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REVIEW

Differential androgen receptor signals in different cells explain why androgen-deprivation therapy of prostate cancer fails

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Prostate cancer is one of the major causes of cancer-related death in the western world. Androgen-deprivation therapy (ADT) for the suppression of androgens binding to the androgen receptor (AR) has been the norm of prostate cancer treatment. Despite early success to suppress prostate tumor growth, ADT eventually fails leading to recurrent tumor growth in a hormone-refractory manner, even though AR remains to function in hormone-refractory prostate cancer. Interestingly, some prostate cancer survivors who received androgen replacement therapy had improved quality of life without adverse effect on their cancer progression. These contrasting clinical data suggest that differential androgen/AR signals in individual cells of prostate tumors can exist in the same or different patients, and may be used to explain why ADT of prostate cancer fails. Such a hypothesis is supported by the results obtained from transgenic mice with selective knockout of AR in prostatic stromal vs epithelial cells and orthotopic transplants of various human prostate cancer cell lines with AR overexpression or knockout. These studies concluded that AR functions as a stimulator for prostate cancer proliferation and metastasis in stromal cells, as a survival factor of prostatic cancer epithelial luminal cells, and as a suppressor for prostate cancer basal intermediate cell growth and metastasis. These dual yet opposite functions of the stromal and epithelial AR may challenge the current ADT to battle prostate cancer and should be taken into consideration when developing new AR-targeting therapies in selective prostate cancer cells.
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Introduction

Prostate cancer is the second leading cause of cancer-related death among men in the United States (Jemal *et al*., 2005). Approximately 80–90% of prostate cancers are dependent on androgens at initial diagnosis. Since the discovery by Huggins and Hodges (1941) that prostate cancer progression is influenced by androgen actions, androgen-deprivation therapy (ADT) to suppress androgens binding to androgen receptor (AR) remains the major treatment regimen for the disease (Denis and Griffiths, 2000). However, ADT ultimately fails, and prostate cancer progresses to a hormone-refractory (androgen-independent) state with advanced metastasis and high morbidity and mortality.

Androgens function mainly through an axis involving the testicular synthesis of testosterone, its transport to target tissues (Roy *et al*., 1999), and the conversion by 5α-reductase to the more active metabolite, 5α-dihydrotestosterone (Shimazaki *et al*., 1965; Anderson and Liao, 1968; Bruchovsky and Wilson, 1968). Testosterone and 5α-dihydrotestosterone exert their biological effects through binding and transactivating AR (Heinlein and Chang, 2002; Heinlein and Chang, 2004; Rahman *et al*., 2004; Wang *et al*., 2005), which involves interaction of AR with various coregulators during prostate development and prostate cancer progression (Heinlein and Chang, 2002; Rahman *et al*., 2004; Wang *et al*., 2005).

AR is expressed throughout prostate cancer progression and its expression persists in the majority of patients with hormone-refractory disease (Cunha *et al*., 1987; Sadi *et al*., 1991; van der Kwast *et al*., 1991; Chodak *et al*., 1992; Hobisch *et al*., 1996; Mohler *et al*., 1996; Buchanan *et al*., 2001), and many AR mutations identified from hormone-refractory prostate tumors are capable of transactivation. These observations suggest that the eventual failure of ADT cannot be attributed simply to the loss of AR function.

Prostate cancer patients undergoing ADT often develop hypogonadism, which is associated with sexual dysfunction, decreased lean body mass and muscle strength, increased adipocity, reduced quality of life, and osteoporosis. They also have a higher risk of developing metabolic syndrome, diabetes, and cardiovascular diseases (Basaria, 2008; Taylor *et al*., 2009). Several studies have indicated that androgen

replacement therapy (ART) of selected patients with castration-resistant prostate cancer led to improved quality of life without any adverse effect on their cancer progression for a considerable follow-up duration. Some of the selected patients even displayed a decrease in their serum prostate-specific antigen levels after ART (see details in section 'ART in prostate cancer patients'), indicating that there might be reduced cancer progression. However, the reasons behind the failure of ADT and why there are differential responses to androgen/AR signals in different prostate cancer patients remain unclear at present.

We will use this review to present our hypothesis based on the recent findings and conclude that AR can function as both a proliferation stimulator and a tumor suppressor depending on its expression in individual prostatic cells to exert its diverse and differential functions for prostate cancer progression. These dual yet opposite functions of AR may thus create a direct challenge to the current ADT and promote new therapies in the battle against prostate cancer.

Human clinical studies with ADT and ART for prostate cancer-differential response to androgen/AR signaling

ADT in prostate cancer patients

The proliferation-stimulating function of AR is at the center of the premise for ADT in the treatment of prostate cancer (Huggins and Hodges, 1941). ADT with either surgical or medical castration usually results in a response rate of 70–80% with a 12–33 months duration of progression-free survival (Bruchovsky, 1993). However, after an average of 24 months, the tumors almost always recur and no longer respond to ADT (Eisenberger *et al*., 1998), even though the prostate tumors still express AR (Visakorpi *et al*., 2005; Mostaghel *et al*., 2007). Interestingly, cell sorting of these ADT-refractory tumors found that the prostatic epithelial basal cell marker, cytokeratin 5 (CK5) (Bruchovsky *et al*., 1990; van Leender *et al*., 2001) increased from 29 to 75%, an observation consistent with the expansion of basal intermediate-like tumor cells observed in transgenic adenocarcinoma mouse prostate (TRAMP) mice with selective knockout of AR (ARKO) in prostatic epithelium (pes-ARKO-TRAMP) and inducible ARKO-TRAMP (ind-ARKO-TRAMP) mice (see details in section 'AR dual functions in prostate cancer progression and metastasis'). Recent clinical findings from 254 prostate cancer patients also indicated that the expression of nestin, which is linked to the metastatic potential of prostate cancer cells, is present only in the metastatic tumors of patients receiving ADT, suggesting that treatment with ADT might result in promotion of prostate metastatic tumors in these patients (Kleeberger *et al*., 2007).

Together, these human clinical data suggest that ADT may be effective for prostate cancer patients only in selective prostate tumor cells and time periods, beyond which tumors will progress into the hormone-refractory stage with a more aggressive metastasis.

ART in prostate cancer patients

Several studies have shown that ART of hypogonadal patients with localized prostate cancer treated with radical prostatectomy or ADT has no adverse effect on prostate cancer progression (Kaufman and Graydon, 2004; Agarwal and Oefelein, 2005; Ferreira *et al*., 2006; Khera *et al*., 2009). Fowler and Whimore, (1981) observed that out of 52 metastatic prostate cancer patients who received ART, 45 exhibited increased cancer progression that could be reversed by androgen withdrawal, whereas 7 experienced symptomatic benefits. Other investigators have observed that in patients with castration-resistant metastatic prostate cancer, ART resulted in either little adverse effect on cancer progression or displayed some biochemical improvement or progression (Mathew, 2008; Morris *et al*., 2009; Szmulewitz *et al*., 2009). These clinical studies suggested that prostate cancer patients have differential responses to androgen/AR signals and ART might be able to improve the quality of life in selective prostate cancer patients with little impact on further progression of their prostate cancers.

Differential androgen/AR signals in various prostate tumor cells from the same patient

The above clinical data led us to hypothesize that differential androgen/AR signals in individual cells of the prostate tumor might exist in the same or different patients, and may explain why ADT of prostate cancer fails. This hypothesis is supported by the isolation of three prostate primary tumor lines, PCa1, PCa2, and PCa3, from the same patient that exhibited differential responses to the androgen/AR signals after orthotopic implantation into androgen-supplemented vs castrated SCID mice (Figure 1) (Wang YZ, unpublished data). Although both PCa1 and PCa2 cells express AR and prostate-specific antigen, PCa1 cells grew more rapidly, whereas PCa2 cells grew more slowly in castrated than in androgen-supplemented mice, suggesting that they are androgen-suppressed and androgen-stimulated tumor cells, respectively. In contrast, the growth of PCa3 cells was similar in castrated vs androgen-supplemented mice, indicating that it is androgen insensitive. These various responses of prostate cancer cells to androgen/AR signals from the same patient are very similar to the responses of hair follicles to androgen/AR signals from the same person (Inui *et al*., 2002): positive for the hair follicles in the mustache area, negative for the hair follicles on the top of the skull, toward the front, and independent for the hair follicles on the back of the head. These contrasting effects of androgen/AR signals in the prostate tumor cells or hair follicles from the same person (Figure 2) strengthen our hypothesis that differential androgen/AR signals exist in various cells of prostate tumors to explain why ADT of prostate cancer fails.

The following sections will summarize various *in vitro* and *in vivo* evidences from mice and human prostate cancer cell lines to support such a conclusion.

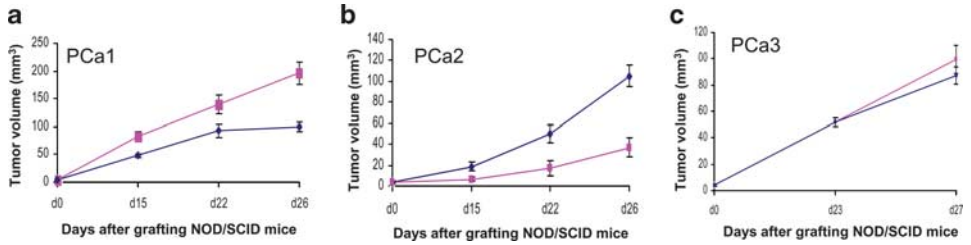

Figure 1 Three tumor sublines from the same prostate cancer patient had differential growth responses to androgen. The growth pattern of the three tumor lines transplanted orthotopically into non-obese diabetic/severe combined immunodeficient (Nod/SCID) mice supplemented with androgen (blue line) and in castrated hosts (purple line) were compared. (**a**) PCa1: an androgen-suppressed subline; (**b**) PCa2: an androgen-stimulated subline; and (**c**) PCa3: an androgen-independent subline.

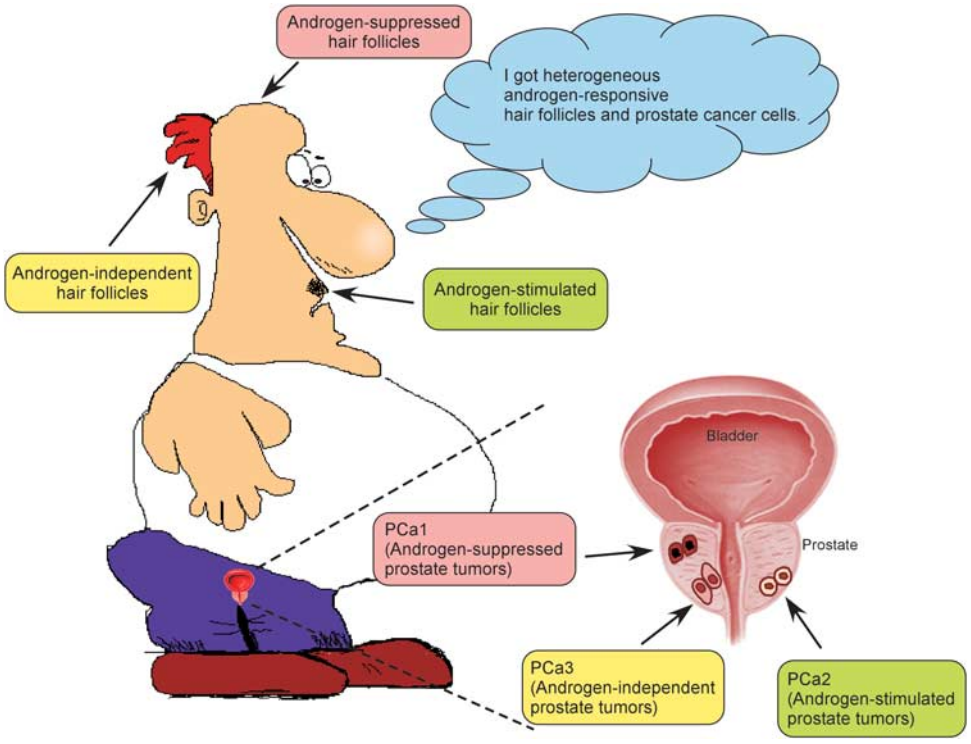

Figure 2 Similar differential responses of hair follicles and prostate cancer cells to androgens/AR signals. Hair follicles in the top of skull to the forehead, back of the head, and mustache areas of the same person have differential responses to androgens, which are similar to the differential responses of three prostate cancer sublines isolated from the same patient shown in Figure 1.

AR dual functions in prostate cancer progression and metastasis

Recent studies using epithelial-specific, fibroblast-specific, and smooth muscle-specific ARKO mice have indicated that during prostate development, AR in stromal cells functions as a proliferation stimulator, whereas AR in epithelial basal intermediate cells functions as a suppressor, and in epithelial luminal cells functions as a survival factor. (The detailed descriptions of these AR dual functions in normal prostate development are described in Supplementary Information). Here, we will focus on discussion of the similar dual AR functions in prostate cancer progression and metastasis.

Huggins and Hodges (1941) provided the first solid *in vivo* evidence for androgen/AR signals to function as a proliferation stimulator for prostate cancer progression, which became a common view supported by the subsequent ADT for treating prostate cancer patients with early success. Nevertheless, how and in which cell(s) AR may function as a proliferation stimulator of cancer cells remain unclear. Here, we summarize recent findings that suggest AR in stromal cells might function as a stimulator, whereas AR in epithelial luminal-like cells might function as a survival factor, and in epithelial basal intermediate-like cancer cells, AR might function as a suppressor of prostate cancer progression and metastasis.

AR in stromal cells: a stimulator for tumor progression and metastasis

Stromal–epithelial interaction remains important for tumor progression and metastasis (Cunha *et al*., 2003; Bhowmick and Moses, 2005; Condon, 2005). During tumor progression, the stromal cells, including fibroblasts, myofibroblasts, endothelial, and immune cells, form a

microenvironment supporting the progression, survival, and metastasis of the tumor. In the normal human prostate, the stroma is constituted mainly of smooth muscle cells expressing AR. In prostatic carcinoma, the tumor stroma is constituted mainly of fibroblastic and myofibroblastic cells (Cunha *et al*., 2003), suggesting that cell-transition changes have occurred in both the stroma and the epithelium during tumorigenesis. Earlier tissue recombination studies by Thompson *et al*. (1993) have indicated that prostatic tumorigenesis indeed requires changes in both the stroma and the epithelium, a notion supported by the results of Cunha and co-workers (Olumi *et al*., 1999; Wang *et al*., 2001; Cunha *et al*., 2003; Ricke *et al*., 2006). Thus, prostate cancer-associated fibroblasts could promote tumor transformation of the epithelial BPH-1 cells that are immortalized with SV40 Tag (Olumi *et al*., 1999; Wang *et al*., 2001). Similarly, urogenital mesenchymes from rat and mouse could also elicite tumor transformation in BPH-1 cells in the presence of testosterone and 17β-estradiol (E2) (Wang *et al*., 2001; Ricke *et al*., 2006). However, cancer-associated fibroblasts in a castrated host and urogenital mesenchyme from testicular feminized mice (a mouse without functional AR) were unable to promote tumor transformation from BPH-1 cells (Ricke WA, personal communication). In addition, knockdown of AR in cancer-associated fibroblasts isolated from tumors of TRAMP mice reduces their ability to promote BPH-1 colony formation in soft agar (Chang C *et al*., unpublished data). These observations indicate that the stromal AR is required for prostate tumor development.

AR-negative epithelial PC-3 cells were co-cultured with human prostatic stromal WPMY1-v or WPMY-ARsi cells (Niu *et al*., 2008a) to investigate the function of the stromal AR in tumor progression and metastasis. The results from the co-culture system indicated that knockdown of AR in WPMY1-ARsi cells resulted in the co-cultured PC-3 cells being less invasive in a Boyden chamber invasion assay than PC-3 cells with vector-transfected WPMY1-v cells (Niu *et al*., 2008a). After orthotopic inoculation in nude mice, PC-3 cells also produced smaller primary and pelvic lymph node (PLN) metastatic tumors when combined with WPMY1-ARsi than with WPMY1-v cells (Niu *et al*., 2008b). Similar *in vivo* androgen/AR-dependent tumor growth were also observed using other AR-negative prostate cancer cell lines (Marques *et al*., 2005; Halin *et al*., 2007), and LNCaP cells might grow more aggressively on orthotopic transplantation after being co-inoculated with rat urogenital mesenchyme or bone marrow fibroblasts (Gleave *et al*., 1991). Together, these *in vitro* and *in vivo* findings suggested that the stromal AR might function as a proliferation stimulator to promote prostate tumor progression and metastasis.

AR in epithelial cells: an anti-apoptosis survival factor and a suppressor of proliferation for cancer epithelial cells

The TRAMP mouse model is a prostate cancer model that develops prostate tumors spontaneously at 10–12 weeks of age (Gingrich *et al*., 1991). TRAMP mice were mated with the floxed AR transgenic mice to obtain TRAMP mice carrying the floxed AR transgene, which in turn were crossed with probasin-Cre mice to generate prostate epithelial-specific (pes)-ARKO-TRAMP mice (Niu *et al*., 2008a, b). Prostatic epithelial cells develop from stem cells through proliferation and differentiation into basal and intermediate cells that finally differentiate into epithelial luminal cells (Litvinov *et al*., 2003; Tokar *et al*., 2005). AR is expressed in about 50% of the basal cells and all of the luminal cells in mouse prostate (Mirosevich *et al*., 1999; Niu *et al*., 2008a). Similar to pes-ARKO mice (Wu *et al*., 2007), knocking out AR from these epithelial cells in pes-ARKO-TRAMP mice and others resulted in increased apoptosis of CK8-positive epithelial luminal cells (18%) as compared with those (2%) from wild-type TRAMP mice at 16 weeks of age (Niu *et al*., 2008a), suggesting that AR in the epithelial luminal cells may function as a survival factor to protect prostate cancer cells from apoptosis.

However, knocking out the epithelial AR in pes-ARKO-TRAMP mice also resulted in increasing numbers of proliferating cells in CK5-positive-basal cells, including the CK5/CK8-double-positive-basal intermediate cells. The consequence of increased apoptosis in luminal cells and increased proliferation in basal intermediate cells then resulted in less differentiated yet larger primary tumors in the ventral prostate of pes-ARKO-TRAMP mice than tumors from 16-week-old TRAMP mice (Niu *et al*., 2008b). The primary prostate tumors of pes-ARKO-TRAMP mice exhibited a higher population of CD44-positive (Liu *et al*., 2004; Bhatia *et al*., 2005) and CK5/CK8-positive (van Leenders and Schalken, 2003; Bhatia *et al*., 2005) intermediate-like cells than those found in wild-type TRAMP mice (Niu *et al*., 2008a). Incidentally, CD44-positive, but AR-negative prostate cancer cells purified from human prostate cancer xenografts were also enriched in tumorigenic and metastatic progenitor cells (Patrawala *et al*., 2006). These results indicated knocking out the epithelial AR might lead to cell population changes with expansion of intermediate-like tumor cells and decreased secretory luminal cells in the prostates of pes-ARKO-TRAMP mice (Niu *et al*., 2008a). These results suggested that epithelial AR might function as a proliferation suppressor in epithelial basal intermediate cells and a survival factor in epithelial luminal cells. As prostate carcinoma arises from epithelial cells, the opposite functions of the epithelial AR in different epithelial cells could then affect prostate cancer progression in TRAMP mice by favoring survival of differentiated tumor epithelium while suppressing proliferation of epithelial basal intermediate cells, thereby retarding tumor progression to a more malignant stage.

AR in epithelial cells: a suppressor of prostate cancer metastasis

The AR signals in prostate cancer epithelial cells also influence tumor metastasis. Thus, the size of metastatic

tumors in PLNs of pes-ARKO-TRAMP mice was larger than those from wild-type TRAMP littermates at the age of 24 weeks. In addition, more prostate cancer metastatic foci were observed within the livers of pes-ARKO-TRAMP than in TRAMP mice (Niu *et al*., 2008a). AR-negative PLN metastatic tumor isolated from pes-ARKO-TRAMP mice was more invasive than those from TRAMP mice *in vitro*. Importantly, restoring the expression of AR by transfection could reduce the invasiveness of PLN tumors from pes-ARKO-TRAMP mice (Niu *et al*., 2008a). An early study also found that poorly differentiated PLN metastatic tumors in castrated TRAMP mice were more aggressive than tumors from intact TRAMP mice (Gingrich *et al*., 1991). Therefore, it may be concluded that the epithelial AR also functions as a suppressor of prostate tumor invasion and metastasis.

Relative influences of the stromal and epithelial AR on prostate cancer progression and metastasis

The ind-ARKO-TRAMP (Niu *et al*., 2008b) mice were then produced to assess the consequence of simultaneously knockdown of both the stromal and epithelial AR that have opposite functions in prostate cancer progression and metastasis. The knockout AR in ind-ARKO-TRAMP mice is mediated by Mx1-Cre, which is interferon inducible and can be activated by injection of polyinosinic–polycytidic acid to induce endogenous interferon and thus activate the Cre recombinase in various tissues (Kühn *et al*., 1995) including the prostate (Wang *et al*., 2006; Niu *et al*., 2008b). After injection of polyinosinic–polycytidic acid in 12-week-old mice, AR mRNA expression in the prostate was found knocked down by 50% in the stroma and 60% in the epithelium in 16-week-old ind-ARKO-TRAMP mice compared with polyinosinic–polycytidic acid-injected control TRAMP mice. Knocking down prostatic AR expression at this early stage resulted in smaller and less-differentiated primary prostate tumors in ind-ARKO-TRAMP than in the control TRAMP mice through 16–24 weeks of age. The tumors of ind-ARKO-TRAMP mice had lower proliferation rates and higher apoptosis rates than tumors of the control TRAMP mice. Moreover, the tumors of ind-ARKO-TRAMP mice had decreased CK8-positive luminal-like cells and increased CK5- and CD44-positive-basal cells and CK5/CK8-double-positive-basal intermediate-like cells than tumors of the control TRAMP mice (Niu *et al*., 2008b).

Despite the fact that both pes-ARKO-TRAMP and ind-ARKO-TRAMP mice had higher epithelial luminal cell apoptosis and expansion of basal intermediate-like cells, pes-ARKO-TRAMP mice produced larger PLN metastatic tumors, whereas knocking down AR at an early stage in ind-ARKO-TRAMP mice produced smaller metastatic tumors than the control mice. These contrasting observations suggest that the stromal AR may have more dominant functions during prostate cancer progression at an early stage.

Metastatic tumors were also compared at the time when primary tumors had reached 1 cm^2 in TRAMP (20 weeks), pes-ARKO-TRAMP (18 weeks), and ind-ARKO-TRAMP (36 weeks) mice. The well-differentiated tumors of TRAMP mice developed small metastatic tumors in the PLN. The poorly differentiated tumors of pes-ARKO-TRAMP mice developed much larger PLN metastatic tumors and metastasized into multiple organs, whereas those of ind-ARKO-TRAMP mice were smallest and they metastasized into the seminal vesicle and liver. Thus, loss of the epithelial AR promotes prostate cancer progression and metastasis as shown in pes-ARKO-TRAMP, whereas concurrent knockdown of the stromal and epithelial AR at an early stage can override these effects of the epithelial ARKO, thereby retarding growth of the tumor and suppressing their metastasis as shown in ind-ARKO-TRAMP mice.

More importantly, ind-ARKO-TRAMP mice with early knockdown of prostatic AR had longer survival time than wild-type TRAMP and pes-ARKO-TRAMP mice (Niu *et al*., 2008b). However, the dominance of the stromal AR over the epithelial AR function diminished when ARKO in ind-ARKO-TRAMP mice was induced after the primary tumor has progressed for some time. Thus, when knockdown of AR in ind-ARKO-mice was induced at 20 weeks of age (and not at 12 weeks of age as mentioned above), the sizes of primary and PLN metastatic tumors developed after 24 weeks of age were similar between ind-ARKO-TRAMP and TRAMP mice, suggesting that the relative influences of the stromal and epithelial AR signals on prostate cancer progression and metastasis can vary with the progression of the tumor (Niu *et al*., 2008b).

Together, these observations not only support the notion that the epithelial AR functions as a tumor suppressor for prostate cancer progression and metastasis, but also indicate that the stromal AR may function as a stimulator of the prostate cancer progression and metastasis.

Dual AR functions in human prostate cancer cell lines

The dual yet opposite AR functions to influence prostate cancer cell proliferation and metastasis are also confirmed in various human prostate cancer cell lines as follows.

PC-3 cells: AR functions as suppressor of proliferation and metastasis

The PC-3 cell line was originally isolated from a human bone marrow prostate metastastic tumor (Kaighn *et al*., 1979). PC-3 cells express CK5 and CK8/18, but not AR. Thus, PC-3 cells are basal intermediate-like tumor cells (van Bokhoven *et al*., 2003) that are highly tumorigenic. Early studies have reported that ectopic expression of AR driven by a strong viral promoter in PC-3 cells resulted in androgen-dependent suppression of cell proliferation (Yuan *et al*., 1993; Garcia-Arenas *et al*., 1995; Heisler *et al*., 1997). This androgen-suppressed cell growth in PC-3 cells transfected with AR was confirmed later by Litvinov *et al*. (2004, 2006) with a modified expression vector for the ectopic AR expression. Interestingly, using

a natural human AR promoter to drive the expression of human AR in PC-3 cells, Altuwaijri *et al.* (2007) observed a slight androgen-induced cell growth in the resultant PC-3-AR9 cells. These observations indicate that ectopic expression of the AR in PC-3 cells may modulate their growth *in vitro* in a manner dependent on the AR-expressing vector. Therefore, the growth properties of prostate cancer cells *in vitro* might not adequately represent the behavior of the cells *in vivo*.

To test whether AR in PC-3 cells may also function as both stimulator and suppressor observed in mouse models, the growth, invasive, and metastatic properties of PC-3 cells carrying the empty vector (PC-3-v cells) and PC-3-AR9 cells were compared. PC-3-AR9 cells were found less invasive in an *in vitro* invasion assay and produced less osteolytic lesions in a bone-wafer resorption assay than PC-3-v cells (Niu *et al.*, 2008a). When these cells were inoculated into the tibia of athymic nude mice, it was found that PC-3-v tumors grew more invasively and aggressively than PC-3-AR9 tumors, suggesting that knockin of a functional human AR in PC-3 cells resulted in suppression of their invasion *in vitro* and *in vivo*.

Furthermore, PC-3-v or PC-3-AR9 cells were orthotopically injected into the anterior prostate of nude mice. Consistent with the above findings, mice injected with PC-3-v cells developed bigger prostate primary tumors that were less differentiated (Niu *et al.*, 2008b) and larger PLN metastatic tumors (Niu *et al.*, 2008a) than mice inoculated with PC-3-AR9 cells. Moreover, when PC-3-v or PC-3-AR9 cells were co-cultured with WMPY1 stromal cells (Webber *et al.*, 1999) and orthotopically transplanted in nude mice, PC-3-AR9 co-cultured cells still produced smaller primary and metastatic tumors than PC-3-v co-cultured cells, suggesting that even in the presence of stromal AR stimulation, the suppressor function of the epithelial AR remains effective. Using transfectants of PC-3 cells with an inducible AR-expressing transgene, Nelius *et al.* (2007) also observed that induction of AR expression in an inducible PC-3-AR$^+$ line resulted in an androgen-dependent decrease in invasion *in vitro* and decreased tumorigenecity because of decreased microvascular density that led to increased tumor cell apoptosis after subcutaneous inoculation in nude mice.

Together, these results showed that loss of prostatic epithelial AR results in the development of more invasive and metastatic prostate tumors and gain-of-AR function reverses these characteristics. Thus, the above human prostate cancer cell observations are consistent with pes-ARKO-TRAMP mice data and strongly indicate that prostatic epithelial AR functions as a suppressor of prostate tumor growth and metastasis.

LNCaP cells: AR could function as proliferation stimulator and suppressor

The LNCaP cells, which were isolated from a lymph node prostate metastatic tumor (Horoszewicz *et al.*, 1983) and express a mutated AR(T877A) (Veldscholte *et al.*, 1990), are CK5-negative and CK8/18-positive and thus are luminal-like tumor cells (van Bokhoven *et al.*, 2003). These cells may respond to androgen/AR signals differentially depending on different environments (Olea *et al.*, 1990) and exhibited variants. For example, the AR-expressing LNCaP-FGC and LNCaP-LNO cell lines isolated from the same lymph node metastatic tumor (Horoszewicz *et al.*, 1983) exhibited different and opposite proliferation responses toward androgen (Olea *et al.*, 1990, Soto *et al.*, 1995). Proliferation of LNCaP-FGC cells was stimulated by a low concentration and suppressed by a high concentration of androgen, whereas proliferation of LNCaP-LNO cells was suppressed by physiological concentrations of androgen. These different cell phenotypes might represent adaptive changes in prostate cancer cells during tumor progression.

The differential responses to androgen seen in various LNCaP cells was confirmed later using androgen-dependent LNCaP cells that were cultured in long-term absence of androgen (Kokontis *et al.*, 1994) or after prolonged numbers of passages (>80) (Igawa *et al.*, 2002) to develop sublines with androgen-independent proliferation. Addition of androgen to these androgen-independent variants of LNCaP cells suppressed proliferation (Kokontis *et al.*, 1998) and promoted apoptosis (Joly-Pharaboz *et al.*, 2000). Such adaptive changes from growth stimulator to suppressor have also been observed in LNCaP xenografts *in vivo* after castration of the host (Thalmann *et al.*, 2000; Zhou *et al.*, 2004; Chuu *et al.*, 2006).

Down-regulation of AR with anti-sense oligonucleotides (Eder *et al.*, 2000) or siRNA (Hååg *et al.*, 2005; Liao *et al.*, 2005) may result in the suppression of cell proliferation or promotion of apoptosis in both androgen-dependent and androgen-independent sublines of LNCaP cells. Eder *et al.* (2002) found that an AR-specific anti-sense oligonucleotide could suppress the growth of LNCaP xenografts. Down-regulation of the AR (through siRNA) in LNCaP cells also suppressed their invasion *in vitro* (Chang C *et al.*, unpublished data).

Interestingly, recurrent androgen-independent tumors developed from orthotopic primary tumors of LNCaP cells after castration of SCID mice hosts exhibited increased proliferation and decreased apoptosis compared with the androgen-dependent primary tumors of LNCaP cells, a change that is associated with decreased AR protein expression (Zhou *et al.*, 2004). Thus, it seems that once progressed to androgen-independent state *in vivo*, LNCaP cells might be relieved from epithelial AR suppression because of down-regulation of the AR.

CWR22Rv1 cells: AR functions as proliferation stimulator and suppressor

The CWR22Rv1 prostate cancer cell line, derived from a recurrent tumor (Nagabhushan *et al.*, 1996) after ADT of a CWR22 xenograft originally established from a human prostate primary tumor (Wainstein *et al.*, 1994), is an AR and CK8/18-expressing tumor cell line (van

Bokhoven *et al*., 2003). CWR22rv1-AR$^{+/-}$ cells were generated after knocking down AR in CWR22rv1 cells by genomic recombination. CWR22rv1-AR$^{+/-}$ cells expressed much less AR with negligible AR transactivation and displayed suppressed growth rate compared with the parental CWR22rv1-AR$^{+/+}$ cells (Chang *et al*., paper in preparation). In contrast, CWR22rv1-AR$^{+/-}$ cells produced bigger primary and PLN metastatic tumors than CWR22rv1-AR$^{+/+}$ cells on orthotopic transplantation (Niu *et al*., paper in preparation), suggesting that the AR functions observed in cell lines *in vitro* might not accurately represent the AR functions *in vivo*.

CWR22rv1-AR$^{+/-}$ cells were more invasive than the parental cells *in vitro*. Using an AR-specific siRNA to knockdown the AR in CWR22rv1-AR$^{+/+}$ cells also rendered the resultant CWR22rv1-AR$^{+/+}$-ARsi cells more invasive than the parental cells transfected with scrambled RNA (Niu *et al*., 2008a), whereas expression of a functional human AR in CWR22rv1-AR$^{+/-}$ cells through a retroviral vector rendered the CWR22rv1-AR$^{+/-}$-human AR cells less invasive than the parental cells transfected with the empty vector (Niu *et al*., 2008a). Together, these observations support the notion that the AR in prostatic cancer epithelial cells functions as a suppressor of prostate cancer metastasis.

PC346C cells: AR functions as proliferation stimulator and suppressor

PC346C, established from a human primary tumor through xenograft (van Weerden *et al*., 1996; van Weerden and Romijn, 2000), is an androgen-dependent prostate cancer cell line expressing wild-type AR and CK8/18 (van Bokhoven *et al*., 2003). After long-term androgen ablation, another androgen-independent subline, PC346DCC, with a 95% decrease in AR expression, was generated from PC346C cells. On orthotopic transplantation, PC346DCC tumors grew more rapidly in intact than in castrated hosts, suggesting stromal influence in addition to androgen-independent growth (Marques *et al*., 2005). Treatment of PC346C cells with the anti-androgen, hydroxyflutamide, results in two new sublines: one (PC346Flu1) with overexpression of the AR and its proliferation suppressed by androgen *in vitro* was more tumorigenic in castrated than in intact nude mice, whereas the other (PC346Flu2) with the AR(T877A) mutation behaved similarly to LNCaP cells (Marques *et al*., 2005). These contrasting results again showed that androgen/AR signals could be either a proliferation stimulator or suppressor in similar human prostate cancer cells.

Disadvantage of using a single human prostate cancer cell line to study prostate cancer progression and metastasis

From the results described above, it should be noted that data obtained solely from *in vitro* studies of human prostate cancer cell lines might not reliably predict the *in vivo* AR functions in prostate cancer progression and metastasis. For example, PC-3 and PC345DCC cells that either lack AR or with minimal AR have been regarded as androgen-independent cells. It is generally believed that a prostate cancer with this type of cells would not respond to ADT. The results presented in sections 'PC-3 cells: AR functions as suppressor of proliferation and metastasis' and 'PC346C cells: AR functions as proliferation stimulator and suppressor', however, indicate these cells are still capable of responding to stromal AR signals for growth and/or metastasis. Furthermore, PC-3 cells with stable transfection of AR cDNA under different promoters may yield different results on androgen treatment (section 'PC-3 cells: AR functions as suppressor of proliferation and metastasis'). CWR22rv1-AR$^{+/-}$ cells also had lower *in vitro* growth rate, yet produced bigger primary and PLN metastatic tumors *in vivo* than its parental AR-positive CWR22rv1-AR$^{+/+}$ cells (section 'CWR22Rv1 cells: AR functions as proliferation stimulator and suppressor').

Finally, several current available human prostate cancer cell lines were generated from long-term culture in the absence of androgen, which may not represent the *in vivo* human prostate condition based on the reports by Titus *et al*. (2005) showing that even after ADT treatment, the human prostate tissues still have about 1–3 nM 5α-dihydrotestosterone, which is approximately 10% of the normal level and is still sufficient to elicit AR transactivation.

Together, these contrasting examples clearly point out the importance of conducting *in vivo* animal studies of prostate tumors to delineate the pathophysiologic functions of AR in prostate cancer progression and metastasis.

AR dual functions in relation to the altered hormone sensitivity in prostate cancer progression during ADT

It has been proposed that cancer arises from neoplastic transformation of stem cells (Reya *et al*., 2001). Thus, prostate cancer can be considered to derive from neoplastic transformation of prostate stem cells to form prostate cancer stem cells that generate progenitor cells, which then progress sequentially into CK5-positive-basal cells, CK5/CK8-positive-basal intermediate cells, and then CK8-positive luminal cells (Figure 3) (Litvinov *et al*., 2003). Recent studies have indicated that prostate cancer progression from prostate cancer stem cells might involve different stem/progenitor cells with different levels of AR expression at different stages with varying proliferation and differentiation potentials (Figure 3). The distribution of these cells in prostate cancer might vary with patients and tumor stages and thus have different sensitivities toward AR-differential signals and may respond to ADT differentially (see Supplementary Information for more details).

Other possible explanations involving differential AR functions in various prostatic cells to influence hormone sensitivity during ADT include (a) AR somatic mutations, (b) altered interactions between AR and AR coregulators, (c) neuroendocrine differentiation of prostate cancer cells, (d) epithelial-mesenchymal transi-

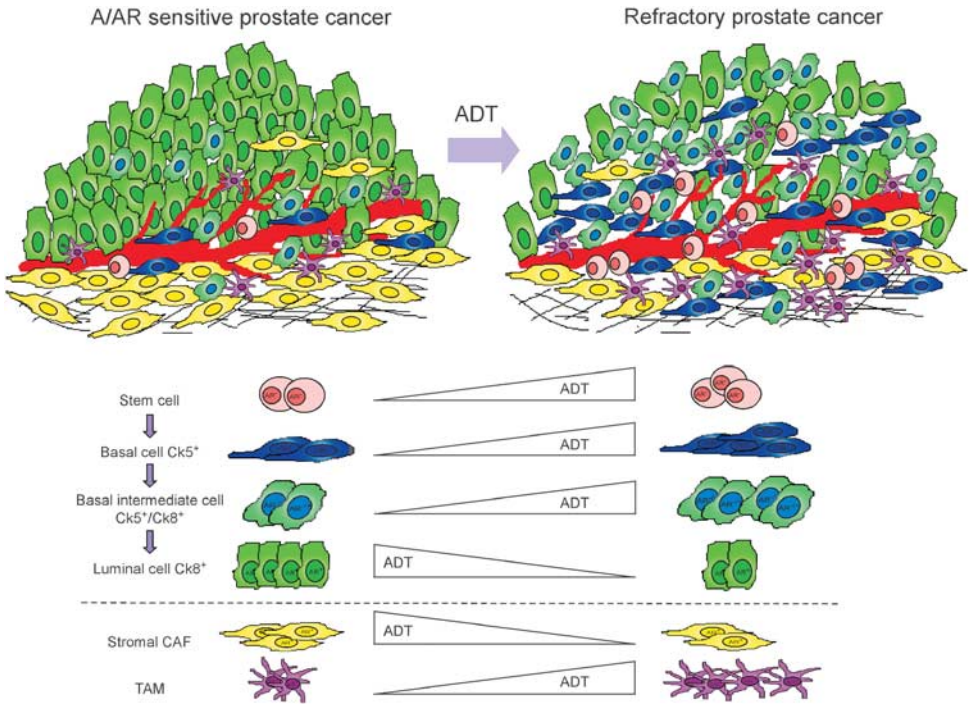

Figure 3 Differential AR signals in different prostate cancer cells on ADT treatment. The prostatic epithelial tumor may be developed sequentially from prostate (cancer) stem/progenitor cells (AR-negative) to transit amplifying cells with basal cell character (CK5-positive/CK8–negative and AR-negative), to intermediate cells with both basal and luminal cell character (CK5-positive/CK8-positive with either AR-positive or AR-negative), and then to luminal cells (CK5-negative/CK8-positive and AR-positive). Targeting epithelial AR through either knockout of epithelial AR or suppression of androgens binding to whole body AR signals as current ADT does, may result in decreased luminal cells and increased basal intermediate cells, which may be due to increased basal transit amplifying cells derived from prostate cancer stem cells and/or transition from luminal cells. ADT may also lead to increasing tumor-associated macrophage (TAM) (Mercader *et al*., 2001) and decreased stromal cells, including the cancer-associated fibroblasts (CAF).

tion (EMT) of prostate cancer cells, (e) development of ligand-independent activation of AR by growth factors or protein kinases, and (f) changes in AR expression between primary and metastatic prostate tumors. All these phenotypic changes undoubtedly would alter androgen/AR signals and affect the outcome of ADT (see Supplementary Information for more detailed descriptions of these changes).

The impact of AR dual functions on current clinical ADT

Challenge to current ADT

The fact that the stromal and epithelial ARs have opposite functions to modulate prostate cancer cell proliferation and metastasis undoubtedly is a main challenge for current ADT to treat prostate cancer patients. As ADT suppresses androgens binding to whole body AR, including both the stromal and epithelial AR, its treatment effect would depend on which cell's AR has a more significant function in a given stage of prostate cancer progression. At early stages of prostate cancer when tumors are dependent predominantly on stromal AR-modulated signals for growth and metastasis, ADT would result in regression of the tumor because of increased apoptosis of the tumor epithelial luminal and stromal cells, with much less effect on cancer stem/progenitor cells and some of their transit amplifying progenitors-basal cells (Figure 3). Subsequently, prostate cancer stem/progenitor cells and their transit amplifying progenitors/basal cells will adapt to the new AR signals during ADT and develop, either with AR somatic mutations, or altered AR to AR coregulators ratio, or increasing neuroendocrine differentiation, and/or EMT into more aggressive tumors that have different responses to AR signals.

The transition of prostate cancer into a more aggressive phenotype under low androgen conditions has been reported during tumorigenesis in Nkx3.1-Pten mutant mice (Banach-Petrosky *et al*., 2007). Continuing suppression of androgen at castration level through ADT would accelerate tumor metastasis because of ablation of the metastasis suppressor function of AR in the basal intermediate cells, thereby resulting in failure of ADT. Consistent with this view is the observation of Kleeberger *et al*. (2007) that the expression of nestin, a tumor metastasis marker, in prostate cancer cells is associated with ADT, and is mainly detected in refractory tumors while almost undetectable in tumors from patients without ADT.

Target the stromal AR at an early stage

It is apparent that ADT will have some beneficial effects on early prostate cancer, but will eventually fail in view of the dual functions of AR signals in prostate cancer

progression (Figure 3). Therefore, it may be better to target the AR at an early stage of prostate cancer when the progression and metastasis of the tumor is predominantly dependent on the stromal AR function. This approach is best exemplified by the timing of inducing ARKO in ind-ARKO-TRAMP mice described in section 'Relative influences of the stromal and epithelial AR on prostate cancer progression and metastasis'
(Niu *et al*., 2008b). Thus, because of the dual functions of AR, treatment of prostate cancer patients with ADT may have good response during the early stage of cancer, but worsen the prognosis during the later stages of cancer progression when they are less dependent on the stromal AR-modulated signals for growth, a scenario apparently observed in castrated TRAMP mice (Johnson *et al*., 2005) as well as in ind-ARKO-TRAMP mice after induction of ARKO at an early or late stage (Niu *et al*., 2008b). This also explains why some patients can benefit from intermittent treatment with cycles of androgen deprivation and supplementation (Akakura *et al*., 1993; Crook *et al*., 1999; Hurtado-Coll *et al*., 2002). For example, combined androgen blockade treatment of advanced prostate cancers provided at best a 5% increase in 5-year survival rate over ADT alone (Schmitt *et al*., 2001), but does not cure the disease. On the other hand, therapies aiming at maximal eradication of cancer cells including the cancer stem cells and their progenitors, such as radical prostatectomy and radiation therapy in conjunction with ADT, should provide a better clinical outcome than ADT alone, particularly in localized prostate cancer.

Strategies targeting the tumor stroma (Bouzin and Feron, 2007; Hofmeister *et al*., 2008) also should be beneficial to prostate cancer patients, as exemplified by the better treatment efficacy of combining therapy with ADT and anti-angiogenic agents targeting the stromal-derived vascular factors (Johansson *et al*., 2007) in the rat Dunning prostate cancer model. Similarly, combining ADT with Trk tyrosine kinase inhibitors targeting the receptor for stroma-derived AR-independent nerve growth factor was reported to prolong tumor regression in the rat prostate cancer model (George *et al*., 1999). However, if the disease recurs after these treatments, the adjuvant ADT may still fail because the recurrent tumor may become less dependent on the stromal AR for growth and more invasive because of abrogation of the metastasis suppressor function of the epithelial AR.

Concomitant treatment of AR antagonism with anti-metastasis agents

As ADT may promote the development of metastatic prostate tumors, it would be better to combine ADT with anti-metastatic agents or agents capable of suppressing EMT to improve the treatment effect. In metastatic tumors of pes-ARKO-TRAMP and PC-3-v cells, the expression of various tumor metastasis-related genes were significantly changed in favor of invasion and metastasis as compared with the tumors of TRAMP mice and PC-3-AR9 cells, respectively. Thus, several pro-metastasis genes such as cyclooxgenase-2, matrix metaloproteinase-9, interleukin-6, and tumor necrosis factor-α were elevated, whereas anti-metastasis genes such as neutral endopeptidase and the cell cycle inhibitor P27 (Kip1) were decreased in the tumors of pes-ARKO-TRAMP mice and PC-3-v orthotopic grafts compared with the tumors of their AR-expressing counterparts (Niu *et al*., 2008a). Part of these effects of AR seemed to be mediated through the suppression of TGFβ1, as expression of AR in prostate cancer cells resulted in suppression of TGFβ1 expression and over-expression of TGFβ1 resulted in increased invasiveness and similarly altered expressions of the metastasis-related genes mentioned above (Niu *et al*., 2008a). Moreover, TGFβ1 is known as an inducer of cancer cells to undergo EMT that is linked to the process of metastasis.

As patients with recurrent prostate cancer usually die of metastatic disease, their survival can be extended if tumor metastasis can be delayed or prevented through additional treatment regimens. Treatments targeting metastasis-related activities (Baritaki *et al*., 2009) or EMT-related genes downstream to the epithelial AR signaling or targeting neuroendocrine factors should improve the clinical results of adjunct ADT. For example, treatments targeting TGFβ1 and/or its receptor have been under clinical trial (Pinkas and Teicher, 2006). In addition, current available agents targeting Akt, COX-2, MMP-9, Vitamin E derivatives, or other relevant anti-metastasis agents in conjunction with ADT as earlier proposed (Miyamoto *et al*., 2005) may have clinical benefits for patients to battle metastatic prostate cancer, and may represent new treatment strategies to combat this deadly disease.

Future direction: Targeting AR in selective prostate cells

It is apparent that successful treatment of prostate cancer will rely on complete eradication of prostate cancer cells. This treatment objective can be achieved by prostatectomy when the disease is localized. Unfortunately, detection of prostate cancer at such an early stage is less frequent than more advanced diseases. As prostate cancer progresses with continuous interactions among various AR-positive stromal, epithelial, and infiltrating cells within the tumor microenvironment (Figure 3) targeting only those cells (such as stromal and/or luminal cells) with AR functions as positive roles to promote prostate cancer progression may be worthy of development in the future to battle prostate cancer. Recent studies (Yang *et al*., 2007; Chang *et al*., paper in preparation) showing ASC-J9 could degrade AR more aggressively in stromal and luminal cells that resulted in suppression of prostate refractory tumors with little side effects or toxicity may shed new light on our hope to treat prostate cancer. Alternatively, developing new nanoparticles carrying anti-AR compounds that only recognize and kill those

prostate cells with AR-positive roles will be another new hope in the future.

Conflict of interest

ASC-J9 was patented by the University of Rochester, the University of North Carolina, and AndroScience Corp., and then licensed to AndroScience Corp. Both the University of Rochester and C Chang own royalties and equity in AndroScience Corp.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Supplementary Information

S1. Dual roles of the AR in normal prostate development

S1a. AR in stromal cells: functions as a proliferation stimulator and survival factor

Previous tissue recombination studies by Cunha *et al* (2004) have indicated that prenatal development of the prostate is dependent on androgen/AR signals from the urogenital mesenchyme (UGM)/stroma to induce prostatic organogenesis and subsequent ductal morphogenesis and to maintain epithelial cell survival, whereas the epithelial AR is required for epithelial terminal differentiation. Prostatic epithelium can also induce UGM to differentiate into smooth muscle. These reciprocal stromal-epithelial interactions remain operative in adult prostate to maintain prostate cellular homeostasis (Cunha *et al*., 2004).

The prostatic stroma is constituted of fibroblasts, smooth muscle cells, endothelial cells, and immune cells (Cunha *et al*., 2004). A recent study of fibroblast-specific ARKO (fsp-ARKO, Yu *et al*, manuscript submitted) mice indicated that reducing AR signals in stromal fibroblasts resulted in under-development of the prostate gland with a decrease in proliferation and substantial increase in apoptosis in the epithelium, which was associated with decreased expression of several stromal paracrine growth factors in fps-ARKO compared to wild type mice. The levels of epithelial differentiation markers were markedly reduced in the prostate of fps-ARKO mice, suggesting loss of luminal

epithelial cells. Smooth muscle cell-specific ARKO (sm-ARKO) male mice have also been generated and found to display decreased epithelial infolding with decreased epithelial cell proliferation and IGF-1 expression in the prostate (Chang *et al.*, unpublished observation), and IGF-1 has been suggested as an important proliferation stimulator and survival factor of prostatic epithelial cells (Ohlson *et al*., 2006). Furthermore, after combining knockout of the stromal AR in a fps- and sm-double ARKO mice, Lai *et al*. (unpublished observation) found increased epithelial apoptosis and decreased proliferation of CK-5^+ basal intermediate cells as well as $CK8^+$ epithelial cells. These observations thus strongly support the consensus view derived from previous tissue recombination studies (Cunha *et al*., 2004) that prostatic stromal AR is an important positive regulator of epithelial cell proliferation and survival in prostatic development and tissue homeostasis.

S1b. AR in epithelial cells: a survival factor for luminal cells and a suppressor for basal intermediate cell proliferation

There are three major types of prostate epithelial cells, luminal cells ($CK5^-$/$CK8^+$), basal intermediate cells ($CK5^+$/$CK8^+$), and basal cells ($CK5^+$/$CK8^-$). The role of epithelial AR in adult prostate could not be delineated through the tissue recombination study using embryonic tissues with a short period of observation (4-6 weeks). By crossing floxed AR mice (Yeh *et al*., 2002) with probasin-Cre mice (Wu *et al*., 2001), Wu *et al* (2007) have generated prostate epithelial specific (pes)-ARKO mice. Consistent with increasing probasin-Cre expression,

the epithelial AR levels gradually decreased in the prostate of pes-ARKO mice beginning at 6-weeks old, and were below detection at 24 weeks of age. The ventral prostates of pes-ARKO mice in comparison with those of wild type mice were enlarged at 24-weeks or older and displayed a progressive decrease in epithelial height, loss of glandular infolding, and an increase in epithelial luminal cell apoptosis through 6-32 weeks of age. As the pes-ARKO mice matured, CK5/CK8-double positive (Wu *et al*., 2007) epithelial basal intermediate cell populations increased during puberty and then remained elevated, while the CK8/ CK18-positive epithelial luminal cell population declined. In contrast, in wild type animals, the basal cell number declined with age, whereas the luminal CK8/18-positive cells population remained stable. These observations strongly suggest that the epithelial AR is an important survival factor for epithelial luminal cells. Thus, the survival of epithelial luminal cells appears to require AR signals being maintained in both stromal and epithelial cells, since lacking either one resulted in apoptosis.

Further examination of pes-ARKO mice at 24-weeks-old indicated that the prostate glands had one layer of undifferentiated epithelial cells with increased proliferation of CK5-positive basal cells. In addition, there was an expansion of CK5/CK8 double positive cells characteristic for basal intermediate cells increasing from <10% to >50% of total epithelial cells in wild type and pes-ARKO mice, respectively.

Knocking-in a functional AR in pes-ARKO mice via transgenic strategy to generate (T857A)-pes-ARKO double transgenic mice showed the normal

prostatic morphology and glandular histology (Wu *et al*., 2007), which further strengthens the above conclusion that epithelial AR is a survivor factor for epithelial cells.

Other studies have reported that stable transfection of AR into AR-negative prostatic epithelial cells also resulted in the suppression of cell growth (Ling *et al*., 2001; Whitacre *et al*., 2002). These observations not only support the view that the AR in prostatic epithelial cells is required for epithelial cell differentiation (Cunha *et al*., 2004), but also suggest that the epithelial AR functions as a suppressor for epithelial basal intermediate cell proliferation and a survival regulator for differentiated epithelial luminal cells.

These two opposite roles of the epithelial AR appear to contribute significantly to cellular homeostasis in the prostate, although the underlying mechanism remains to be elucidated.

S2. Adaptive phenotype changes to altered hormone sensitivity in prostate cancer progression during ADT

S2a. AR dual roles in prostate cancer stem cell progression

The observation that the prostate in adults can undergo involution-regeneration upon castration-androgen supplementation for many cycles (English *et al*., 1987) have led Issacs and Coffey (1989) to propose that the prostate gland is regenerated from a population of prostate stem cells (PSC) in the remnant basal compartment that give rise to proliferating progenitors of

transit amplifying intermediate cells. These cells may then proliferate and differentiate into basal and luminal cells. It has been proposed that cancer arises from neoplastic transformation of stem cells (Reva *et al*., 2001). Thus, prostate cancer can be considered to derive from neoplastic transformation of PSC to form prostate cancer stem cells (PCSC) that generate progenitor cells, which in turn progress into epithelial tumor (Litvinov *et al*., 2003).

Recent studies have made considerable progress toward identifying PSC (Collins and Maitland, 2006; Lawson and Witte, 2007) and PCSC (Lawson and Witte, 2007). The putative human PSC are a subpopulation (~1%) of basal cells, which can be enriched by selecting integrin $\alpha_2\beta_1^{hi}$ cells (Collins *et al*., 2001). The $\alpha_2\beta_1^{hi}$ cells could be further separated into the $\alpha_2\beta_1^{hi}$/CD133$^+$ putative stem cells and $\alpha_2\beta_1^{hi}$/CD133$^-$ transient amplifying intermediate cell populations (Richardson *et al*., 2004), with the latter expressing the AR protein at a low level detectable only in the presence of a proteasome inhibitor or after stimulation with an androgen (Heer *et al*., 2007). The $\alpha_2\beta_1^{hi}$/CD133$^+$ putative stem cells have high proliferation potential *in vitro* and are capable of forming acini-like structures with expression of differentiation markers, including AR, prostate acid phosphatase (PAP), and CK-18 upon subcutaneous inoculation with prostate stromal cells into immunodeficient nude mice (Richardson *et al*., 2004). This observation is consistent with the current hypothesis of prostatic development, in which PSC proliferate in response to stromal AR signaling, progress sequentially through progenitor cells to CK5-positive basal cells and CK5/CK8-positive intermediate cells, with progressive increase in AR expression and decrease in proliferation,

until they are terminally differentiated into luminal epithelial cells, a process also consistent with the dual roles of the AR (as a suppressor for basal cells proliferation and a survivor factor to prevent luminal cell apoptosis) observed in the pes-ARKO mice (see **Section S1** for detail).

The $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ cells isolated from human prostate tumors express various basal cell markers, have high proliferative potential, and capability of androgen-dependent differentiation to express AR, CK18, and PAP *in vitro* (Collins *et al*., 2005). The $CD44^+/\alpha_2\beta_1^{hi}/CD133^-$ cells isolated from the same tumors possessed less proliferation potential and were regarded as tumor transient amplifying cells. Although purified $CD44^+$ cancer cells are AR-negative, they were able to give rise to $CD44^-$ and AR^+ cells, suggesting the capability of differentiation at least to some extent. Interestingly, $CD44^+/\alpha_2\beta_1^{hi}/CD133^-$ tumor cells are highly tumorigenic and are present in a high proportion in PC-3 cells, while nearly absent in LNCaP cells (Patrawala *et al*., 2006). Similarly $CD44^+/CD24^-$ prostate cancer cells, that express several “stemness” genes and are considered as stem/early progenitor cells, are highly tumorigenic (Hurt *et al*., 2008). Recently we have isolated $CD44^+/CD133^+/CK5^+$ PCSC/early progenitor cells from DU145 and PC-3 prostate cancer cells and observed that transfection of a functional AR into these AR-negative cells resulted in decreases in cell renewal and promotion of their differentiation into $CK8^+$ luminal-like cancer cells (Chang *et al*., unpublished observation). This observation indicates that expression of AR in these cells will suppress their renewal and elicit them to differentiate into AR-positive cancer cells.

Together, these results suggest that prostate cancer progression from PCSC involve different stem/progenitor cells with varying AR expression and differential abilities to proliferate and differentiate. In addition, the distribution of tumor stem/progenitor cells in prostate cancer might vary with patient and tumor stage and thus will have a differential response to ADT.

S2b. AR somatic mutations

More than 70 AR somatic mutations have been identified in prostate tumors (Gottlieb *et al*., 2004). Most of these mutations involve single base changes resulting in substitution of single amino acid residue and occur more frequently in androgen-independent metastases than primary tumors (Taplin *et al*., 1995; Tilley *et al*., 1996; Marcelli *et al*., 2000; Buchanan *et al*., 2001; Linja and Visacorpi, 2004; Chen *et al*., 2005). Many of these AR mutants displayed gain-of-function with increased sensitivity toward androgens, DHEA (Shi *et al*., 2002), E2, progesterone, corticosteroids, and/or anti-androgens (Fenton *et al*., 1997; Buchanan *et al*., 2001; Shi *et al*., 2002; Chen *et al*., 2005). There are also AR mutants with decreased transactivation (Shi *et al*., 2002).

Some AR mutants also result in nonsense codons or alternative splicing leading to the expression of truncated AR proteins lacking the C-terminal ligand binding domain with significantly altered transactivation (Lapouge *et al*, 2007; Guo *et al*., 2009). The nonsense AR mutants were detected at high frequency in metastatic prostate tumors and could be coexisted with other AR mutants in the tumor, particularly the promiscuous T877A mutation (Alvarado *et al*., 2005). In

addition, an alternatively spliced AR of 80 kDa (designated as AR3) was shown to be constitutively active independent of androgen and capable of stimulating growth of androgen-independent prostate cancer cells both *in vitro* and *in vivo*. AR3 level is increased upon ADT and in malignant cells compared to benign prostate tissues. AR3 nuclear localization is increased in hormone-resistant tumors compared to hormone-naïve tumors, and the increase appears to correlate well with PSA recurrence after radical prostatectomy. Ablation of AR3 from prostate cancer cells suppressed cell proliferation without affecting apoptosis (Guo *et al*., 2009). Thus, AR3 may represent another form of the AR whose expression can be adaptive to prostate cancer progression.

Somatic mutations of the AR also occur spontaneously in tumors of TRAMP mice at high rates, which are increased after castration (Han *et al*., 2001), suggesting an adaptive response. In human prostate cancer, AR mutants are detected more frequently in hormone-refractory disease and after treatment with anti-androgens (Linja and Visacorpi, 2004), also suggestive of an adaptive change. It is interesting to note that expression of the murine AR mutant, AR(E231G), in mouse prostate resulted in development of prostate intraepithelial neoplasia, which progressed into invasive and metastatic diseases (Han *et al*., 2005), suggesting AR could also function as a proto-oncogene to promote tumor development.

S2c. Neuroendocrine differentiation

Human prostate cancer cells are capable of reversible neuroendocrine-like differentiation upon androgen deprivation (Shen *et al*., 1997; Burchardt *et al*., 1999), treatment with anti-androgen bicalutamide *in vitro* (Vias *et al*., 2007), or silencing AR expression (Wright *et. al*., 2003). Neuoendocrine differentiation also occurred in human prostate cancer xenografts upon castration of the host (Jongsma *et al*., 1999; Jongsma *et al*., 2002; Huss *et al*., 2004). Similarly, in rodent prostate cancer models, ADT led to formation of highly proliferative and poorly differentiated neuroendocrine-like tumors with varying extents of AR expression (Masumori *et al*., 2001; Kaplan-Lefko *et al*., 2003; Huss *et al*., 2007). These neuroendocrine-like tumors is more frequently found in castrated than intact TRAMP mice, and could be elicited to become more differentiated tumor upon testosterone supplementation (Johnson *et al*., 2005). Neuroendocrine cells are found in 50% to 100% of prostate cancers and metastasis, and their number is correlated with the stage, Gleason grade, cell proliferation, and microvessel density of the tumor as well as survival of the patients following recurrence of prostate cancer after ADT (Aprikian *et al*., 1994; Speights *et al*., 1997; Ather and Abbas, 2000; Grobholz *et al*., 2000; Bollito *et al*., 2001; Segawa *et al*., 2001). Jin *et al* (2004) reported that a mouse prostate neuroendocrine tumor allograft (NE-10) was able to support the continuous growth of LNCaP xenograft tumors with increasing expression of AR in the castrated host. Moreover, secretions of NE-10 cells were able to stimulate LNCaP cell proliferation at low an androgen concentration *in vitro*. These observations suggest that increase in

neuroendocrine differentiation after ADT could provide paracrine/autocrine growth factors to influence prostate cancer progression and metastasis.

S2d. AR roles in EMT during prostate cancer progression

A growing body of recent evidence links epithelial-mesenchymal transition (EMT)-like process to tumor progression and metastasis (Thiery, 2002; Mareel and Leroy, 2003). During EMT tumor epithelial cells that dissociated from tumor epithelium, may invade into the neighboring stroma as individual cells and acquire many mesenchymal cell characteristics including increased invasiveness and resistance to apoptosis (Shook and Keller, 2003; Condeelis and Pollard, 2006; Guarino *et al*., 2007; Guarino 2007; Hugo *et al*., 2007). Such EMT may also involve the modulation of TGFβ1, IGF-1, or Snail transcription factor in prostate cancer cells (Graham *et al*., 2008; Zhau *et al*., 2008; Odero-Marah *et al*., 2008; Zhang *et al*., 2009; Klarman *et al*., 2009; Zhu and Kyprianou, 2010) and *in vivo* (Xu *et al*., 2006; He *et al*., 2010). Zhu and Kyprianou (2010) further reported that a low concentration of DHT was able to elicit EMT phenotype and Matrigel invasion in PC-3 cells with little endogenous AR, and addition of AR in PC-3 cells led to suppression of DHT-induced EMT and Matrigel invasion. However, DHT failed to induce EMT or affect invasion in LNCaP cells expressing high levels of functional AR, and knocking-down AR in these cells may then induce EMT. These results suggest that the AR in luminal-like prostate cancer epithelial cells functions as a suppressor of EMT and invasion or metastasis.

We have examined the primary and metastatic tumors of wild type TRAMP and pes-ARKO-TRAMP mice and found that the tumors of pes-ARKO-TRAMP mice expressed higher levels of mesenchymal markers and less epithelial markers characteristic of EMT than tumors of wild type TRAMP mice (Niu *et al*., manuscript in preparation). Comparison of the tumor cells in primary cultures also indicated that pes-ARKO-TRAMP tumor cells had increased EMT phenotype with respect to cell morphology, detachment, motility, and invasion over those of TRAMP tumors. Similar comparative observations were made with orthotopic xenografts of CWR22rv1-$AR^{+/+}$ and CWR22rv1-$AR^{+/-}$ cells, with the results showing epithelial AR functions as a suppressor of prostate cancer EMT and metastasis.

Finally, It is interesting to note that overexpression of Snail in LNCaP cells not only induced EMT but also neuroendocrine differentiation (McKeithen *et al*., 2010), suggesting that these two processes might occur concurrently in prostate cancer and pes-ARKO-TRAMP mice.

S2e. Altered AR-AR coregulators interactions

Altered anti-androgen sensitivity and modulated AR transactivation during ADT can be seen via interaction with various coregulators (Heinlein and Chang, 2004; Rahman *et al*., 2004; Wang *et al*., 2005). Several AR somatic mutants have altered interaction with AR coactivatiors (Duff *et al*., 2005; Li *et al*., 2005) to affect AR transactivation and change of ligand sensitivity. In addition, among >80 AR coregulators, several are found to be up-regulated in advanced prostate

cancer (Wang *et al*., 2002; Nishimura *et al*., 2003; Hu *et al*., 2004; Culig and Bartsch, 2006; Kahl *et al*., 2006; Fujimoto *et al*., 2007; Yang *et al*., 2007a,b), a number of which are capable of increasing androgen sensitivity and ligand promiscuity of wild type AR and/or some AR mutants (Heinlein and Chang, 2004; Rahman *et al*., 2004) resulting in considerable gain-of-function so that other hormones, such as estrogen, may also be able to activate AR in the absence of testosterone/DHT. The activities of AR coregulators can be further modulated via interaction with their interacting proteins. For example, Pyk2 suppresses ARA55-enhanced AR transactivation (Wang *et al*., 2002), tansgelin/SM22α or hnRNP A1 suppresses ARA54-enhanced AR transactivation (Yang *et al*., 2007a,b), and PSA/KLK3 activates ARA70-induced AR transactivation (Niu *et al*., 2008c). Interruption the interaction between these AR coregulators and their interacting proteins, such as PSA may lead to suppression of AR-mediated cell growth in a selective manner that depend on the existence of both AR coregulators and their interacting proteins.

S2f. Ligand-independent AR activation via growth factors or tyrosine kinases

The levels of several growth factors, including EGF, IGF-1, and IL-6, and/or their receptors have been found elevated in human hormone-refractory prostate cancers (Di Lorenzo *et al*., 2002; Lorenzo *et al*., 2003; Kruecki *et al*., 2004; Bartlett *et al*., 2005; George *et al*., 2005). These growth factors are able to activate AR transactivation in the absence of androgen or enhance the

androgen-induced AR transactivation via altered interaction with AR coregulators (Ueda *et al*., 2002; Culig 2004; Gregory *et al*., 2004) that might be mediated by their receptor protein kinase cascades (Culig, 2004). For example, ectopic overexpression of the Her2/neu/erbB-2, an EGF receptor family protein tyrosine kinase, in the androgen-dependent prostate cancer cells was found to induce AR transactivation (Craft *et al*., 1999; Yeh *et al*., 1999) and promote androgen-independent growth. Several serine/threonine protein kinases including MAPK, Akt/PKB, protein kinase C, and cAMP-activated protein kinase A (PKA) have also been reported to activate androgen-independent AR transactivation through phosphorylation of AR or its coregulators (Lin *et al*., 2001; Ueda *et al*., 2002; Culig 2004; Gregory *et al*., 2004; Craft *et al*., 1999; Yeh *et al*., 1999). In addition, several non-receptor tyrosine kinases including Src, FAK, and Etk/BMX were proposed to mediate IL-6- and bombesin-induced AR transactivation (Lee *et al*., 2001; Lee *et al*., 2004). Interestingly, Guo *et al.* (2006) reported that various hormone-refractory prostate cancer cell xenografts and human prostate cancer specimens exhibited elevated AR tyrosine phosphorylation and activated Src (tyrosine-phosphorylated Src) over their hormone-sensitive counterparts. However, some of these *in vitro* cell line studies were carried out in the absence of androgen, a condition that does not exist in human prostate that still has 1-3 nM of DHT at the hormonal refractory stage (Titus *et al*., 2005) and still capable of activating the AR without involving growth factors or protein kinases. Therefore, further *in vivo* evidence may be needed before the final conclusion that AR can be activated in a ligand-independent manner.

S2g. AR expression in human primary vs metastatic prostate tumor

Another phenotypic change that might influence hormone sensitivity in prostate cancer is the change in AR expression levels during tumor progression. Niu *et al*. (2008a), have evaluated AR expression in primary prostate tumors (97 cases) and prostate metastases (28 cases) and observed a significant difference between AR expression in primary tumors (91.75%) and metastatic tumors (67.86%), ($P<0.01$). These clinical data are consistent with an early study (Li *et al*., 2004) showing that AR expression was significantly decreased in metastatic prostate cancers as compared to primary prostate cancers or adjacent normal prostates (mean 1.30 vs. 3.49, $p<0.01$). Such a decrease in AR expression would decrease its suppressor role and favor EMT and prostate cancer metastasis.

Together, the above described phenotypic changes may contribute to the hormone sensitivity in prostate cancer progression during ADT that may well be influenced by the dual roles of the AR.

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