

Promotion of Bladder Cancer Development and Progression by Androgen Receptor Signals

Hiroshi Miyamoto, Zhiming Yang, Yei-Tsung Chen, Hitoshi Ishiguro, Hiroji Uemura, Yoshinobu Kubota, Yoji Nagashima, Yu-Jia Chang, Yueh-Chiang Hu, Meng-Yin Tsai, Shuyuan Yeh, Edward M. Messing, Chawnshang Chang

- Background** Males have a higher incidence of bladder cancer than females, but the reason remains unknown. Unlike prostate cancer, human bladder cancer is not generally considered to be dependent on hormone activity. We investigated the possible involvement of androgens and the androgen receptor (AR) in bladder cancer.
- Methods** We used *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) to induce bladder cancer in wild-type male and female mice, with and without castration in males, and in AR knockout (ARKO) male and female mice, with and without dihydrotestosterone (DHT) supplementation in males. We also treated human bladder cancer cell lines, including TCC-SUP and UMUC3, and mouse xenograft models established from these same lines with androgen deprivation therapy (antiandrogen treatment or castration), AR–small-interfering RNA (AR-siRNA), or the anti-AR molecule ASC-J9, which causes selective degradation of the AR.
- Results** More than 92% of wild-type male and 42% of wild-type female mice treated with BBN eventually developed bladder cancer, whereas none of the male or female ARKO mice did. Treatment with BBN induced bladder cancer in 25% of ARKO mice supplemented with DHT and in 50% of castrated wild-type male mice. Androgen deprivation of AR-positive human bladder cancer cells by androgen depletion in vitro or castration in mice and/or by treatment with the antiandrogen flutamide in vitro or in vivo, as well as AR knockdown by AR-siRNA or by ASC-J9, suppressed cell proliferation in vitro and xenograft tumor growth in vivo.
- Conclusions** Our findings implicate the involvement of both androgens and the AR in bladder cancer. Targeting AR and androgens may provide novel chemopreventive and therapeutic approaches for bladder cancer.

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Men have a substantially higher risk of bladder cancer than women (1). Excessive exposure of men to cigarette smoke and industrial chemicals, both of which include amines, has been suggested to result in the development of bladder cancer (2). However, sex-related differences in the risk of bladder cancer have been shown to persist in the absence of exposure to known carcinogenic factors (2). In animal experimental models, males are more likely than females to develop bladder cancer induced by certain chemical carcinogens (e.g., aromatic amines, such as *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine [BBN]) (3). In contrast, a recent study (4) showed that certain other carcinogens, such as the arsenical metabolite dimethylarsinic acid, are more toxic to the female bladder than the male bladder in rats. This finding is consistent with epidemiologic evidence suggesting that women are more susceptible to arsenic-induced bladder cancer than men (5). Thus, the basis for the sex-specific difference in bladder cancer incidence is not understood.

A potential mediator of sex-specific differences is the androgen receptor (AR). The AR, a member of the nuclear receptor superfamily, is a ligand-dependent transcriptional factor that mediates

the biologic effects of androgens (6,7). Expression of the AR has been detected in normal bladder epithelium (8) and in bladder carcinomas from both male and female patients (9). However, little is known about AR function in the bladder or about androgen metabolism in the bladder urothelium. Early studies (10) showed that levels of cytochrome P450 CYP4B1, which is present at higher levels in male bladder than female bladder and activates

Affiliations of authors: Departments of Pathology and Urology, University of Rochester Medical Center, Rochester, NY (HM, ZY, YTC, YJC, YCH, SY, EMM, CC); Departments of Urology (HI, HU, YK) and Molecular Pathology (YN), Yokohama City University Graduate School of Medicine, Yokohama, Japan; Reproductive Medicine Institute, Chang Gung University Memorial Hospital, Kaohsiung, Taiwan (MYT).

Correspondence to: Chawnshang Chang, PhD, Departments of Pathology and Urology, University of Rochester Medical Center, 601 Elmwood Ave, Box 626, Rochester, NY 14642 (e-mail: chang@urmc.rochester.edu).

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amines to genotoxic substances, are reduced by castration but recover following androgen supplementation. This finding raised the possibility that androgens might affect the sex difference in incidence of BBN-induced bladder cancer in animals by modulating the P450 system. In addition, the 5 α -reductase inhibitor finasteride, which blocks the conversion of testosterone to dihydrotestosterone (DHT), only slightly suppressed the development of BBN-induced bladder cancer in rats, whereas castration had a much stronger effect (11). This difference suggests that DHT may not be more potent than testosterone in inducing bladder cancer. However, none of the previous studies in bladder cancer have, to our knowledge, targeted the AR directly, and bladder cancer in humans is not generally considered to be dependent on hormone activity. Moreover, androgen deprivation therapy, which is frequently used in prostate cancer treatment (12–15), or other approaches that target the AR have never been considered as a therapeutic option for human bladder cancer.

Based on these epidemiologic and experimental observations, we hypothesized that androgens and/or the AR may play a role in bladder cancer initiation. We first examined the ability of BBN to induce bladder cancer in mice that did and did not carry a functional AR gene. We then used AR-positive bladder cancer cell lines and their mouse xenograft models in conjunction with androgen deprivation therapy or modulation of AR activity by AR–small-interfering RNA (AR-siRNA) or the anti-AR molecule ASC-J9 [5-hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one] to investigate the potential role of AR signals in bladder cancer progression.

Methods

Chemicals and Cell Lines

We obtained DHT from Sigma (St. Louis, MO), hydroxyflutamide (HF) from Schering (Kenilworth, NJ), and 90-day release pellets (placebo, DHT 1.5 mg/pellet, and flutamide 0.5 mg/pellet) from Innovative Research of America (Sarasota, FL). We obtained ASC-J9 from AndroScience Corporation (San Diego, CA). We maintained human urothelial carcinoma [5637, J82, HT1197, HT1376, T24, TCC-SUP, and UMUC3 from the American Type Culture Collection (Manassas, VA) and 647V originally isolated by Elliott et al. (16)] and prostatic adenocarcinoma (LNCaP, CWR22R, and PC3) cell lines in appropriate medium (Life Technologies, Bethesda, MD; RPMI-1640 medium for 5637, LNCaP, and CWR22R; Dulbecco's modified Eagle's medium for J82, HT1197, HT1376, TCC-SUP, UMUC3, 647V, and PC3; McCoy's 5A medium for T24) supplemented with 10% fetal bovine serum (FBS).

Plasmids and Stable Cell Lines

We used reporter plasmids (mouse mammary tumor virus [MMTV]-luciferase [Luc] and [ARE]4-Luc [provided by Dr Michael L. Lu, Harvard Medical School]) and a retrovirus vector pMSCV/U6 (Clontech, Palo Alto, CA)-AR-siRNA as described in our previous studies (17,18). To establish stable cell lines expressing the AR-siRNA or scrambled control-siRNA, pMSCV/U6-AR-siRNA or pMSCV/U6-control-siRNA was transfected, using SuperFect reagent (Qiagen, Chatsworth, CA),

CONTEXT AND CAVEATS

Prior knowledge

The incidence of bladder cancer is higher in males than females. The reason for the difference is unknown, but it is possible that androgens or the androgen receptor are involved in the development of bladder cancer.

Study design

Cell line and mouse model (both xenograft and carcinogen-induced tumors) study.

Contribution

Proliferation of human bladder cancer cells was reduced both in vitro and in vivo by treatments that reduce androgen activity as well as by treatments that block androgen receptor function. The development of bladder cancer in mice treated with a known bladder carcinogen was reduced in male mice with lowered levels of the androgen receptor or of androgens.

Implications

If bladder cancer development or progression is indeed influenced by both androgens and the androgen receptor, then it is possible that bladder cancer could be prevented or treated by targeting these molecules or related signaling pathways.

Limitations

Only two cell lines were used. The relevance of the mouse models to bladder cancer in humans is not yet known.

into Phi-NX packaging retrovirus producer cells (developed by the Nolan Lab, Stanford University). The target cells (i.e., TCC-SUP, UMUC3, and 5637) were then cultured in the presence of the viral supernatant, and infected cells were selected with puromycin (Sigma).

N-butyl-*N*-(4-hydroxybutyl)nitrosamine-Induced Mouse Bladder Cancer Model

We created male and female AR knockout (ARKO) mice in the background of the mosaic founder strain (C57BL/6-129Sv) as described previously (19). Animal care was in accord with institutional guidelines. Androgen receptor knockout mice (male: $n = 48$; female: $n = 24$) and their wild-type littermates (male: $n = 48$; female: $n = 24$, all 5–6 weeks old) were supplied ad libitum with tap water containing 0.05% BBN (TCI America, Portland, OR) in opaque bottles for 12 weeks and thereafter with tap water without BBN. The drinking water was prepared fresh twice a week, and consumption was recorded to estimate BBN intake. Negative control mice (wild-type males; $n = 24$) did not receive BBN. Wild-type male mice received surgical castration ($n = 24$) or sham surgery ($n = 24$) at 5 weeks of age before starting BBN treatment. Slow-releasing pellets (DHT 1.5 mg per mouse) were injected into half of the ARKO male mice ($n = 24$) at 5 weeks of age and replaced every 90 days. All mice were killed by administration of pentobarbital followed by rapid cervical dislocation at 20 ($n = 4$ in each group), 30 ($n = 8$ in each group), or 40 ($n = 12$ in each group) weeks of age, and urinary tract specimens (i.e., kidney, ureter, and bladder) were harvested. These specimens were preserved in phosphate-buffered 10% formalin, embedded in paraffin, sectioned,

stained with hematoxylin–eosin, and examined microscopically to identify carcinoma or other pathological changes. We also used paraffin-embedded sections for immunohistochemical analysis. Part of each specimen was rapidly frozen in liquid nitrogen and stored at -80°C for subsequent RNA analysis.

Immunohistochemical Analysis of Cell Proliferation and Apoptosis

Sections (5- to 7- μm thick) from paraffin-embedded mouse urinary tract tissue and xenograft tumors (see below) were deparaffinized in xylene and rehydrated in a graded ethanol series. Sections were then incubated in 3% hydrogen peroxide to block endogenous peroxidase, with a protein-blocking solution containing preimmune rabbit serum, and finally with a 1:500 dilution of primary antibody (rabbit polyclonal anti-proliferating cell nuclear antigen [PCNA]; Biotechnology, Santa Cruz, CA). We then incubated the samples with a 1:200 dilution of peroxidase-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA). The slides were rinsed with phosphate-buffered saline (PBS), incubated with diaminobenzidine, and finally counterstained with hematoxylin. The terminal deoxynucleotidyltransferase-mediated UTP end-labeling (TUNEL) assay was performed on additional rehydrated sections from paraffin-embedded mouse urinary tract tissue and xenograft tumor using the Fluorescein-FragEL DNA Fragmentation Detection Kit (Calbiochem, La Jolla, CA), according to the manufacturer's instructions, followed by counterstaining for DNA with 4',6-diamidino-2-phenylindole (DAPI). The percentage of apoptotic cells was determined by fluorescence microscopy. Cell proliferation and apoptotic indices were determined according to the percentage of PCNA- and TUNEL-positive cells, respectively, in 1000 cells counted on each specimen by a single observer who was unaware of the treatment group for the tissue.

Reverse Transcription–Polymerase Chain Reaction

Total RNA from mouse bladder and xenograft tumor tissues and cultured cells was extracted by the acid guanidinium–phenol–chloroform method as described (20) or by using Trizol reagent (Life Technologies). Isolated RNAs were reverse transcribed to cDNA using random hexamers as described (20,21) and amplified by polymerase chain reaction (PCR) using a primer set specific for the AR gene (21). Real-time quantitative PCR analysis of AR, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and matrix metalloproteinase (MMP)-9 was also performed on the tissue samples from mouse xenograft tumor, using iCycler (Bio-Rad, Hercules, CA), as described (21). Polymerase chain reaction primers designed using Beacon Designer 2 (Premier Biosoft, Palo Alto, CA) amplified bFGF: 5'-AAGAGCGACCCTCACATCAAGCTA-3' and 5'-AAGAAACTCATCCGTAAC-3', VEGF: 5'-GGAACACCGACAAACCCA-3' and 5'-TCCCCAAAGCACAGCAAT-3', and MMP-9: 5'-GGGACGGCAATGCTGATGG-3' and 5'-TGGTGGCGCACCAGCGGTAGCCGTC-3'. β -actin was used as an internal control. Polymerase chain reaction conditions for all primer sets were initial denaturation at 95°C for 10 minutes and 30 (for semi-quantitative reverse transcription–polymerase chain reaction [RT–PCR]) or 45 (for real-time RT–PCR) cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds.

Western Blot of Androgen Receptor Expression

Western blotting analysis was performed in bladder cancer cell lines, using a polyclonal AR antibody, N20 (Santa Cruz), as described previously (22). An antibody for β -actin (Santa Cruz) was used as the internal control. Equal amounts of protein (75 μg) obtained from cell extracts were separated in 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) by electroblotting, using a standard protocol. Specific antibody binding was detected using an alkaline phosphatase detection system (AP-Detection kit, Bio-Rad).

Reporter Gene Assay

Cell transfections and luciferase assays were performed as previously described (17,22). Briefly, bladder cancer cells at a density of 50%–60% confluence in 12-well tissue culture plates were transfected with 1.5 μg of plasmid DNA using SuperFect transfection reagent (Qiagen) according to the manufacturer's instructions. After 2 or 3 hours, the medium was replaced with medium supplemented with charcoal-stripped FBS in the presence of ligands (testosterone, DHT, HF, and/or ASC-J9) for 24 hours. Cells were harvested, lysed, and assayed for luciferase activity. Luciferase activity in cell extracts was determined using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) and luminometer (TD-20/20, Turner BioSystems, Sunnyvale, CA).

Cell Proliferation Assay

We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (thiazolyl blue, Sigma) assay to assess cell growth as described previously (22). We seeded cells in 6-well tissue culture plates at a density of 2×10^4 – 5×10^4 cells per well in medium supplemented with charcoal-stripped FBS containing ligands (testosterone, DHT, HF, and/or ASC-J9). After 6 days of treatment (changing media every 2 days), we added 200 μL of MTT stock solution (5 mg/mL) to each well with 2 mL of medium for 3 hours at 37°C . We then added 1–2 mL of 0.04 N HCl in isopropanol, incubated for 5 minutes at room temperature, and measured the absorbance at a wavelength of 570 nm with background subtraction at 660 nm.

Mouse Xenograft Models

Bladder cancer cell lines (TCC-SUP, UMUC3, and 5637) were harvested, washed twice with PBS, and resuspended in Matrigel (BD Biosciences, San Jose, CA) at a final concentration of 1×10^7 cells/mL. Cells (1×10^6 cells in 100 μL per site) were then injected subcutaneously into the right and left flanks of 6-week-old male athymic nude mouse. Treatment (castration or sham surgery, flutamide [0.5 mg per mouse] or placebo pellet injection, and the recently developed anti-AR molecule ASC-J9 (23) [50 mg/Kg] or dimethyl sulfoxide [DMSO] injection) was initiated at the same time as tumor cell injection (for tumor incidence studies, $n = 8$ mice in each group) or when the sizes of all tumors reached 40 mm^3 (for tumor progression studies, $n = 12$ tumors from 6 mice in each group). Hormonal pellets were replaced as required (every 90 days). Tumors were measured twice a week using calipers and tumor weight was calculated by the following formula: tumor weight (mg) = tumor length (mm) \times [tumor width (mm)]² \times 0.5 (24), until tumor size reached 40 mm^3 (for tumor incidence study) as described

previously (13) or until tumors exceed 10% of the animal's body weight, at which time the mice were killed (for tumor progression study).

Statistical Analysis

Differences in tumor incidence among groups in the BBN-induced carcinogenesis study and in the tumorigenicity study in nude mice were analyzed by Fisher's exact test. Differences in mean values (i.e., in tumor size, level of gene expression, and amount of cell proliferation or apoptosis) among different groups were analyzed by two-way analysis of variance (ANOVA) with repeated measures (to correct for within-mouse correlation) or Student's *t* test. *P* values less than .05 were considered to be statistically significant. All statistical tests were two-sided.

Results

Androgens and Androgen Receptor in Bladder Carcinogenesis

In a previous study (19), we generated ARKO mice in which exon 2 of the AR gene, which encodes the second zinc finger of the AR DNA-binding domain, is disrupted by a Cre-lox P system. The urinary tract (i.e. kidney, renal pelvis, ureter, bladder, and urethra) in both male and female ARKO mice is morphologically similar to that of their wild-type littermates, although male ARKO mice have hypospadias with castration levels [approximately 95% reduction compared with wild-type male littermates (25)] of serum testosterone. For this analysis, we treated ARKO mice and their wild-type littermates with the strong genotoxic bladder carcinogen BBN at 0.05%. Previous studies showed that wild-type mice given drinking water containing BBN at 0.01%–0.05% for 10–20 weeks develop bladder carcinomas at a high frequency (30%–100%) by 40 weeks

(3,26). We also treated some wild-type male mice with surgical castration and some ARKO male mice with DHT injection to modify androgen levels.

We studied bladder cancer development in a total of 168 mice that were divided into seven groups (24 mice/group): 1) male wild-type mice treated with BBN, 2) male castrated wild-type mice treated with BBN, 3) male ARKO mice treated with BBN, 4) male ARKO mice treated with DHT and BBN, 5) female wild-type mice treated with BBN, 6) female ARKO mice treated with BBN, and 7) untreated male wild-type mice. There was no substantial difference in consumption of drinking water with BBN among groups 1 through 6, indicating that intake of BBN was similar. None of the untreated mice (i.e., those in group 7) developed bladder tumors by 40 weeks of age. The incidence of BBN-induced bladder tumors in the other six groups is shown in Table 1, and representative lesions are shown in Fig. 1, A. Invasive bladder carcinoma developed in all the BBN-treated wild-type male mice examined at 30 weeks of age, whereas only 42% of BBN-treated wild-type female mice examined at 40 weeks of age had developed bladder tumors, confirming the difference in incidence of bladder cancer between male and female wild-type mice treated with BBN (3). Preneoplastic lesions (hyperplasias or papillomas) also developed in 75% of wild-type male mice treated with BBN and examined at 40 weeks. In 50%–67% of bladders from the other wild-type mice (castrated males and females), preneoplastic bladder lesions were seen at 30–40 weeks. By contrast, BBN did not induce any bladder carcinomas in ARKO male or female mice, with only two cases (17%) of hyperplasia in the bladder in each group at 40 weeks. None of the mice in any group developed renal pelvic, ureteral, or metastatic tumors.

Together, these results not only confirm the sex differences in BBN-induced bladder cancer incidence but also indicate that the

Table 1. Incidence of BBN-induced bladder tumors in mice at various time points*

Age	20 wk			30 wk			40 wk		
	Group	Preneoplastic		No. of mice	Preneoplastic		No. of mice	Preneoplastic	
No. of mice		lesions, No. (%)	Carcinoma, No. (%)		lesions, No. (%)	Carcinoma, No. (%)		lesions, No. (%)	Carcinoma, No. (%)
WT male	5†	2 (40)	0 (0)	7	5 (71)	7 (100)	12	9 (75)	11 (92)
Castrated WT male	3	0 (0)	1 (33)	8	5 (63)	0 (0)‡	12	8 (67)	6 (50)§
ARKO male	4	0 (0)	0 (0)	8	0 (0)	0 (0)‡	12	2 (17)¶	0 (0)‡
ARKO male + DHT	4	0 (0)	0 (0)	8	3 (38)	1 (0)#	12	5 (42)	3 (25)**
WT female	4	0 (0)	0 (0)	8	4 (50)	0 (0)‡	12	8 (67)	5 (42)††
ARKO female	4	0 (0)	0 (0)	8	0 (0) ,‡‡	0 (0)‡	12	2 (17)¶,§§	0 (0)‡,

* BBN = *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; WT = wild-type; ARKO = androgen receptor knockout; DHT = dihydrotestosterone.

† Includes a mouse that died at 24 weeks without a bladder tumor.

‡ Statistically significantly different from WT male group by Fisher's exact probability test ($P < .001$).

§ Statistically significantly different from WT male group by Fisher's exact probability test ($P = .0343$).

|| Statistically significantly different from WT male group by Fisher's exact probability test ($P = .0070$).

¶ Statistically significantly different from WT male group by Fisher's exact probability test ($P = .0061$).

Statistically significantly different from WT male group by Fisher's exact probability test ($P = .0012$).

** Statistically significantly different from WT male group by Fisher's exact probability test ($P = .0014$).

†† Statistically significantly different from WT male group by Fisher's exact probability test ($P = .0136$).

‡‡ Statistically significantly different from WT female group by Fisher's exact probability test ($P = .0385$).

§§ Statistically significantly different from WT female group by Fisher's exact probability test ($P = .0180$).

|||| Statistically significantly different from WT female group by Fisher's exact probability test ($P = .0186$).

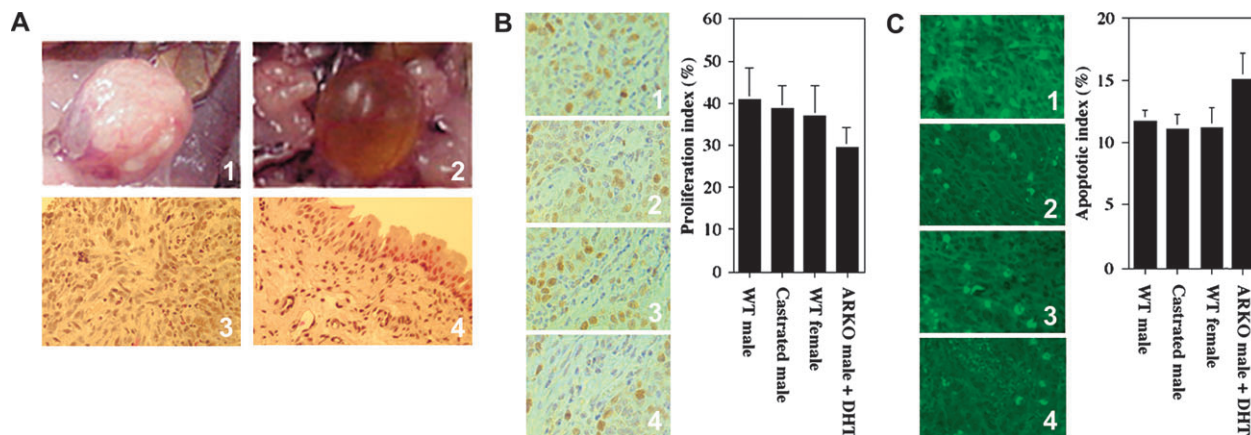


Fig. 1. Chemical induction of bladder tumors in wild-type and androgen receptor knockout (ARKO) mice. Six groups of mice—1) wild-type male, 2) castrated wild-type male, 3) ARKO male, 4) ARKO male with dihydrotestosterone (DHT) supplement, 5) wild-type female, and 6) ARKO female—were treated with 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in drinking water, starting at 5–6 weeks of age. **A**) Representative lesions from a wild-type male mouse with invasive carcinoma (**1,3**) and an ARKO male mouse without a bladder tumor (**2,4**) both at 40 weeks of age are shown in macroscopic (**1,2**) and histopathologic (**3,4**; magnification = $\times 100$) views. **B**) Immunohistochemical analysis (left panels) of proliferating cell nuclear antigen (PCNA) in representative bladder tumors harvested, at 40 weeks of age, from wild-type (WT) male mice (**1**; $n = 4$), castrated male mice (**2**; $n = 4$), wild-type female mice (**3**; $n = 4$), and ARKO male mice treated with DHT supplement by slow-

releasing pellet (1.5 mg per mouse per 90 days) injection (**4**; $n = 3$) (magnification = $\times 400$). Graph at right shows proliferation index, the percentage of PCNA-positive cells in 1000 cells in each tumor. Means and 95% confidence intervals from 15 tumors (one tumor from each mouse) are shown. **C**) Fluorescence images (left panels) generated by terminal deoxynucleotidyltransferase-mediated UTP end-labeling (TUNEL) analysis of the same bladder tumors as analyzed in (B) harvested from wild-type male mice (**1**; $n = 4$), castrated male mice (**2**; $n = 4$), wild-type female mice (**3**; $n = 4$), and ARKO male mice with DHT supplement (**4**; $n = 3$) (magnification = $\times 400$). Total cell population was visualized by staining with DAPI. Graph at right shows apoptotic index, the percentage of TUNEL-positive cells in 1000 cells in each tumor. Means and 95% confidence intervals from 15 tumors (one tumor from each mouse) are shown.

AR might play a role in bladder carcinogenesis in that statistically significant differences in bladder cancer incidence were observed between wild-type male mice and ARKO male mice at 30 (100% versus 0%, $P < .001$) and 40 (92% versus 0%, $P < .001$) weeks and between wild-type female mice and ARKO female mice at 40 weeks (42% versus 0%, $P = .0186$). It is noteworthy that female mice with or without AR have similar urinary tract characteristics and serum hormone levels [e.g., of testosterone, 17β -estradiol, progesterone, luteinizing hormone, and follicle-stimulating hormone (27)]. These results may suggest that the AR is essential for induction of bladder cancer. Unexpectedly, treatment of male ARKO mice with both BBN and DHT led to the development of carcinoma and preneoplastic lesions in 25% and 42% of the mice, respectively, at 40 weeks. Additionally, 50% of BBN-treated castrated male wild-type mice developed invasive carcinoma by 40 weeks. The difference in incidence between DHT-treated and untreated ARKO mice suggests that androgens may be involved in BBN-induced bladder cancer through mechanisms that are independent of the AR. These contrasting findings suggest that androgens (via the AR and non-AR pathways) and AR (via androgen-mediated and non-androgen-mediated signals) both contribute to bladder carcinogenesis.

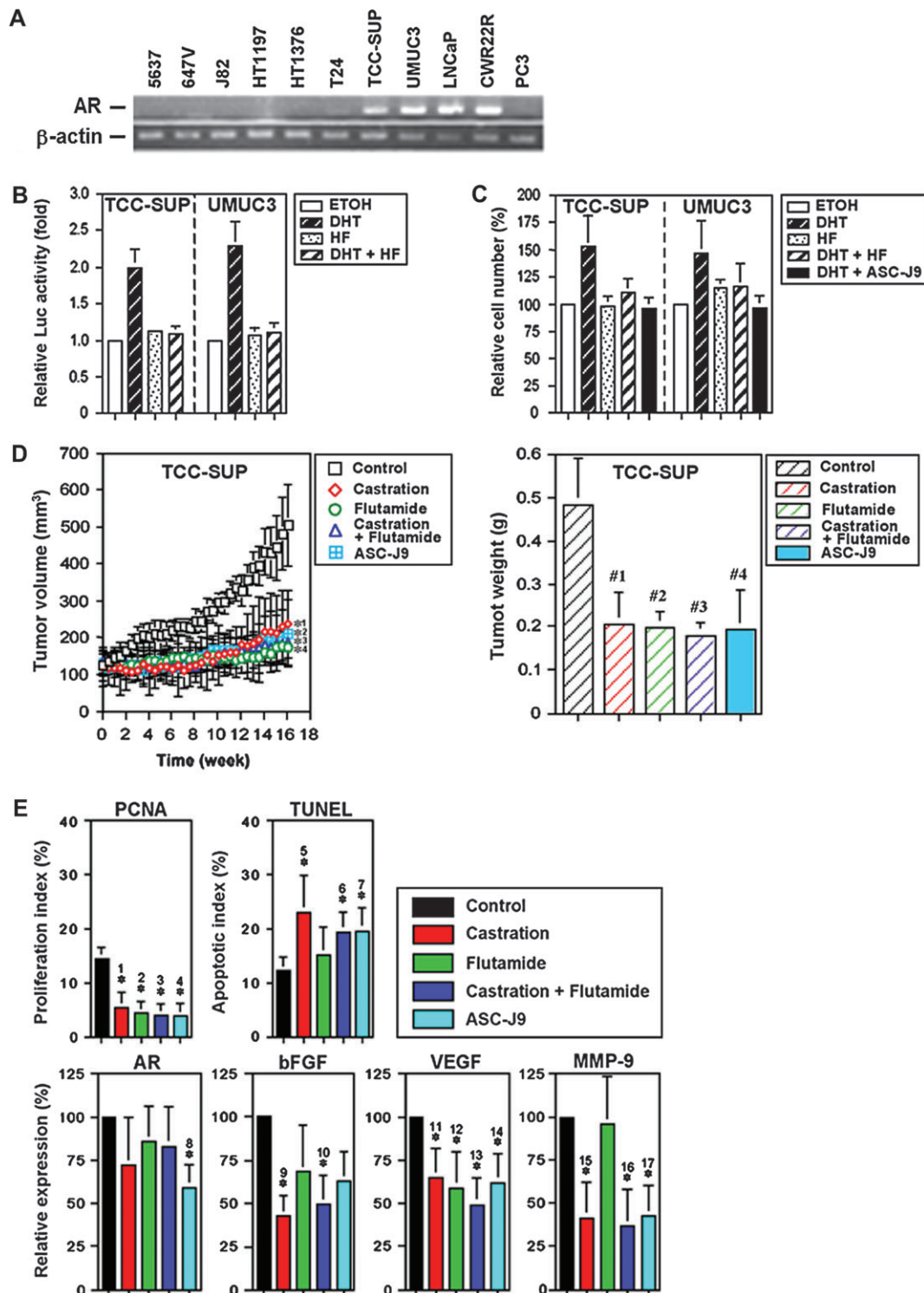
Androgens and Androgen Receptor in Bladder Cancer Progression

Having shown that androgens and the AR both appear to influence BBN-induced bladder carcinogenesis, we next investigated whether either or both also influence bladder cancer progression. To do so, we assessed cell proliferation, by immunostaining for PCNA, and apoptotic cell death, using the TUNEL method, in bladder tumors harvested from mice treated with BBN (groups 1–6). The prolifera-

tion and apoptotic indexes—defined as the percentages of PCNA- and TUNEL-positive cells, respectively, in 1000 cells in each tumor—were statistically significantly different between normal epithelia from 11 mice in groups 1 ($n = 1$), 2 ($n = 2$), 3 ($n = 2$), 4 ($n = 2$), 5 ($n = 2$), and 6 ($n = 2$) and invasive bladder carcinomas from 15 mice in groups 1 ($n = 4$), 2 ($n = 4$), 4 ($n = 3$), and 5 ($n = 4$) (proliferation index: mean = 37.4% versus mean = 1.1%, respectively; difference = 36.3%; 95% confidence interval [CI] = 28.2 to 44.3; $P < .001$ and apoptotic index: mean = 9.6% versus mean = 12.4%, respectively; difference = 2.8%; 95% CI = 1.3 to 4.3; $P < .001$). There were no differences in proliferation or apoptosis among BBN-induced carcinomas from wild-type male, castrated male, and wild-type female mice (Fig. 1, B and C). The similar levels of these parameters may reflect the similar expression levels of AR as detected by RT-PCR (data not shown) among these tumors, although androgen levels in the mice might vary. In contrast, BBN-induced bladder tumors in DHT-implanted male ARKO mice had a lower proliferation index (mean = 29.6% [ARKO/DHT] versus 37.4% [others]; difference = 7.8%; 95% CI = -4.7 to 20.3; $P = .1211$) and a higher apoptotic index (mean = 15.2% [ARKO/DHT] versus 12.4% [others]; difference = 2.8%; 95% CI = 0.4 to 5.2; $P = .062$) than those from the other three groups (Fig. 1, B and C). Cancer progression may correlate with higher proliferation rate and lower apoptotic rate, both of which were found in AR-expressing tumors compared with AR-negative tumors. Thus, these findings suggest that the AR contributes to bladder cancer cell proliferation and apoptosis in BBN-induced bladder cancer and support the hypothesis that AR activation also promotes bladder cancer progression.

To further test this hypothesis, we identified bladder cancer cell lines that may express a functional AR. A recent study (28) used

Fig. 2. Effects of androgen and antiandrogen on bladder cancer cell lines. **A)** Androgen receptor (AR) expression was analyzed in untreated bladder cancer cell lines (5637, 647V, J82, HT1197, HT1376, T24, TCC-SUP, and UMUC3) and prostate cancer cell lines (LNCaP, CWR22R, and PC3) by reverse transcription-polymerase chain reaction (RT-PCR), and PCR products were separated on an agarose gel. Polymerase chain reaction products derived from β -actin mRNA served as an internal control. **B)** Androgen receptor transactivation in bladder cancer cells. TCC-SUP or UMUC3 cells transfected with MMTV-Luc were cultured for 24 hours in the presence of ethanol (ETOH), 1 nM dihydrotestosterone (DHT) (androgen), and/or 5 μ M hydroxyflutamide (HF) (antiandrogen), as indicated, and luciferase activity was analyzed in a luminometer. Luciferase activity is presented relative to that of ETOH treatment in each cell line (set as 1-fold). Each value represents the mean and 95% confidence interval from at least three independent experiments. **C)** Bladder cancer cell proliferation. TCC-SUP or UMUC3 cells were cultured in the presence of ETOH, 1 nM DHT, 5 μ M HF, and/or 1 μ M ASC-J9 for 6 days, as indicated. Proliferation was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, and cell numbers are presented relative to cell number with ETOH treatment (set as 100%). Each value represents the mean and 95% confidence interval from at least three independent experiments. **D)** Growth of xenograft tumors in mice. TCC-SUP cells were implanted subcutaneously into the right and left flanks of male nude mice, and after 4 weeks, treatment (castration or sham surgery, injection of flutamide or placebo pellet, and/or intraperitoneal injection of ASC-J9 [50 mg/Kg dissolved in 10 μ L dimethyl sulfoxide {DMSO} + 90 μ L corn oil] or control [10 μ L DMSO + 90 μ L corn oil] every other day) began. Tumor volume ($n = 12$ tumors in each group, two tumors from each mouse) was monitored twice a week for 16 weeks (**left panel**). *1, castration group, $P = .0289$; *2, ASC-J9 group, $P = .0201$; *3, castration + flutamide group, $P = .0173$; *4, flutamide group, $P = .0163$ by repeated measures analysis of variance (ANOVA) test (versus control group). The mice were then killed, and the tumors were harvested and weighed (**right panel**). #1, $P = .0395$; #2, $P = .0250$; #3, $P = .0167$; #4, $P = .0445$ by repeated measures ANOVA test (versus control group). All the values represent the means and 95% confidence intervals. **E)** The same tumor specimens ($n = 6$ in each group, one tumor from each mouse) were analyzed for proliferation index (percentage of proliferating cell nuclear



antigen-positive cells in 1000 cells), apoptotic index (percentage of terminal deoxynucleotidyltransferase-mediated UTP end-labeling (TUNEL)-positive cells in 1000 cells), and relative expression levels of AR, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and matrix metalloproteinase (MMP)-9 as detected by real-time RT-PCR. For gene expression analyses, expression of each specific gene was normalized to that of β -actin. *1, $P = .0182$; *2, $P = .0020$; *3, $P = .0026$; *4, $P = .0042$; *5, $P = .0452$; *6, $P = .0365$; *7, $P = .0441$; *8, $P = .0125$; *9, $P = .0090$; *10, $P = .0218$; *11, $P = .0371$; *12, $P = .0414$; *13, $P = .0029$; *14, $P = .0213$; *15, $P = .0076$; *16, $P = .0058$; *17, $P = .0041$ by t test (versus control group). All the values represent the means and 95% confidence intervals of at least three determinations.

RT-PCR to show that two bladder cancer cell lines, 253J and T24, express the AR. We therefore assessed levels of AR mRNA expression in eight bladder cancer lines, including T24, using RT-PCR. This analysis showed AR expression in two of these lines, the human urothelial carcinoma lines TCC-SUP and UMUC3 (Fig. 2, A). Only a weak AR mRNA signal was detected in the T24 cell line after 30 cycles of PCR.

We next investigated the functional activity of AR in TCC-SUP and UMUC3 cells by transfecting both lines with the androgen response element reporter plasmid (MMTV-Luc). We treated transfected cells with DHT and/or the antiandrogen HF and assayed luciferase activity as a reporter of AR-mediated transcriptional activity. As shown in Fig. 2, B, DHT treatment of the TCC-SUP and UMUC3 cell lines increased luciferase activity by 2.0- and 2.3-fold (95% CI = 1.7 to 2.3 and 1.9 to 2.7), respectively, over mock treatment. Hydroxyflutamide alone had only marginal agonist activity by itself but clearly inhibited DHT induction of luciferase activity. In contrast, in T24 and other bladder cancer cells with undetectable AR mRNA, DHT exhibited only a marginal effect on AR transactivation (data not shown). Similar results

were obtained when MMTV-Luc was replaced with a luciferase reporter containing synthetic androgen response elements ([ARE]4-Luc, data not shown). These results suggest that TCC-SUP and UMUC3 cells possess a functional AR.

We then used the MTT assay to test the effects of androgen (DHT) and antiandrogen (HF) on cell growth of bladder cancer lines. As shown in Fig. 2, C, DHT treatment increased the growth of TCC-SUP cells (by 55%, 95% CI = 28% to 82%) and UMUC3 cells (by 45%, 95% CI = 18% to 73%) and HF antagonized, at least partially (67%–75% reduction), the DHT effect in both lines. In contrast, in all other AR-negative bladder cancer cell lines, DHT and HF had only marginal effects (<10% changes) on cell growth (data not shown, also see Fig. 3, B). These results, together with AR transactivation data, indicate that androgen treatment increases proliferation of AR-positive bladder cancer cells.

Therapeutic Effects on Bladder Cancer Progression of Targeting Androgens or the Androgen Receptor

Based on the finding that androgens transactivate the AR in bladder cancer cells and induce proliferation of AR-positive bladder cancer

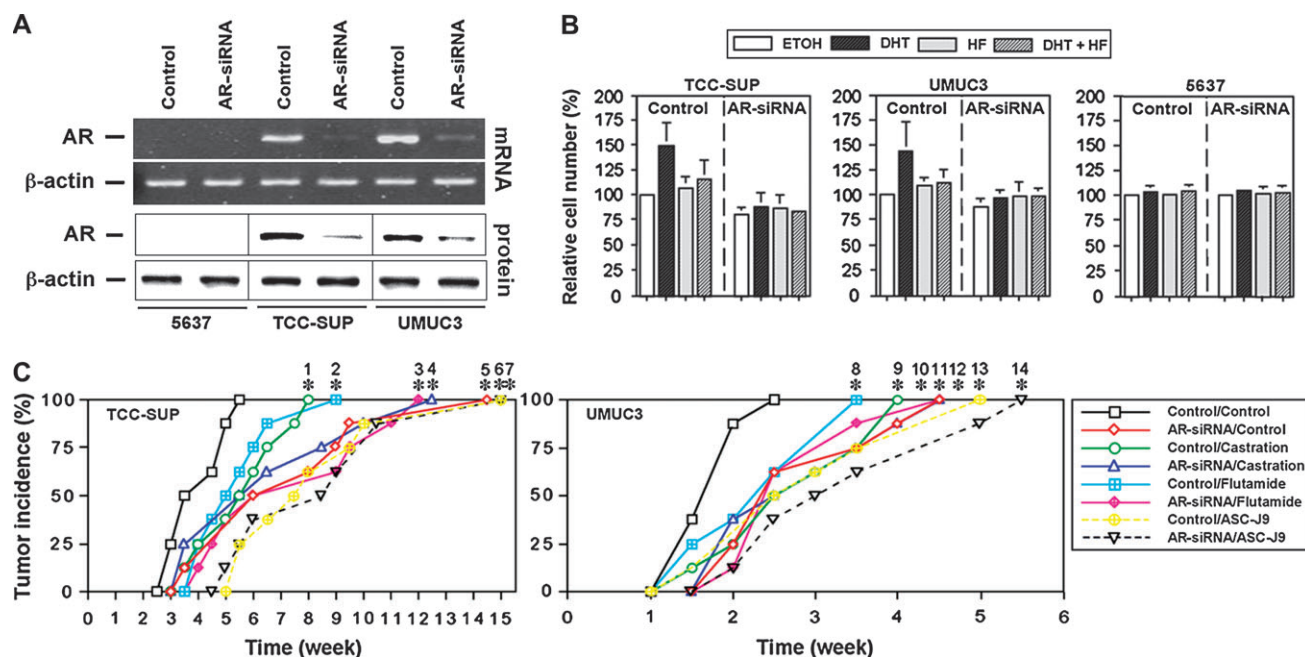


Fig. 3. Effect of AR-small-interfering RNA (AR-siRNA) expression on bladder cancer cell growth in vitro and in vivo. **A)** Bladder cancer cells (5637, an AR-negative line, and TCC-SUP and UMUC3, both AR-positive lines) were transfected with vectors containing either scrambled siRNA (control) or AR-siRNA. Androgen receptor mRNA levels in stable transfectants were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (upper 2 lanes), and protein levels were analyzed by immunoblot (lower 2 lanes) analyses. **B)** The scrambled control-siRNA- and AR-siRNA-transfected cells (one best subline for each combination) were cultured in the presence of ethanol (ETOH), 1 nM dihydroxytestosterone (DHT) (androgen), and/or 5 μM hydroxyflutamide (HF) (antiandrogen) for 6 days. Cell proliferation was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, and cell numbers are presented relative to cell number with ETOH treatment in each control line (set as 100%). Each value represents the mean and 95% confidence interval from at least three independent experiments for each subline. **C)** Scrambled control-siRNA and AR-siRNA-transfected TCC-SUP and UMUC3 cells were implanted subcutaneously into the flanks (right: control-siRNA; left: AR-siRNA) of male nude mice.

Treatment (castration or sham surgery, injection of flutamide or placebo pellet, and/or intraperitoneal injection of ASC-J9 [50 mg/Kg dissolved in 10 μL dimethyl sulfoxide (DMSO) + 90 μL corn oil] or vehicle control [10 μL DMSO + 90 μL corn oil] every other day) began at the time of tumor cell implantation. Tumor incidence (in n = 8 mice in each group) was determined as positive when size reaches 40 mm³. Values represent the percentage of the mice, in each group, that developed tumors. *1, TCC-SUP/control-siRNA/castration, *P* = .0166; *2, TCC-SUP/control-siRNA/flutamide, *P* = .0459; *3, TCC-SUP/AR-siRNA/flutamide, *P* = .0082; *4, TCC-SUP/AR-siRNA/castration, *P* = .0088; *5, TCC-SUP/AR-siRNA/control, *P* value less than .001; *6, TCC-SUP/control-siRNA/ASC-J9, *P* value less than .001; *7, TCC-SUP/AR-siRNA/ASC-J9, *P* value less than .001; *8, UMUC3/control-siRNA/flutamide, *P* = .0485; *9, UMUC3/control-siRNA/castration, *P* = .0258; *10, UMUC3/AR-siRNA/control, *P* = .0116; *11, UMUC3/AR-siRNA/castration, *P* = .0140; *12, UMUC3/AR-siRNA/flutamide, *P* = .0250; *13, UMUC3/control-siRNA/ASC-J9, *P* = .0038; *14, UMUC3/AR-siRNA/ASC-J9, *P* = .0011 by Fisher's exact test (all comparisons versus the group of control-siRNA with control treatment).

cells, we used mouse xenograft models to investigate whether targeting androgens and/or the AR could suppress bladder cancer progression *in vivo*. We used three therapeutic approaches: 1) androgen deprivation therapy via castration and/or antiandrogen (i.e., flutamide) treatment, 2) treatment with the anti-AR compound ASC-J9, and 3) treatment with AR-siRNA.

Targeting Androgens by Androgen Deprivation Therapy.

Bladder cancer cells (TCC-SUP, UMUC3, and 5637) were implanted subcutaneously into the right and left flanks of 6-week-old male nude mice ($n = 6$ per line). After 2 (for UMUC3) to 4 (for TCC-SUP and 5637) weeks, when the estimated volumes of all tumors for each cell line reached 40 mm³, we performed castration or sham surgery and implanted the mice with either slow-releasing flutamide or placebo pellets. Tumor sizes were monitored until the tumors exceeded 10% of the animal's body weight, at which time the mice were killed (i.e., after 6–16 weeks of treatment). As shown in Fig. 2, D, TCC-SUP tumors in mice treated with castration and/or antiandrogen were statistically significantly smaller than those in the control mice at 16 weeks. When the tumors in the treatment groups were harvested, their weights were found to be reduced by 57% to 63%. Similar results (40% to 54% reduction in tumor size in treatment groups at 6 weeks) were obtained in UMUC3 xenograft tumors, whereas minimal effects (up to 12% reduction at 12 weeks) of androgen deprivation therapy were seen in the AR-negative 5637 xenograft tumors (data not shown).

We also analyzed tumor specimens harvested when the mice were killed to evaluate cell proliferation (by PCNA immunostaining), apoptosis (by TUNEL assay), and angiogenesis or metastatic ability (expression of bFGF, VEGF, and MMP-9 by real-time RT-PCR). As shown in Fig. 2, E, androgen deprivation therapy led to statistically significantly (except for an apoptotic index in the flutamide treatment group) decreased proliferation (62%–73% reduction) and increased apoptosis (19%–81% induction) in TCC-SUP tumors. Levels of angiogenic factors and metastasis-related factors in tumors were also reduced by this treatment. These results suggest that androgen blockade suppresses androgen-sensitive bladder cancer progression.

Targeting Androgen Receptor by Treatment With the Anti-Androgen Receptor Compound ASC-J9.

The recently developed compound ASC-J9 (23) directly targets the AR by dissociating AR coregulators from the AR, leading to selective degradation of the AR protein (29). The effect of this compound on the growth of AR-positive bladder cancer cells was examined both *in vitro* and *in vivo*. As expected, ASC-J9 inhibited DHT-simulated growth of TCC-SUP and UMUC3 cells (Fig. 2, C). In the TCC-SUP mouse xenograft model, intraperitoneal injections of ASC-J9 suppressed growth (58% reduction [95% CI = 41%–76%] in tumor size over the course of 16 weeks of treatment) by a similar amount as other androgen deprivation strategies (57%–63%; Fig. 2, D). Analysis of harvested tumors showed that, like castration and/or treatment with flutamide, treatment with ASC-J9 decreased the proliferation index, increased the apoptotic index, and reduced levels of angiogenic factors and metastasis-related factors (Fig. 2, E). In addition, treatment with ASC-J9, but not androgen deprivation, reduced AR

expression by 39% (95% CI = 28% to 51%). These results suggest that directly targeting the AR can, like targeting androgens, suppress androgen-sensitive bladder cancer progression.

Targeting Androgen Receptor With Small-Interfering RNA.

We first established stable sublines of TCC-SUP and UMUC3 cells transfected with a retrovirus vector expressing AR-siRNA, which efficiently knocks down the AR in mammalian cells (18). As shown in Fig. 3, A, levels of AR mRNA and protein were substantially lower in AR-siRNA-expressing TCC-SUP and UMUC3 cells than in cells transfected with scrambled control-siRNA-expressing vector.

We then investigated the effects of the siRNA-induced reduction in AR mRNA and protein levels on proliferation of the stable sublines. Each stable subline was cultured with DHT and/or HF for 6 days, and cell growth was assessed by MTT assay. In control TCC-SUP or UMUC3 AR-positive cells, DHT induced cell proliferation and HF antagonized the DHT effect (Fig. 3, B), as also indicated in Fig. 2, C. However, AR knockdown by the AR-siRNA resulted in slower cell proliferation (20% reduction [95% CI = 12 to 32] in TCC-SUP and 15% reduction [95% CI = 6 to 24] in UMUC3) without androgen treatment (Fig. 3, B). Dihydrotestosterone and HF did not affect the growth of AR-positive TCC-SUP or UMUC3 and AR-negative 5637 sublines that express AR-siRNA.

Having found evidence that AR activation promotes BBN-induced mouse bladder cancer development and bladder cancer cell proliferation both *in vitro* and *in vivo*, we finally investigated whether the AR signaling in bladder cancer cells influences tumorigenicity by using the stable sublines with AR-siRNA or scrambled control-siRNA transfection in mouse xenograft models. Each stable subline (TCC-SUP/control-siRNA, TCC-SUP/AR-siRNA, UMUC3/control-siRNA, and UMUC3/AR-siRNA) was injected subcutaneously into the right (control-siRNA-expressing cells) and left (AR-siRNA-expressing cells) flanks of each male nude mouse ($n = 8$ for each treatment group), and treatment (by castration, by flutamide pellet implantation, or with ASC-J9) began immediately. These experiments allowed us not only to compare the development of AR-positive and AR-negative bladder tumor cells but also to evaluate the effects of androgen deprivation therapy in mice that carry a functional AR gene. As shown in Fig. 3, C, AR knockdown (by AR-siRNA expression in tumor cells) or ASC-J9 treatment of the mouse substantially prolonged the latency of tumor formation, compared with tumor development of respective cells with control-siRNA expression and control treatment of the host. Androgen deprivation also suppressed tumorigenicity of AR-expressing (control-siRNA-transfected) bladder cancer. By contrast, castration and flutamide treatment had little effect on the development of AR-siRNA-expressing tumors.

Discussion

Previous epidemiologic and preclinical findings formed the basis of our hypothesis that androgens and/or the AR play roles in bladder cancer development. We first confirmed previous observations in animal models with BBN (3,11) showing that this carcinogen induces bladder cancer more frequently and more rapidly in males

than in females and that castration retards or reduces the occurrence of BBN-induced bladder cancer. We further showed that bladder carcinomas were found in approximately half of castrated male or wild-type female mice but in none of the male or female mice lacking a functional AR. Knockout of the AR also had a substantial inhibitory effect on premalignant changes in the mouse bladder. These results advance previous observations using androgen deprivation therapy only (11) and suggest that AR signals may also contribute to bladder carcinogenesis through androgen-independent mechanisms.

We also noted that there was a progressive increase in the incidence of preneoplastic lesions from 30 to 40 weeks in BBN-treated wild-type females and ARKO males and females but not in other groups (wild-type males and castrated wild-type males in which $\geq 50\%$ of mice developed carcinoma at 40 weeks). These findings may reflect a delay in the development of, rather than a decrease in the actual incidence of, BBN-induced carcinoma in wild-type females and ARKO males and females. To investigate this possibility, a longer (>40 weeks) observation will be necessary. It would also be of interest to examine the consequences of excess androgens in males and to compare effects of supplemental testosterone and DHT in BBN-treated wild-type and ARKO mice (male and female) to determine which is more potent in inducing bladder cancer.

It has been suggested that androgens and the AR might regulate the P450 system (10), thereby leading to differential activation of BBN in male and female mice. However, the large difference in BBN-induced bladder cancer incidence between wild-type (42% at 40 weeks) and ARKO (0%) females, both of which have similar levels of sex hormones (27) and therefore are likely to have similar P450 activity, may be evidence for the importance of non-P450-involved AR functions in bladder carcinogenesis. This conclusion is further supported by the findings from the other AR knockdown approaches we used (i.e., AR-siRNA and the anti-AR molecule ASC-J9 in mouse xenograft models), which showed an effect of AR reduction on the growth of non-BBN-induced bladder cancer. Moreover, we found that two bladder cancer cell lines express a functional AR and that AR knockdown, as well as androgen deprivation therapy in male mice, results in a substantial delay in tumor formation by these lines.

In the BBN mouse model, the differences in cancer incidence between castrated males (50% at 40 weeks) and ARKO males (0%) and between DHT-supplemented ARKO males (25% at 40 weeks) and ARKO males and females (0%) suggest the involvement of non-androgen-mediated AR signals and androgen-mediated non-AR signals, respectively, in inducing bladder carcinogenesis. Indeed, nonclassical AR signal pathways have been reported in prostate cancer (14). For example, peptide growth factors or protein kinases could induce AR activity through signal transduction pathways. On the other hand, androgen-mediated non-AR pathways, which might include activation of other steroid hormone receptors, including estrogen receptor, by DHT or its metabolites, have also been suggested (30). Taken together, our data imply that AR as well as androgens could be essential targets for controlling bladder cancer development.

Expression of the AR has been detected by immunohistochemistry in normal bladder epithelium as well as bladder smooth mus-

cle and neuronal cells (8). Early studies using androgen-binding assays also suggested the expression of AR in bladder tumors (31,32). Laor et al. (31) demonstrated higher AR content in 13 bladder cancers than in 8 normal bladders as well as in 10 low-grade tumors than in 3 high-grade tumors. Noronha and Roa (32) detected AR activity in all six bladder cancer specimens examined, with no association between receptor levels and tumor characteristics. More recently, two studies showed AR expression in more than half of bladder cancers using immunohistochemistry (9,33). Zhuang et al. (33) detected AR expression in seven of eight bladder transitional cell carcinoma specimens, but expression was not associated with tumor grade or stage. Boorjian et al. (9) found that 26 of 49 bladder cancers expressed the AR and that its loss in tumors was associated with advanced tumor stage but not with tumor grade. Our preliminary study of bladder cancer tissue specimens from 33 patients, using a quantitative RT-PCR method, also showed no association between AR expression and tumor grade or stage (Ishiguro et al., unpublished data). Interestingly, we found some evidence that recurrence-free survival in patients with high AR-expressing tumors tended to be lower than that in patients with low AR-expressing tumors. However, no study has, to our knowledge, demonstrated a strong relationship between AR status and tumor recurrence or patient survival. In addition, AR has not been identified as a marker for classification or prognosis for bladder cancer in any of several microarray-based gene expression analyses (34,35).

We also investigated whether androgens regulate bladder cancer progression through the AR. Cell proliferation assays and mouse xenograft models revealed that androgen increases the growth of AR-positive bladder cancer cells. Conversely, androgen depletion and/or antiandrogen treatment suppressed cancer progression. It is well known that prostate cancer cells generally require androgens for growth and regress in response to androgen deprivation (12,14). Our present results indicate that proliferation of some bladder cancers is also androgen sensitive. Furthermore, because AR knockdown in AR-expressing bladder cancer cell lines by siRNA also decreased cell proliferation, even in androgen-depleted conditions, it is possible that AR signals (via androgen-mediated and non-androgen-mediated pathways) might contribute to the promotion of bladder cancer progression.

Despite more than 50 years of efforts to understand the roles of androgen and the AR in prostate cancer, the detailed mechanisms of how these molecules modulate prostate cancer progression are still poorly understood, and it is unlikely that androgens and the AR modulate prostate cancer progression through any single molecule or pathway. We found that androgen deprivation reduced the expression of several molecules involved in angiogenesis or metastasis (i.e., bFGF, VEGF, MMP-9) in bladder cancer xenografts and that the changes were consistent with xenograft tumor progression or regression. It will be important to identify AR-regulated genes in bladder cancer. The identification of such genes could lead not only to the elucidation of the role of the AR in bladder cancer but also to the development of useful markers for detecting bladder cancer and monitoring its recurrence or progression.

There are potential limitations associated with using mouse models. The phenotype of ARKO male mice is similar to that of

wild-type female mice (19). Although both wild-type and ARKO mice have morphologically identical bladders, whether they differ in functionality and dynamics needs to be determined. Therefore, it may not be perfectly appropriate to compare the susceptibility of these mice to bladder tumor induction using the chemical carcinogen BBN. It may also be important to test other carcinogens that are known to be more toxic to the female bladder than the male bladder [e.g., arsenical metabolites (4,5)]. Thus, the relevance of the mouse models we used in this study to bladder carcinogenesis in humans remains uncertain. In addition, only two out of the eight human bladder carcinoma cell lines tested likely possess a functional AR, and these two lines may not be representative of various urothelial carcinomas in patients.

Nonetheless, the results presented here have the potential to provide the basis for the development of new preventive or therapeutic approaches for bladder cancer, via targeting androgens and the AR. Several agents and strategies used for treating and preventing prostate cancer (14) may be adopted for this purpose and merit consideration for clinical testing. More important, our findings provide the first evidence indicating the involvement of both androgens (via the AR pathway and non-AR pathway) and the AR (via androgen-mediated signals and non-androgen-mediated signals) in bladder cancer. Targeting both androgens (by castration and/or antiandrogen treatment) and the AR (by siRNA technology or small molecules that degrade AR protein, such as ASC-J9) may be necessary to achieve maximal inhibitory effects on bladder cancer. Further dissection of detailed mechanisms by which androgens affect both the AR and non-AR pathways and by which the AR is modulated by both androgens and nonandrogens to promote the initiation and/or progression of bladder cancer will be important.

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Notes

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