beta$  signaling pathway.

3. *Proline-rich tyrosine kinase 2 (PYK2)*. PYK2/cell adhesion kinase  $\beta$  (CAK $\beta$ )/related adhesion focal tyrosine kinase (RAFTK)/focal adhesion kinase 2 (FAK2) can be activated in response to multiple stimuli, including integrin stimulation, treatment with growth factors, activation of PI3K, and increases in intracellular calcium (259–262). The phosphorylation and activation of PYK2 allows the recruitment of the adapter proteins growth factor receptor binding protein (Grb2) and Shc, resulting in activation of the MAPK pathway (263). In addition, a direct phosphorylation target of PYK2 is ARA55/Hic-5 (172, 264, 265), a protein known to function as an AR coregulator in prostate cancer cells (172). In contrast to Her2- and TGF $\beta$ -induced signal transduction, PYK2 modulates AR transactivation through phosphorylation of an AR coregulator.

The phosphorylation of ARA55 by PYK2 may contribute to the regulation of prostate epithelial cell growth and AR transcriptional activity. The normal prostate expresses ARA55 mRNA (172), and phosphorylated PYK2 is found in normal prostatic epithelial cells (266). ARA55 was initially characterized as a coactivator of AR (172). However, PYK2-mediated phosphorylation of ARA55 blocks the interaction between AR and ARA55, reducing AR transcription in prostate cancer cell lines (267). Clinically, a progressive reduction in PYK2 expression is observed with increasing tumor grade in prostate cancer samples (266). Significantly, 19 of 19 prostate tumors with Gleason scores of 7 to 9 showed a complete loss of PYK2 immunoreactivity (266). A decrease in activated PYK2 would be expected to result in a decrease in ARA55 phosphorylation, allowing ARA55 to enhance AR transcription and contribute to prostate cell proliferation. However, PYK2 and ARA55 may also regulate prostate cancer cell growth independently of AR. Overexpression of PYK2 induces apoptosis in several epithelial, fibroblastic, and multiple myeloma cell lines in the absence of exogenous androgen (268–270). Expression of a dominant negative mutant of

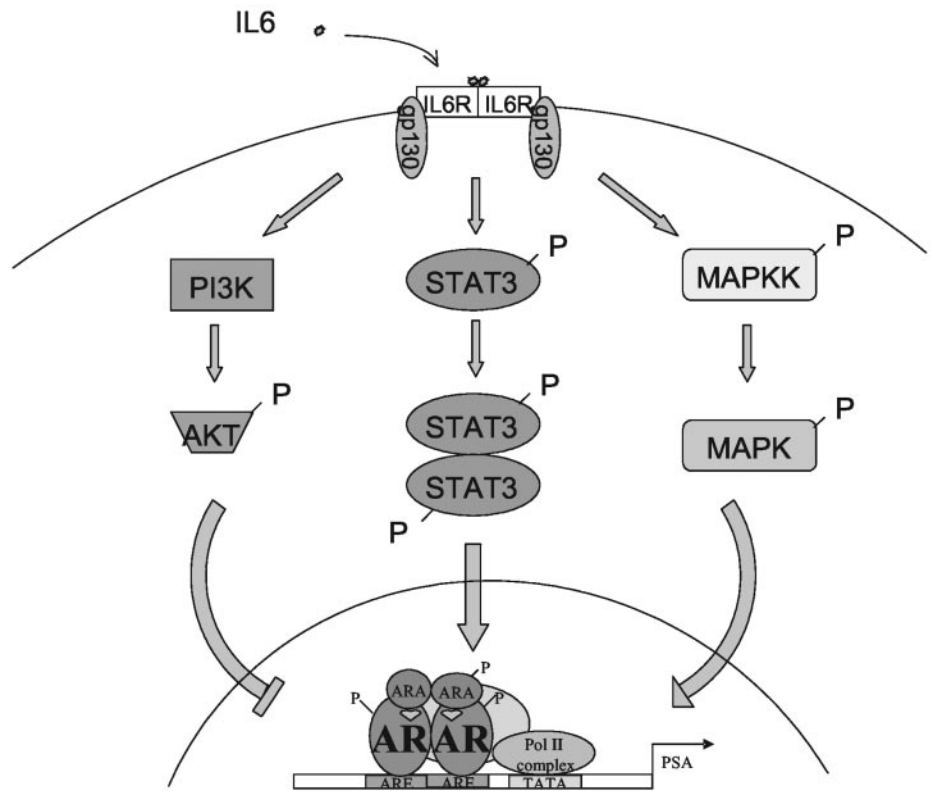
PYK2 in AR-negative PC-3 cells reduces cellular proliferation (271). ARA55 overexpression in the AR-negative cell line NIH3T3 reduces cell spreading on fibronectin (272), and an increase in ARA55 expression is associated with senescence in fibroblasts and TGF $\beta$ -induced senescence in osteoblastic cells (265, 273). These observations suggest that PYK2 and ARA55 may regulate cell motility and cell death through multiple mechanisms.

4. *IL-6*. The cytokine IL-6 functions to regulate cellular differentiation, proliferation, or growth inhibition in a cell type-specific manner (274). Elevated serum levels of IL-6 have been found in patients with metastatic prostate cancer (275–277), particularly those with hormone refractory disease (275, 278), suggesting that IL-6 may play a role in the progression of prostate cancer.

The receptor for IL-6 is composed of an IL-6 specific subunit, IL-6R, and a signal transducing subunit, gp130. IL-6 binding to IL-6R induces the formation of a multimeric complex containing two IL-6R and two gp130 molecules (279, 280). The formation of this complex results in autophosphorylation of the Janus tyrosine kinases (JAK1, JAK2, and TYK2), which in turn phosphorylate gp130 (281, 282). Phosphorylated gp130 is able to recruit the transcription factors STAT1 (signal transducer and activator of transcription 1) and STAT3 to the complex, resulting in their phosphorylation. The phosphorylated STAT proteins form homo- or heterodimers and translocate to the nucleus where they function as transcriptional regulators (283, 284). In addition to activation of the JAK-STAT pathway, IL-6 also induces the MAPK pathway through two distinct mechanisms. IL-6 mediated activated JAK is able to phosphorylate Shc, the upstream activator of Ras (282, 285). Alternatively, IL-6 has been shown to induce gp130 and Her2 association and phosphorylation resulting in MAPK activation in LNCaP cells (286, 287). STAT1 and STAT3 are phosphorylated at serine residues by members of the MAPK signal cascade (283, 288–291). The MAPK-mediated phosphorylation of STAT3 influences the tyrosine phosphorylation status and contributes to maximal transcriptional activation (283, 288, 291). The PI3K pathway is also stimulated in LNCaP and PC-3 cells by IL-6 (292, 293). In these cell lines, IL-6 increases the interaction between the p85 subunit of PI3K and gp130 and enhances p85 phosphorylation (293). Inhibition of IL-6-induced PI3K activity by wortmannin causes apoptosis in LNCaP cells (293), suggesting that this pathway may contribute to prostate cancer cell survival.

Studies of the effect of IL-6 on prostate cancer cell growth and transcriptional activation of AR have yielded conflicting results. One possible reason for the divergent observations is the number of different pathways induced by IL-6 that can influence AR transcription, as shown in Fig. 3. Several investigators have found that PC-3 and DU145 cells are unaffected or show slight growth inhibition in response to IL-6 treatment (294, 295). IL-6 has also been reported to result in growth inhibition and neuroendocrine differentiation of LNCaP cells (292, 294, 296). In DU145 cells, the transcriptional activity of transfected AR is not influenced by IL-6 treatment. However, inhibition of PI3K activity using LY294002 results in IL-6 enhancement of AR transcription in the presence of androgen (297). AR can be phosphorylated by Akt, a downstream target of PI3K (231, 298). Lin *et al.* (298)

FIG. 3. Differential modulation of AR activity by IL-6 via various signal transduction pathways. STAT3 interacts with AR to enhance AR activity. IL-6 can activate MAPK and enhance AR transactivation or activate PI3K/Akt to suppress AR function. Alternatively, Her2 may enhance AR activity via PI3K/Akt pathways. The degree of influence that IL-6 has on AR activity may be a result of some or all of the above signaling pathways working in concert. MAPKK, MAPK kinase; P, phosphorylation.



found that DHT-induced transcription by AR was inhibited by constitutively active Akt in DU145 cells (Fig. 2). Similarly, inhibition of PI3K enhanced AR transcription (298).

This effect is due at least in part to a decrease in the interaction between AR and AR coactivators, including ARA70 (298). Recent findings demonstrate that ARA70 expression is increased in prostate tumors compared with normal prostate, and that androgen ablation enhances ARA70 expression both *in vitro* and in a prostate cancer xenograft model (S. Yeh, and C. Chang, unpublished observations). A higher ratio of ARA70 to AR may induce the AR agonist activity of antiandrogens, such as hydroxyflutamide (HF), which are commonly used in combined androgen blockade treatment of later stage prostate cancer. Therefore, increased ARA70 expression may contribute to the onset of antiandrogen withdrawal syndrome (S. Yeh, and C. Chang, unpublished observations).

The effect of Akt may also be due to association between AR and the Akt bridging protein APPL (299, 300) (Fig. 2). APPL inhibits DHT-induced transcription through a mechanism dependent on Akt function (300). Akt-mediated inhibition of AR is in contrast to the observation showing that Her2 can stimulate AR transcription through PI3K and Akt (231). It is possible that the divergent effects of Akt on AR are due to differences in the cell lines used or cell culture conditions under which the assays were performed. Differences in cell culture conditions within the same cell line, as well as cell line passage number have been shown to alter steroid responsiveness (299, 301) and may also contribute to differential effects of kinase cascades. Another possibility is that the relative strength of the PI3K induction is different in the

two experimental designs and contributes to a different AR transcriptional endpoint. This is similar to the observed differences in cellular response depending on the relative strength and duration of MAPK stimulation (302). Extracellular signals that result in a prolonged or strong activation of the PI3K pathway would therefore be expected to inhibit AR transcriptional activity. IL-6 treatment may represent one such condition. Expression of a constitutively active Her2 would be expected to result in a strong stimulation of MAPK but possibly a weaker activation of PI3K than would be provided by transfection of a constitutively active Akt. Although the effect of Akt on AR phosphorylation is the same in the two experimental systems, the strength of the PI3K signal or the balance between MAPK and PI3K stimulation may influence AR-interacting proteins that contribute to the AR transcriptional response.

In contrast to the negative effect of IL-6 on AR transcription and LNCaP cell growth, other investigators have observed stimulation of cell growth in DU145, PC-3, and LNCaP cells with IL-6 treatment (303, 304). AR transcriptional activity can be enhanced by IL-6 in LNCaP cells and in DU145 cells transfected with AR (297, 305–307). The observed increase in AR transactivation with IL-6 treatment is blocked by the MAPK inhibitors PD98059 and U0126, suggesting that the IL-6-MAPK pathway is required for enhancement of AR transcriptional activity (305, 306, 308) (Fig. 3). As discussed above, growth factor stimulation of the MAPK pathway may enhance AR transcription through direct phosphorylation of AR or AR coactivators. IL-6 stimulation of LNCaP cells has recently been shown to promote SRC-1 phosphorylation by MAPK (309). IL-6 stimulation of

AR transactivation may also be mediated through the coactivator p300, although the mechanism has not yet been determined (310). AR transcription may also be enhanced through interaction with phosphorylated STAT3, as shown in Fig. 3 (305, 307). STAT3 has been found to coimmunoprecipitate with AR in cells treated with IL-6 or overexpressing JAK1 (305, 307). Transfection of a dominant negative mutant of STAT3 into LNCaP cells abrogates IL-6 enhancement of AR activation (305, 307).

The reasons for these divergent results are not completely understood. It has been suggested that altered levels of intracellular kinases exist in the prostate cancer cell lines used by different laboratories (311). However, in the studies that found IL-6-induced prostate cell growth inhibition, with one exception (292) IL-6 was added to media containing 3–10% fetal calf serum (294–296, 312). In the studies in which IL-6 was found to stimulate prostate cancer cell growth or enhance AR transcriptional activity, IL-6 and androgen were added to serum free or defined media (303–307). This suggests that a factor may be present in fetal calf serum that reverses the growth stimulatory effect of IL-6 on prostate cancer cells. Because the proliferative effect of IL-6 is mediated via the MAPK pathway and the inhibitory effect by PI3K, it is possible that fetal calf serum factors contribute to a stronger induction of PI3K with IL-6 treatment. The identification of such a putative factor could be of potential therapeutic benefit in the treatment of prostate cancer.

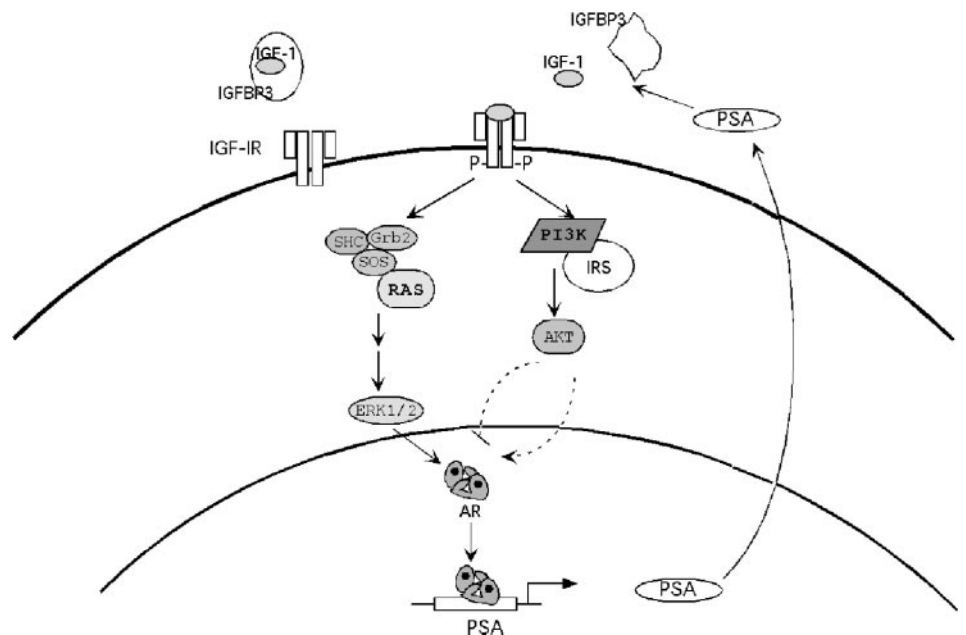
In light of the conflicting cell culture models, the mechanism resulting in the association between elevated serum IL-6 and hormone refractory metastatic prostate cancer remains unclear. It is possible that in advanced prostate cancer, secondary mutational events result in loss of growth inhibition by IL-6 (294, 312). Alternatively, elevated exposure of the tumor to IL-6 may result in constitutive activation of STAT3 and other IL-6 mediated signaling pathways. IL-6 can function as an antiapoptotic factor in hepatocytes (313), but it is not known whether it serves a similar function in prostate

cells. Constitutive STAT3 activation could contribute to cancer progression by enhancing AR transcriptional activity in conditions of low circulating androgens as found in patients treated with androgen ablation therapy (305, 306). Constitutive activation of STAT3 has been observed in a number of tumor types, including multiple myelomas, squamous cell carcinomas, and mammary carcinomas (314). Inhibition of STAT3 in multiple myeloma-derived cells and in a head and neck squamous cell carcinoma xenograft enhanced apoptosis, suggesting that STAT3 may be an important mediator of cell survival in some cancer types (314, 315) and raising the possibility that the AR-STAT3 interaction could promote survival of prostate cancer cells.

5. *IGF-I*. IGF-I mediates mitogenic and antiapoptotic signals through the IGF-I receptor (IGF-IR) (316). In the serum, IGF-I is bound predominantly to IGFBP-3, which inhibits IGF-I activity (317). The AR target gene PSA is capable of proteolytic cleavage of IGFBP-3, resulting in the release of IGF-I and potentially increasing IGF-I bioavailability (141). The activated IGF-IR can transduce signals through both the MAPK and PI3K pathways (Fig. 4). The phosphorylated IGF-IR can directly interact with and phosphorylate Shc, resulting in the recruitment of the complex containing Grb2/SOS (Ras guanylnucleotide exchange factor) and activation of Ras and the MAPK cascade (318). The activated IGF-IR is also able to phosphorylate insulin receptor substrate-1, a protein that functions to recruit the p85 subunit of PI3K to the receptor. This enables the phosphorylation of PI3K and activation of the PI3K/Akt signaling pathway (319).

Alteration in the IGF system is observed in many human cancers, including prostate cancer (320). Epidemiological studies have suggested that an elevation of serum IGF-I, especially when associated with a decrease in serum IGFBP-3, is associated with a 2- to 4-fold increase in prostate cancer risk (reviewed in Refs. 317 and 321). It remains to be determined whether the serum levels of IGF-I and IGFBP-3 indicate a general increased bioavailability of IGF-I to which

FIG. 4. Signal transduction by IGF-I. IGF-I is prevented from binding to the IGF-IR through interaction with selected IGFBPs, shown here as IGFBP-3. Proteolytic cleavage of IGFBP-3 by PSA releases IGF-I and allows binding to IGF-IR, thereby activating the MAPK and/or PI3K pathways. The ability of Akt to enhance or inhibit AR transcription may be dependent on signal strength or other cellular factors and is therefore represented by *dashed lines*.





the prostate is sensitive, or whether the serum level of these factors correlates to local prostatic production (321). In contrast to the inhibitory effect of IGFBP-3 on IGF-I signaling, IGFBP-5 promotes the effect of IGF-I (317). An increase in IGFBP-5 is associated with prostate cancer progression (322, 323) and in LNCaP cells promotes the mitogenic effect of IGF-I through the PI3K pathway (324). In transgenic mice, overexpression of IGF-I in the prostate epithelia results in prostatic hyperplasia by 2–3 months of age that progressively develops into adenocarcinoma (325). However, castration resulted in similar levels of prostatic apoptosis in wild-type and transgenic mice, suggesting that elevation of IGF-I alone does not confer androgen-independent growth (325).

As indicated in the preceding sections, both MAPK and the PI3/Akt kinase pathways have been reported to modulate AR transcriptional activity. However, divergent results have been obtained for the effect of IGF-I treatment of prostate cancer cells on AR transactivation. Culig *et al.* (326) observed that IGF-I enhanced AR transcription in LNCaP and DU145 cells in the absence of exogenous androgen. In PC-3 cell sublines that have been stably transfected with AR and undergo androgen-induced apoptosis, IGF-I treatment reduced the apoptotic effect of androgen (298), suggesting that IGF-I inhibits AR activity. It is unclear whether these differing results reflect the ability of Akt to mediate both activation (231) and suppression of AR (55) in different cellular contexts.

## V. Prostate Cancer Progression Associated with Relaxation of AR Ligand Specificity

In addition to becoming hypersensitive to residual testosterone and DHT after androgen ablation, AR may acquire the ability to become transcriptionally activated by adrenal androgens, estrogen, progesterone, cortisol, and antiandrogens. This relaxation of ligand specificity may be due to mutation of AR. However, several AR coactivators have been identified that have the capacity to broaden the range of ligands that can induce AR transactivation. The mutation of AR and/or a permissive coactivator milieu could contribute to the failure of androgen ablation by allowing endogenous hormones or the therapeutic antiandrogens to function as AR agonists and thus promote the proliferation of prostate cancer cells.

### A. AR mutations

In untreated prostate cancer, the presence of AR mutations is generally found to increase with cancer stage. AR mutations are found in 0–4% of latent and stage B prostate tumors (327–329). Although one study has found AR mutations in 18 of 74 (23%) of latent prostate cancers from Japanese men, a population at low risk of developing clinically detectable prostate cancer, the significance of this observation is not yet clear (327). However, AR mutations are observed in 21–44% of metastatic tumors sampled before therapy (328, 330). The observation that AR mutations are relatively frequent in tumors before hormonal therapy suggests that hormonal therapy itself does not drive mutagenesis of AR, although it may contribute to the selection of specific mutations after the initiation of therapy. Supporting this idea, a novel AR mutation has been identified

through long-term culture of LNCaP-FCG cells in androgen-depleted medium with the antiandrogen bicalutamide (331). The surviving LNCaP sublines, through the mutant AR molecules, used bicalutamide as a transcriptional agonist, whereas HF remained an antagonist.

As shown in Table 1, the majority of the AR mutations identified from prostate carcinoma samples are point mutations resulting in a single amino acid substitution. These mutations are predominantly localized to the AR LBD (Table 1 and Refs. 332 and 333). Although the effect of many of the identified mutations has not yet been investigated *in vitro*, the predominant effect of the amino acid substitution is to increase the number of ligands capable of inducing AR transactivation. There are relatively few reports of tumors that contain multiple AR mutations (Table 2). AR mutations are typically identified by sequencing RT-PCR products representing short regions of the AR cDNA. It is therefore difficult to determine whether these samples carry a single AR allele with multiple mutations or whether the tumor is heterogeneous for different mutant AR alleles. The prevalence of tumors carrying a single mutant AR allele that permits activation by a broad range of compounds suggests that once such a mutation arises, it rapidly comes to predominate in the tumor.

The two most frequent functional consequences of AR mutations isolated from metastatic prostate cancer are mutations that enable antiandrogens to function as AR agonists and mutations that allow AR transcription by the adrenal androgens dehydroepiandrosterone (DHEA) and androstenediol (Table 1). The prevalence of these functional types suggests that they provide a greater selective advantage for cancer progression than other mutational types. Mutations that result in the ability of antiandrogens to function as AR agonists are predominantly found in hormone refractory metastatic prostate cancer. The most frequently occurring mutation of this type is the AR T876A mutation, which has been reported to occur in 25% (6 of 24 patients) (334) and 31% (5 of 16 patients) (335) of hormone refractory metastases after combined androgen blockade, although this frequency is not a universal observation (336). This mutation is also found in the AR of the prostate cancer cell line LNCaP (337). The AR T876A mutation allows the antiandrogens HF and cyproterone acetate to activate AR transcription and stimulate the proliferation of LNCaP cells (337, 338). However, in addition to antiandrogens, the AR T876A mutant can be activated by DHEA (152, 339), androstenediol (175), estradiol, and progesterone (174, 337–339). The ability of the AR T876A mutant to be activated by a range of endogenous hormones may contribute to the observation that patients with tumors carrying this mutation rarely show a reduction in progression upon the withdrawal of flutamide (335). In addition to the AR T876A mutation, several other mutations that are associated with the acquisition of an agonistic response to antiandrogens have been isolated in single tumors from prostate cancer patients (328, 329, 336, 340) (Table 1). With the exception of the AR A720T mutation (152, 341), these mutants can be activated by both an antiandrogen and adrenal androgens (Table 1).

Excluding the AR T876A mutant, AR mutations that confer enhanced transcriptional sensitivity to adrenal androgens

TABLE 2. Compound AR mutations associated with prostate cancer

No.	Exon	Position	Change	Characteristics	Ref.
1	4	646	AGC→AAC S→N	Isolated from pleural fluid of a hormone refractory metastasis. Patient was initially treated by orchiectomy	336
	5	723	GGC→GAC G→D		
	8	879	CTG→CAG L→Q		
	8	895	GCA→ACA A→T		
2 <sup>a</sup>	1	54	TTG→TCG L→S	Isolated from a TURP section from a patient prior to hormonal therapy.	330
	8	890	TTT→CTT F→L		
3 <sup>a</sup>	1	262	ATG→ACG M→T	Isolated from a TURP section from a patient prior to hormonal therapy.	330
	2	573	CTC→CCC L→P		
4 <sup>a</sup>	1	64	CAG→CGG Q→R	Isolated from a TURP sample prior to hormonal therapy.	330
	7	829	CTT→CCT L→P		
5 <sup>a</sup>	4	670	CAG→CGG Q→R	Isolated from a TURP sample prior to hormonal therapy.	330
	6	790	TCT→CCT S→P		
6 <sup>a</sup>	5	750	TGG→TAG W→Stop	Detected in an archival latent prostatic tumor sample from a Japanese man.	327
	5	753	TCC→CTC F→L		
7 <sup>a</sup>	5	747	GCC→ACC A→T	Isolated from a pelvic lymph node metastasis. The two exon 7 mutations were never found in the same AR template and were considered to be in separate AR molecules.	328
	7	864	TCC→CCC S→P		
	7	866	CAG→TAG Q→Stop		
	8	918	CAG→CGG Q→R		
8 <sup>a</sup>	1	386	CCG→CTG P→L	Isolated from a bone marrow metastasis from a patient with hormone refractory prostate cancer after treatment with flutamide	407
	5	755	AAT→GAT N→D		
9 <sup>a</sup>	1	218	AAT→GAT N→D	Isolated from a bone marrow metastasis from a patient with hormone refractory prostate cancer after treatment with flutamide	407
	8	876	ACT→GCT T→A		

TURP, Transurethral resection of the prostate.

<sup>a</sup> Multiple AR mutations were detected in a tumor sample, but the methodology used cannot exclude the possibility that the tumor contained multiple AR genes carrying separate point mutations instead of a single AR with multiple mutations.

have been identified in up to 30% of metastatic prostate cancer samples (328–330, 336, 340). This suggests that adrenal androgens alone or in combination with antiandrogens may contribute to the progression of prostate cancer and the failure of androgen ablation therapy in patients with tumors carrying AR mutations. Combined androgen blockade results in a 49% decrease in serum DHEA and a 41% decrease in androstenediol (57). Therefore, abundant adrenal androgens are still present after androgen ablation and may contribute to selection of adrenal androgen-responsive AR mutations. Patients with tumors carrying adrenal androgen-responsive AR mutations would be expected to particularly benefit from P-450 inhibitors such as ketoconazole and aminoglutethimide that inhibit adrenal androgenesis. An AR germline polymorphism, AR R725L, has been associated with a 6-fold increase in prostate cancer risk in a Finnish

population (342). This mutation is associated with an increased transcriptional response to DHEA (152), and it is possible that increased AR transcriptional activity by adrenal androgens in addition to testicular androgens contributes to prostate cancer risk in these individuals.

### B. Role of coactivators in ligand activation

The interaction between AR and coactivators can contribute to the range of ligands that can induce AR transcription. One of the most extensively studied coactivators that is capable of relaxing the ligand specificity of AR is ARA70 (167). ARA70 has been found to enable the antiandrogens HF and bicalutamide to activate wild-type AR (343). Elevated expression of ARA70, as seen in a model of hormone refractory prostate cancer (Ref. 168; and S. Yeh, and C. Chang, unpub-

lished observations), may contribute to the failure of combined androgen blockade by enabling antiandrogens to function as AR agonists even in the context of a nonmutated AR. However, ARA70 can also enable adrenal androgens to activate AR. In DU145 cells, ARA70 can permit transcription of wild-type AR in the presence of 1 nM androstenediol, the serum level of androstenediol present after combined androgen blockade (57, 175). In the case of the AR T876S mutant, originally isolated from a hormone refractory prostate cancer metastasis, ARA70 can enhance AR T876S transcriptional activity in the presence of physiological levels of DHEA and androstenediol (57, 175). In the absence of ARA70, the AR T876S mutant shows only marginal transcriptional activity in response to adrenal androgens (175). The effect of an elevation in ARA70 expression in prostate cancer may therefore be similar to some types of AR mutation in that it allows AR transcriptional activity in response to antiandrogens and adrenal androgens.

In addition to ARA70, supervillin and a mutant of  $\beta$ -catenin have also been found to permit AR transcription in response to adrenal androgens. Supervillin, a recently identified AR-associated protein, has been reported to promote AR transcription in COS-1 cells by approximately 2-fold in the presence of 10 nM androstenediol (344).  $\beta$ -Catenin mutations have been found in 5% of prostate cancer samples (345). One prostate cancer-associated mutation of  $\beta$ -catenin, S33F, has been found to enhance AR transcription in prostate cancer cells in response to 1–10 nM androstenedione (346), which is within the physiological range for prostate cancer patients before and after androgen ablation (57). Therefore, alteration in the expression of specific coactivators, or mutation of coactivators, may allow adrenal androgens to activate AR transcription.

In addition to adrenal androgens, ARA70,  $\beta$ -catenin S33F, and the coregulator ARA55 have also been found to permit AR transcription in response to estradiol. Several authors have demonstrated that ARA70 can permit the transcription of wild-type AR, AR T876A, and AR E231G in prostate cancer cell lines in response to 1–10 nM estradiol (174, 179, 347, 348). ARA70 promotes the estradiol induction of AR at least in part by retarding the dissociation of estradiol from AR (349). The  $\beta$ -catenin S33F mutant has similarly been found to enhance AR transactivation in response to similar estradiol concentrations (346). Stable transfection of LNCaP cells with ARA55 has been found to enable transcription of endogenous LNCaP AR in response to 100 nM estradiol (350). This estradiol-mediated induction of AR could be inhibited by the expression of a dominant negative mutant of ARA55 (350).

Although the serum level of estradiol in adult males is substantially lower than that required to permit AR transcription in the presence of ARA70, bovine prostatic fluid can contain up to 0.5 nM estradiol (351), suggesting that local concentrations in the prostate may enable estradiol to induce AR-ARA70 transcription. An elevated level of aromatase, the enzyme responsible for metabolizing testosterone to estrogen, has been observed in some prostate cancer samples (352), although this is not a universal observation (353). Although the role of endogenous estradiol in prostate cancer is not yet understood, pharmacological doses of estrogens have been used therapeutically for prostate cancer patients to sup-

press pituitary LH release and lower serum androgen levels (354, 355). Estrogen therapy is generally not considered to be a treatment of choice due to cardiovascular side effects (355), although it is sometimes used as a secondary hormonal therapy after the failure of androgen ablation (45). It remains to be determined whether AR coregulators can influence prostate cancer progression during prolonged estrogen treatment in these patients. The predominant estrogen used in prostate cancer therapy is diethylstilbestrol (DES) (45). Although ARA70 permits AR transcriptional activation by estradiol, it does not allow transactivation in response to DES (174). Therefore, ARA70 alone is unlikely to contribute to prostate cancer progression in DES-treated patients, although it may contribute to proliferation of prostate cancer cells in response to endogenous hormones.

### C. Antiandrogen withdrawal syndrome

In a proportion of patients with disease progression after combined androgen ablation therapy, PSA decline and tumor regression occurs after discontinuation of antiandrogen treatment. This response is referred to as antiandrogen withdrawal syndrome. Although antiandrogen and hormonal withdrawal has been most extensively investigated with discontinuation of the nonsteroidal antiandrogen flutamide, withdrawal responses have also been found to occur with bicalutamide, nilutimide, the steroidal antiandrogen megestrol acetate, and the synthetic estrogen DES (reviewed in Ref. 45). Patients on antiandrogen monotherapy apparently do not manifest a withdrawal response (356, 357), possibly due to the ability of testicular androgens to promote tumor growth after the discontinuation of antiandrogen treatment. Cumulatively, a flutamide withdrawal response as determined by a decline in PSA occurs in 47% of cases (95% confidence interval, 38–54%) and a measurable regression occurs in 22% (95% confidence interval, 9–42%) of patients (356). The median duration of response is 4 months (45).

Although the mechanisms through which withdrawal responses occur are not yet completely understood, several possibilities have been proposed. One potential mechanism involves tumor AR mutations that enable antiandrogens to function as AR agonists. However, only 20% of patients with the most prevalent somatic mutation of AR that confers flutamide agonist activity, the AR T876A mutation, respond to flutamide withdrawal (335). The majority of AR mutations that can be activated by antiandrogens can also be induced by adrenal androgens and other endogenous hormones (Table 1) and therefore may resist antiandrogen withdrawal. The AR coactivators that have been characterized as permitting transcription of a wild-type AR in response to antiandrogens have similarly been reported to enhance the ability of adrenal androgens to induce AR activity (175, 346). Simultaneous withdrawal of flutamide and treatment with suppressors of adrenal androgen synthesis such as ketoconazole or aminoglutethimide have been reported to result in a decline in serum PSA in a slightly greater percentage of patients (46%–62%) than withdrawal of flutamide alone (358–360). However, it is possible that patients with certain AR mutations or coactivator imbalances may be more likely to respond to adrenal suppression with antiandrogen with-

drawal. Antagonist-bound steroid receptors can bind to the corepressors nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) that prevent transcriptional activation (2, 361, 361a). More recently, it has been shown that the NCoRs SMRT and DAX-1 interact directly with AR (362, 363). Additionally, overexpression of SMRT blocked DHT-induced AR transactivation and suppressed the inhibition of AR activity mediated by the antiandrogen flutamide (362). Likewise, DAX-1 blocked agonist-induced AR activity while also suppressing partial antagonist induction of AR-mediated transcription (363). In the breast cancer cell line MCF-7, stimulation of cell growth by the antiestrogen tamoxifen in mouse xenograft experiments is associated with a decrease in nuclear receptor corepressor expression (364). It is possible that the ability of antiandrogens to stimulate prostate cancer growth may be due in part to a similar decline in corepressor activity.

Another possible mechanism contributing to antiandrogen withdrawal syndrome involves increased agonist activity of antiandrogens, such as HF (Refs. 365 and 366; and Y. C. Hu, and C. Chang, unpublished observations), in the presence of coactivators such as ARA70, CBP [coactivator cAMP response element-binding protein (CREB) binding protein] (366a) and gelsolin (367). Increased expression of ARA70 or gelsolin in prostate cancer tissue, as well as upon androgen deprivation *in vitro* and *in vivo*, combined with known promotion of antiandrogen-mediated induction of AR transactivation by these coactivators suggests a mechanism by which increased ARA70 or gelsolin may reach a threshold that can promote the agonist activity of antiandrogens. Further evidence exists in the demonstration that a dominant negative mutant of ARA70 suppresses the agonist activity of ARA70, allowing the antiandrogenic function of HF to resume (368). Additionally, the transcriptional coactivator CBP, once overexpressed in the prostate cancer cell line DU145, enhanced HF, but not bicalutamide, induction of AR transactivation (369). Furthermore, the actin-severing protein gelsolin has been shown to interact with AR; increase in expression in LNCaP cells, LNCaP xenografts, and human prostate tumors after androgen ablation; and enhance AR transcriptional activity in the presence of either androgen or HF (367).

A third potential mechanism through which antiandrogen withdrawal may occur is by the mediation of cellular effects via antiandrogens independent of AR. We have recently found that HF induces the MAPK signal transduction pathway and stimulates the proliferation of the AR-negative prostate cancer cell line DU145. In this system, activation of MAPK results in the phosphorylation and activation of cyclin D1, resulting in cell cycle progression and stimulation of cell growth (370). While these mechanisms may contribute to the withdrawal response, antiandrogen withdrawal is transitory and does not occur in approximately 50% of patients with hormone refractory prostate cancer treated with combined androgen ablation (45, 356). As discussed above, the progression of prostate cancer is associated with multiple changes in growth factor sensitivity that may allow cancer progression independent of antiandrogen treatment. The ability of nonandrogenic growth factor to promote tumor

growth may therefore contribute to the failure of antiandrogen withdrawal.

## VI. Nongenomic Androgen Action

The classical mechanism of androgen action is through induction of AR transcriptional activity. In addition to this transcriptional or genomic mode of action, an increasing body of evidence suggests that androgens and other steroid hormones can exert rapid, nongenomic effects at physiological concentrations (371–373). Nongenomic steroid activity involves the rapid induction of second messenger signal transduction cascades, including MAPK, cAMP, and alteration in intracellular calcium levels. The induction of second messenger signaling through nongenomic steroid action is insensitive to inhibitors of transcription and translation. Typically, these effects occur within 5 min, considered to be too rapid to involve changes in transcription and translation. Membrane steroid receptors distinct from the classical nuclear steroid receptors are thought to mediate many nongenomic steroid actions, although to date only the membrane progesterone receptor has been cloned (374, 375). However, nongenomic action may also occur through nuclear steroid receptors functioning in the cytoplasm. For example, some nongenomic estrogen effects can be mediated through a membrane-associated form of estrogen receptor  $\alpha$  (376, 377). Although a membrane receptor complex for  $17\alpha$ -alkylated androgens has recently been purified (378), the best characterized mechanisms of the nongenomic action of testosterone and DHT occur through activation of Src kinase by AR in the cytoplasm and by androgen acting through SHBG. The nongenomic action of residual testosterone and DHT may contribute to prostate cancer progression after androgen ablation therapy by contributing to the modulation of AR transcriptional activation or the function of other transcription factors.

Src is a tyrosine kinase that is normally targeted to the inner cell membrane by myristylation and palmitoylation. The tyrosine kinase activity of Src is normally autoinhibited by interaction between the tyrosine kinase domain and the Src homology 2 (SH2) and SH3 domains. Disruption of this intramolecular interaction by protein binding or phosphorylation results in the activation of Src kinase. In response to DHT or R1881, AR can interact with the SH3 domain of Src (379, 380). The ligand-induced association between AR and Src stimulates Src kinase activity within 1 min in LNCaP cells (379). Two members of the MAPK signaling cascade, Raf-1 and ERK-2, are phosphorylated in response to androgen within 2 and 5 min, respectively (379). Androgen induction of the Src/Raf/ERK pathway is abrogated by inhibition of Src kinase activity or treatment with antiandrogens (379, 380). These studies suggest that androgens acting through AR can stimulate MAPK signaling through a nongenomic mechanism. The physiological role of this nongenomic stimulation of MAPK has not been fully determined. In LNCaP cells, inhibition of Src kinase or MAPK activity prevents androgen-induced cell cycle progression (379). However, this effect occurs over a period of days, and therefore it is difficult to determine the contribution of nongenomic an-

drogen action and secondary transcriptional effects on the cell growth and proliferation machinery. One possible target of MAPK induction is AR itself. Phosphorylation of AR by ERK-2 is associated with an increase in AR transcriptional activation and an increased ability to recruit ARA70 (213). The SRC family of coactivators is also a target of MAPK phosphorylation, which increases the ability of the coactivators to recruit additional coregulatory complexes to DNA-bound steroid receptors (222–224). Therefore, it is possible that DHT can induce an autocrine stimulation of AR transcriptional activity through the MAPK pathway, resulting in the phosphorylation of AR and the enhanced recruitment of coactivators. Residual prostatic DHT remaining after androgen ablation could contribute to this enhancement of AR transcriptional activity and diminish the effect of the androgen ablation therapy.

Recently, Ras/MAPK signaling has been linked to androgen sensitivity in both androgen-sensitive and -insensitive human prostate cancer cell lines. Stable transfection of Ras mutants that activate Ras/MAPK resulted in hypersensitivity of LNCaP cells to androgen, effectively reducing the level of androgen needed for cell growth, expression of PSA, and maintenance of cell tumorigenicity (381). In contrast, inhibition of Ras signaling restored androgen sensitivity to the LNCaP-derived hormone-refractory C4–2 cell line (382).

Androgens may also stimulate second messenger signaling through binding to SHBG and the SHBG receptor. The majority of serum DHT, and approximately half of serum testosterone, is bound to SHBG with the remainder bound to albumin (383, 384). Only approximately 1–2% of serum testosterone and DHT are not complexed with serum proteins (384). In addition to androgen, SHBG also binds estradiol (384). Although the SHBG receptor has not yet been cloned, it is thought to be either a G protein-coupled receptor or functionally linked to one (385, 386). Binding of the androgen-SHBG to the SHBG receptor results in the rapid induction of cAMP in LNCaP and MCF-7 cells, resulting in activation of protein kinase A (PKA) (387–389). In serum free media or in media from which the SHBG has been removed, the readdition of SHBG is necessary for androgen to induce cAMP (388). In organ culture of human prostate explants, the DHT induction of PSA can be mimicked by stimulation of the SHBG-PKA pathway (389). PSA expression in this system can be induced by SHBG and estradiol, whereas SHBG or estradiol alone had no effect. Cyproterone acetate and HF blocked the estradiol-SHBG induction of PSA, suggesting that AR transcription is induced in the absence of exogenous androgen (389). Similar results have been obtained in a canine prostate explant model (390). These observations suggest that AR transcription may be enhanced through either DHT or estradiol binding to SHBG and activating PKA through the SHBG receptor. However, the mechanism of PKA stimulation of AR transcription in response to activation of the SHBG receptor has not yet been determined. AR itself does not contain a consensus PKA phosphorylation site and is not directly phosphorylated by PKA (391). The AR coregulator GT198 has been shown to be directly phosphorylated by PKA, and induction of PKA increases the ability of GT198 to enhance AR transcriptional activity (392). Therefore, PKA may enhance AR transcription primarily through

modification of AR coregulators. Although serum levels of SHBG have not been consistently correlated with prostate cancer risk (34), it is possible that up-regulation of the SHBG receptor in prostate cancer cells could contribute to AR-mediated proliferation in part through the nongenomic action of androgen or therapeutic estrogen treatment.

## VII. Summary and Future Directions

Histological analysis of primary and metastatic prostate cancer indicates that AR is expressed throughout prostate cancer progression and in hormone refractory cancer. Although AR expression is heterogeneous within tumor foci (8, 9, 46), the absence of completely AR-negative hormone refractory tumors suggests that lack of AR expression does not confer a selective advantage to cancer survival or growth. Similarly, the majority of prostate cancers at all stages of progression express a wild-type AR, although AR mutations are found to occur more frequently with increasing tumor grade and stage (328, 330). Of the tumor-associated mutant ARs analyzed to date, only three have been characterized as lacking transactivation function (Table 1), also suggesting that inactivation of AR does not confer a growth or survival advantage to prostate cancer cells. The acquisition of AR mutations that permit AR transcription in response to antiandrogens and/or other endogenous ligands such as adrenal androgens may represent a significant mechanism for the failure of androgen ablation therapy. The AR T876A mutation allows both antiandrogens and other endogenous steroids to function as transcriptional agonists and is found by some authors in approximately 30% of hormone refractory prostate cancers (334, 335). Coactivators such as ARA70 that enhance the transcription of both wild-type and some mutant ARs in response to antiandrogens, adrenal androgens, and estradiol (174, 175, 343) may also contribute to the progression of prostate cancer to a hormone refractory state. Although growth factors functioning through signal transduction cascades have not yet been demonstrated to influence the ligands able to activate AR, phosphorylation of AR influences AR-coregulator interaction and may contribute to prostate cancer progression in tumors with an altered coactivator milieu. However, growth factors also influence multiple other mitogenic and antiapoptotic pathways and may therefore influence prostate cancer progression through mechanisms that do not involve AR.

However, the heterogeneity of AR expression within individual tumor foci suggests that AR expression may not be required for the survival of each individual prostate cancer cell. Transformation of prostate epithelia is associated with a shift from paracrine growth factor regulation to autocrine growth factor production (38, 393). It is possible that AR-positive cells are necessary within a prostate tumor mass to sustain local growth factor conditions. However, relatively little is known about the role of AR in growth factor production in prostate cancer cells. Strong activation of Akt kinase results in a switch from stimulation of AR activity to degradation of AR (55, 298). It is possible that the combined growth factor stimulation of some prostate cancer cells within the tumor mass may cause very high levels of active

Akt resulting in levels of AR expression that are difficult to detect immunohistologically, but may still function to promote prostate cancer cell survival. Alternatively, the discovery of two Ets family transcription factors that are capable of regulating PSA expression independently of AR suggests that for at least some AR target genes, AR is not required for regulation. Although the Ets transcription factors PDEF and ESE2 are expressed in the normal prostate (96, 97), it is not yet known whether either of these factors can induce PSA expression in prostate cancer cells in the presence of AR antagonistic ligands or in AR-negative prostate cancer cell lines. Similarly, the expression of PDEF or ESE2 has not yet been examined immunohistologically in prostate cancer samples, and in particular those cells of prostate cancers that are AR negative and PSA positive. It has not yet been determined whether alteration of expression of a specific set of transcription factors in prostate cancer cells can enable prostate cancer progression independent of AR function or inhibition by androgen ablation therapies.

The induction of AR transcriptional activity has been implicated alternately in apoptosis and proliferation of prostate epithelial cells. The ability of physiological concentrations of androgens to induce apoptosis has been observed in some, but not all, LNCaP sublines that have been selected for growth in androgen-depleted media (75, 77, 394). Similarly, stable transfection of PC-3 cells with AR generates sublines that are growth inhibited by androgen (78, 79). No consistent differences have been identified between LNCaP and PC-3-AR sublines that are inhibited by androgen and those sublines that proliferate in response to androgen (75, 78, 79, 394). Although AR has been implicated in the induction of apoptosis in selected prostate cancer cell lines, the primary role of AR in human prostate cancer patients is to control proliferation. Androgen ablation results in a reduction of proliferation in 88% of prostate tumor foci but does not induce apoptosis in the majority of tumors (64). Similar results have been obtained in the Dunning rat model of prostate carcinoma (66, 67). These observations suggest that the ability of androgens to regulate apoptosis is lost early in the progression of prostate cancer. It has been proposed that prostate cancer cells that survive androgen deprivation may be forced to undergo apoptosis with androgen treatment (73). Although prostate cancer cell lines have been selected to undergo apoptosis in response to physiological levels of androgen (for example, see Refs. 78 and 394), treatment of prostate cancer patients with physiological or pharmacological doses of androgen results in a negative outcome in the majority (97%) of patients (44).

Although AR activity is apparently important throughout prostate cancer progression, particularly in the regulation of prostate cancer cell proliferation, aspects of the interaction between AR and growth factor-mediated signal transduction cascades remain to be clarified. Although elevated serum levels of IL-6 are found in patients with hormone refractory prostate cancer (275, 278), divergent effects of IL-6 on the growth of prostate cancer cell lines and AR transcriptional activity have been described (292, 295, 303, 306). As shown in Fig. 3, IL-6 mediates signaling through JAK/STAT, MAPK, and PI3/Akt kinase. It is possible that subtle alterations in the relative strength of these pathways, possibly

under the influence of different cell culture conditions, contributes to the different observations between research groups. IL-6 induction of MAPK and STAT3 has been found to enhance AR transcription (305–307). However, IL-6-mediated induction of PI3K in LNCaP cells has been found to have no effect on AR activity (395), to inhibit AR activity, or to result in apoptosis (293). It is possible that the relative strength of STAT3 phosphorylation compared with PI3K phosphorylation contributes to the overall effect on AR transcription and cellular proliferation. AR is generally considered to be a direct target of Akt, a downstream kinase from PI3K (Fig. 2). However, Akt-mediated phosphorylation of AR has been reported to result in different transcriptional effects. We have observed that Akt inhibits AR transcription, as determined by transfection of constitutively active or dominant negative mutants of Akt and PI3K in DU145 cells (298). Mutation of serine 210 or 790, the predicted Akt phosphorylation sites, relieved Akt inhibition of AR (298). The Akt-mediated inhibition of AR may occur through ubiquitination and proteolytic degradation of AR (55). In contrast, Wen *et al.* (231) found that constitutively active Akt enhances AR transcription in LNCaP cells, and inhibition of Akt activity by transfection of a dominant negative mutant of Akt or transfection of PTEN inhibits AR activity. The phosphatase PTEN inhibits PI3K and thus Akt activity (Fig. 2). PTEN suppresses AR transcription, consistent with a stimulatory effect of Akt phosphorylation on AR (239, 240). The reason for these divergent results is unclear. The use of different cell lines and reporter genes may contribute to the differing observations. Even within the same cell line, differences in culture conditions and cell density can alter steroid responsiveness (301) and may also contribute to the different effects of phosphorylation. These factors may contribute to the divergent effect of Smad3 on AR transactivation. Treatment of DU145 or PC-3 cells with TGF $\beta$ , or transfection of DU145 cells with Smad3, has been reported to enhance AR transcription (256). In contrast, TGF $\beta$  or Smad3 transfection was found to inhibit AR transcription in PC-3 and CV-1 cells (255). It is possible that Smad3 alone may enhance AR activity but that a Smad3-Smad4 heteromer may repress AR function (257). However, it is unclear whether the PC-3 cells used by the different groups contain differing levels of Smad4.

Approximately half of the patients with prostate cancer progression after combined androgen ablation with antiandrogens show a decline in serum PSA after withdrawal of the antiandrogen. The majority of AR mutations that enable antiandrogens to function as AR agonists also permit AR activation by adrenal androgens and/or other endogenous hormones (Table 1). In addition, the AR coactivator ARA70 is able to allow antiandrogens to induce wild-type and mutant AR transcription (343), but this coactivator also permits the induction of AR transcription in response to adrenal androgens (175). In attempts to identify new antiandrogens with reduced agonist effects compared with currently used compounds, Miyamoto *et al.* (396) discovered that the DHEA metabolite 3 $\beta$ -acetoxyandrost-1,5-diene-17-ethylene ketal blocks androgen binding to both wild-type and mutant forms of AR and suppresses LNCaP cell growth and PSA expression. Additionally, Le *et al.* (397) discovered that 3,3'-

diindolymethane (DIM), a plant-derived antiandrogen, has strong activity in human prostate cancer cells. DIM was found to suppress LNCaP cell proliferation and inhibit DHT-stimulated DNA synthesis as well as PSA promoter expression. The mechanism through which DIM acts was also shown to involve repression of androgen-induced AR nuclear translocation.

It is possible that at least some antiandrogens can exert a biological effect independently of AR. In the case of HF, we have observed that it can stimulate the growth of AR-negative DU145 cells through activation of MAPK (370). It remains to be determined whether HF stimulation of MAPK can contribute to antiandrogen withdrawal. In addition to the potential role of AR mutations, alteration in AR coregulator abundance, and the nongenomic action of HF, it is possible that other undiscovered mechanisms may also contribute to antiandrogen withdrawal.

The involvement of AR in the regulation of prostate cancer cell proliferation has important implications for the development of additional treatment strategies for hormone refractory disease. It has been suggested that inhibition of AR through mechanisms other than ligand manipulation might provide additional therapeutic benefit (398). Because AR activity can be influenced by phosphorylation by MAPK and Akt, modulation of these kinase pathways together with combined androgen ablation may delay prostate cancer progression.

Inhibition of AR transcription, translation, protein stability, or nuclear translocation would be expected to reduce the net transcriptional activity of AR. The development of hormone refractory prostate cancer is generally thought to occur through mechanisms that modulate AR function. Therefore, the combination of a therapy that regulates the availability of AR protein with androgen ablation might be expected to provide a longer period of androgen sensitivity. A reduction in the bioavailability of AR may reduce the impact of an elevation of AR coactivators or AR mutations that allow transcription in response to multiple ligands. Relatively few compounds are known to influence AR protein availability. A number of naturally occurring compounds or their derivatives have been found to regulate AR bioavailability in prostate cancer cell lines. These compounds include silymarin, vitamin D<sub>3</sub>, vitamin E derivatives, and polyphenols such as resveratrol and epigallocatechin gallate (398). Although the mechanisms through which these compounds influence AR activity have not been fully elucidated and further investigation of the effects of such compounds in animal models of prostate cancer is needed, it is possible that they may become therapeutically useful when combined with androgen ablation therapy. Resveratrol and vitamin E succinate are known to inhibit the expression of AR and androgen-induced proliferation of LNCaP cells (399, 400). Additionally, resveratrol treatment has been shown to down-regulate PSA, the androgen coactivator ARA24, and nuclear factor  $\kappa$ B p65, supporting the potential usefulness of this compound as a prostate cancer chemopreventative agent (401).

Some dietary compounds linked to prevention or inhibition of prostate cancer may function at least in part through modulation of GH signaling. For example, the flavonoid silibinin and the vitamin D analog EB1089 have been found

to increase the levels of IGF1 (402, 403). Although inhibition of AR through androgen deprivation and AR protein availability would be expected to reduce the proliferation rate of prostate cancer cells, it is unclear whether apoptosis would be induced. Inhibition of proliferation, while delaying or preventing further progression, still could allow further cancer progression through mechanisms that do not involve AR. Further experimental investigation of this form of combinatorial therapy is necessary to determine its ultimate therapeutic utility.

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