TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Decreased Tumorigenesis and Mortality from Bladder Cancer in Mice Lacking Urothelial Androgen Receptor

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Much fewer mice lacking androgen receptor (AR) in the entire body develop bladder cancer (BCa). However, the role of urothelial AR (Uro-AR) in BCa development remains unclear. In the present study, we generated mice that lacked only Uro-AR (Uro-AR−/−) to develop BCa by using the carcinogen BBN [N-butyl-N-(4-hydroxybutyl)-nitrosamine] and found that Uro-AR−/− mice had a lower incidence of BCa and a higher survival rate than did their wild-type (WT; Uro-AR+/+) littermates. In vitro assay also demonstrated that Uro-AR facilitates the neoplastic transformation of normal urothelial cells to carcinoma. IHC staining exhibited less DNA damage, with much higher expression of p53 and its downstream target protein PCNA in Uro-AR−/− than that found in WT urothelium, which suggests that Uro-AR may modulate bladder tumorigenesis through p53-PCNA DNA repair signaling. Indeed, Uro-AR−/− mice with the transgene, simian vacuolating virus 40 T (SV40T), in the urothelium (Uro-SV40T-AR−/−) had a similar incidence of BCa as did their WT littermates (Uro-SV40T-AR+/+), and p53 was inactivated by SV40T in both genotypes. Use of the AR degradation enhancer ASC-J9 led to suppression of bladder tumorigenesis, with few adverse effects in the BBN-induced BCa mouse model. Together, these results provide the first direct in vivo evidence that Uro-AR has an important role in promoting bladder tumorigenesis and BCa progression. Targeting AR with ASC-J9 may provide a novel approach to suppress BCa initiation. (Am J Pathol 2013, 182: 1811 e1820; http://dx.doi.org/10.1016/j.ajpath.2013.01.018)

In the United States, bladder cancer (BCa) is the fourth leading cancer in men and the eleventh in women.1 In men, the incidence of BCa is approximately threefold higher than in women, which suggests that sex hormone signals may contribute to bladder tumorigenesis; however, detailed mechanisms remain unclear.

Expression of androgen receptor (AR) has been well studied and has been detected in both benign urothelium2–4 and BCa2–10 via immunohistochemistry (IHC). However, the reported range of AR expression in human benign urothelium and BCa varies considerably among reports, from 0% to 86% and from 0% to 78%, respectively.2–5,7–10 The huge variation in AR expression among reports may be due to different methods of tissue preparation (eg, tissue processing and embedding in paraffin) and staining (eg, antibody, staining protocol, and criteria for positivity).11 Most reports have suggested a higher level of AR expression in BCa than in benign urothelium; however, some reports have reached the opposite conclusion. Therefore, the correlation between AR expression and BCa remains controversial.

Two animal models that target androgen-AR signals in BCa, ie, castrated and AR knockout (ARKO) mouse models, have provided evidence for the roles of androgen-AR signaling in cancer development with distinct mechanistic explanations. Using ARKO mice, Miyamoto et al12 reported...
that the loss of AR in the entire body led to a reduced incidence of BBN-induced BCa, which suggests that AR might promote bladder tumorigenesis. Others\textsuperscript{13,14} have found that surgical castration and high-dose antiandrogen (flutamide) therapy reduced bladder tumorigenesis in rats. Imaoka et al\textsuperscript{14} have suggested that castration-induced down-regulation of P450 CYP4B1, a key P450 enzyme that converts BBN \([N\text{-butyl-N-(4-hydroxybutyl)}]-\text{nitrosoamine}\) to the carcinogen BCPN \([N\text{-butyl-N-(3-carboxy-propyl)}]-\text{nitrosoamine}\), is the primary mechanism. Therefore, it remains unclear whether urothelial AR (Uro-AR) has any role in promoting bladder tumorigenesis.

To answer this question and explore the possibility of targeting Uro-AR as a therapeutic approach to BCa, we generated conditional Uro-ARKO mice (Uro-AR\textsuperscript{−/−}) that lacked AR only in urothelium. We found that Uro-AR could promote bladder tumorigenesis via modulation of p53-PCNA signals. The AR degradation enhancer ASC-J9, which degrades Uro-AR, can suppress bladder tumorigenesis.

Materials and Methods

Chemicals and Cell Line

Dihydrotestosterone and the carcinogen 3-methylcholanthrene (MCA) were purchased from Sigma-Aldrich (St. Louis, MO), and the p53 inhibitor pifithrin-\(\alpha\) (PFT) from Enzo Life Sciences, Inc. (Farmingdale, NY). ASC-J9 was obtained from AndroScience Corp. (San Diego, CA). A human normal urothelial cell line, SV-HUC-1, was purchased from ATCC (Manassas, VA) and maintained in F-12K medium (Kaighn’s modification of Ham’s F-12 medium) with 10% fetal bovine serum. A human kidney epithelial cell line, 293T, was purchased from ATCC and maintained in Dulbecco’s modified Eagle’s medium (GIBCO Corp., Grand Island, NY) with 10% fetal bovine serum.

Plasmids and Stable Cell Lines

To generate an AR overexpressive stable clone of the SV-HUC-1 cell line, the lentivirus vectors pWPI-AR or pWPI-control were co-transfected with pMD2.G and pAX2 into 293T cells. After 48 hours of transfection, the cultured medium of 293T cells was harvested and mixed with fresh F-12K culture medium (ratio, 1:1) and 8 \(\mu\)g/mL polybrene (Millipore Corp., Billerica, MA), then incubated with SV-HUC-1 cells for 24 hours. The viral-infected SV-HUC-1 cells contained green fluorescent protein, and the SV-HUC-AR or SV-HUC-vector stable cells were obtained via flow cytometry sorting.

Generation of Urothelial ARKO Mice and Induction of BCa by BBN

All animal procedures were approved by the University Committee on Animal Resources of the University of Rochester and in accord with NIH guidelines. Male Uro-ARKO (Uro-AR\textsuperscript{−/−}) mice were generated by mating uroplakin II promoter—driven Cre (UPII-Cre) male mice\textsuperscript{15} with flox-AR female mice.\textsuperscript{16} After genotyping, offspring male mice were subjected to BBN-induced BCa experiments. The protocol for BBN-induced BCa was used, as in a previous study.\textsuperscript{12} Uro-AR\textsuperscript{−/−} and Uro-AR\textsuperscript{−/+} mice were given, ad libitum, drinking water containing 0.05% BBN (TCI America, Inc., Portland, OR), which was prepared twice a week, and BBN consumption was recorded. At age 6 weeks, mice were given drinking water containing BBN for 12 weeks, and thereafter were given water without BBN. Mice were sacrificed at age 20 or 30 weeks, and bladders were harvested and preserved in paraffin for later histologic diagnosis and IHC.

Generation of Uro-ARKO Mice with Uroplakin Promoter—Driven SV40T Transgene

To generate Uro-ARKO mice with spontaneous BCa, we first mated UPII-Cre mice with UPII-SV40T mice,\textsuperscript{17} which harbor SV40T in the urothelium, to induce BCa. After genotyping, the male offspring with both UPII-Cre and UPII-SV40T transgenes were chosen to mate with flox-AR female mice for generation of Uro-ARKO male mice with UPII-SV40T (Uro-SV40T-AR\textsuperscript{−/−}). Mice were sacrificed at 15, 20, and 30 weeks of age. Urinary tract specimens were harvested for later analysis, similarly as in the BBN-induced BCa mouse model.

Neoplastic Transformation of SV-HUC-1 Cells

The protocol for neoplastic transformation of SV-HUC-1 was used, as described in a previous study.\textsuperscript{18} In brief, \(1 \times 10^6\) SV-HUC-1 cells were seeded in 100-mm culture dishes. After seeding for 48 hours, the culture medium was replaced with serum-free F12-K containing 1 nmol/L dihydrotestosterone and 5 \(\mu\)g/mL carcinogen MCA, with or without the p53 inhibitor PFT, at 30 \(\mu\)mol/L, and incubated for 48 hours. After the first 24 hours of MCA exposure, 1% fetal bovine serum was added to the medium. After 48 hours of MCA exposure, the medium was replaced with 1% fetal bovine serum—F12-K, and cells were cultured until confluent and then subcultured with 1:3 split ratio. After two additional MCA exposures as above, the SV-HUC-1 cells were cultured for 6 weeks to complete neoplastic transformation.

Western Blot Analysis

Western blot analysis was performed as previously described.\textsuperscript{19} In brief, 50 \(\mu\)g protein from each cell sample was resolved via electrophoresis in \(10\%\) or \(15\%\) SDS-PAGE gels and transferred to polyvinylidene difluoride membrane (Millipore Corp.). Specific proteins were detected using antibodies against human AR (C-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), \(\gamma\)-H2AX (Ser139; Upstate Biotechnology, Inc., Lake Placid, NY), and \(\beta\)-actin (N-21; Santa Cruz Biotechnology).
Histology and IHC

Bladders were fixed in 10% buffered formalin (Sigma). After standard tissue processing, bladder tissues were embedded in paraffin. Tissue sections were treated with H&E for general histologic evaluation. All histologic data were diagnosed by our licensed pathologist (H.M.). We used the ABC kit and DAB kit (both from Vector Laboratories, Inc., Burlingame, CA) to detect AR, γ-H2AX, p53, PCNA, and p21 using antibodies (from Santa Cruz Biotechnology) specific for mouse AR (C-19), γ-H2AX (Ser139), p53 (FL-393), PCNA (FL-261), and p21 (F-5). The terminal deoxynucleotidyl transferase—mediated TUNEL staining assay was performed using a Fluorescein-FragEL DNA Fragmentation Detection Kit (Calbiochem Corp., La Jolla, CA). Cell proliferation and apoptotic index were determined using the percentage of PCNA- and TUNEL-positive cells in 100 cells from each specimen.

Suppression of Bladder Tumorigenesis in Mice via AR Degradation Enhancer ASC-J9

The AR degradation enhancer ASC-J9 was used to examine the suppression of bladder tumorigenesis induced by BBN. Six-week-old FVB male mice were subjected to BBN challenge for 12 weeks to induce BCa. When mice were aged 16 weeks, they were divided into two groups, vehicle control and ASC-J9, and subjected to daily treatment via i.p. injection (75 mg/kg body weight). At age 24 weeks, mice were sacrificed, and bladders were harvested for histologic diagnosis.

Statistical Analysis

Differences in mean values between the two groups were analyzed using the two-tailed Student’s t-test. Differences in tumor incidence between the two groups were analyzed using Fisher’s exact test. Difference in survival rate with BCa between the two groups were analyzed using the log-rank (Mantel-Cox) test. P < 0.05 was considered statistically significant.

Results

Generation and Characterization of Mice Lacking Uro-AR

To study the role of Uro-AR in bladder tumorigenesis, we generated a new mouse model (Uro-AR\(^{-\gamma}\)) in which AR is deleted only in urothelial cells by mating male UPII-Cre mice with female flox-AR mice. The detailed mating strategy is described in Supplemental Figure S1A. The tail genotyping results demonstrated that the new Uro-AR\(^{-\gamma}\) mice carried all transgenes, such as UPII-Cre and flox-AR allele (Supplemental Figure S1B). IHC staining of the bladder from wild-type (WT; Uro-AR\(^{+/\gamma}\)) and Uro-AR\(^{-\gamma}\) mice using antibody against AR C-terminal showed that partial deletion of AR was restricted to the urothelial cells and was not found in the adjacent stromal cells (Supplemental Figure S1C). Furthermore, the serum testosterone levels were similar between 20-week-old Uro-AR\(^{-\gamma}\) mice and their WT littermates (P = 0.479) (Supplemental Figure S1D), indicating that altered phenotypes, including bladder tumorigenesis and animal mortality, found in the Uro-AR\(^{-\gamma}\) mice were not due to the

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**Figure 1**  Decreased BBN-induced bladder tumorigenesis in mice lacking Uro-AR. A: Incidence of hematuria in Uro-AR\(^{+/\gamma}\) (n = 22) and Uro-AR\(^{-\gamma}\) (n = 11) mice. Urine was collected from each mouse, and erythrocyte concentrations were detected using test strips. Positive response indicates presence of blood in the urine. Uro-AR\(^{-\gamma}\) mice showed a significantly higher incidence of hematuria than did Uro-AR\(^{+/\gamma}\) mice at age 20 weeks under 12-week BBN challenge. B: Incidence of BCa in Uro-AR\(^{+/\gamma}\) (n = 16) and Uro-AR\(^{-\gamma}\) (n = 8) mice. After 12 weeks of BBN treatment, Uro-AR\(^{+/\gamma}\) and Uro-AR\(^{-\gamma}\) mice were sacrificed at age 20 weeks, and bladders were harvested, stained with H&E, and diagnosed by licensed pathologists. Uro-AR\(^{-\gamma}\) mice revealed a significantly higher incidence of BCa than did Uro-AR\(^{+/\gamma}\) mice (50% versus 0%). C: H&E staining for morphologic features of benign and tumorous bladder. Left panel is representative of normal bladder, and right panel is representative of BCa (circled) in a Uro-AR\(^{-\gamma}\) mouse. For statistical analysis, differences in hematuria and tumor incidence between the two groups were analyzed using Fisher’s exact test. Original magnification, ×400. Scale bars: 50 μm.
change in circulating testosterone. Furthermore, there was no difference in consumption of drinking water containing BBN between Uro-AR+/y and Uro-AR−/y male mice (P = 0.304) (Supplemental Figure S1E).

Decreased BBN-Induced Bladder Tumorigenesis in Mice Lacking Uro-AR

To detect bladder tumorigenesis at the early stage, we first used a chemical reagent dipstick to monitor hematuria, as described previously in the Uro-AR+/y and Uro-AR−/y mice at age 20 weeks. There was a statistically significant difference in the detection of hematuria between Uro-AR+/y and Uro-AR−/y mice (65% versus 19%; P = 0.026) (Figure 1A). We then examined bladder tumorigenesis in Uro-AR+/y and Uro-AR−/y mice at age 20 weeks via histologic diagnosis and found that 50% of Uro-AR+/y mice had noninvasive (stage pTa) urothelial carcinoma, whereas Uro-AR−/y male mice were all cancer free (Figure 1, B and C) (P = 0.022). These results suggested that Uro-AR was able to promote BBN-induced bladder tumorigenesis.

AR Promotes Bladder Tumorigenesis by Facilitating Neoplastic Transformation of Normal Urothelial Cells

We further confirmed the role of Uro-AR in bladder tumorigenesis via a neoplastic transformation assay. We first established the human normal urothelial cell (SV-HUC-1) lines with stable transfection of AR (SV-HUC-AR) and vector control (SV-HUC-V). The mRNA and protein levels of SV-HUC-AR and SV-HUC-V cells were examined using real-time PCR and Western blot analysis (Figure 2, A and B). These stable cells were then subjected to neoplastic transformation with MCA for induction of colonies in the soft agar assay. The results showed that SV-HUC-AR cells form more colonies in soft agar than do SV-HUC-V cells (Figure 2C), which suggests that AR is able to promote neoplastic transformation of normal urothelial cells. Together, results from the in vivo BBN-induced bladder tumorigenesis mouse model (Figure 1) and the in vitro neoplastic transformation assay (Figure 2) indicated that Uro-AR was able to promote bladder tumorigenesis.

Increased p53-PCNA–Mediated DNA Damage Repairing Signals in Urothelium in Mice Lacking Uro-AR

To dissect the mechanism(s) by which Uro-AR promotes BBN-induced bladder tumorigenesis, we first examined DNA damage in benign urothelium from 20-week-old Uro-AR+/y and Uro-AR−/y mice after treatment with BBN. IHC staining with the DNA damage marker γ-H2AX showed that Uro-AR+/y mice exhibited more γ-H2AX signals in the benign urothelium than did Uro-AR−/y mice (Figure 3A). Similar results were obtained in the urothelial cell line. SV-HUC-AR expressed a higher level of γ-H2AX protein than did SV-HUC-V cells under chemical carcinogen MCA treatment (Figure 3B). This suggests less DNA damage under BBN or MCA challenges in the benign urothelium that lost AR, because p53, a known tumor suppressor involved in DNA repair signaling and p53 deficiency, has been linked to bladder tumorigenesis. We then examined p53 expression in benign urothelium and found that urothelium from Uro-AR−/y mice expressed a significantly higher level of p53 than that from Uro-AR+/y mice (Figure 3C). We also found that expression of PCNA, a key player in DNA excision repair that is upregulated by p53, was higher in urothelium from Uro-AR−/y mice than from Uro-AR+/y mice (Figure 3D). Together, data from Figure 3, A–D, suggested that Uro-AR could modulate

**Figure 2** AR promotes bladder tumorigenesis by facilitating the neoplastic transformation of normal urothelial cells. A: mRNA expression of AR in the stable cell line SV-HUC-1. mRNA samples were isolated from SV-HUC-V and SV-HUC-AR stable cells and analyzed for expression quantification using real-time PCR. SV-HUC-AR cells expressed higher AR mRNA levels than did SV-HUC-V cells. B: AR protein expression in SV-HUC-V and SV-HUC-AR cells. Western blot analysis revealed that, compared with SV-HUC-V cells, SV-HUC-AR cells expressed abundant AR protein. C: Colony formation of neoplastic transformed SV-HUC-V and SV-HUC-AR cells in soft agar. After the carcinogen MCA induced neoplastic transformation, the same number of SV-HUC-V and SV-HUC-AR cells were seeded into soft agar for colony formation to examine the degree of neoplastic transformation. SV-HUC-AR stable cells revealed significantly more colonies than did SV-HUC-V cells, which indicated that AR may promote neoplastic transformation of normal urothelial cells. Difference in colony formation numbers between the two groups was analyzed using the two-tailed t-test. Results are expressed as the means ± SEM of three experiments.
After 12-weeks of BBN treatment, 20-week-old Uro-AR+/y and Uro-AR−/y mice were sacrificed, and bladders were harvested for histologic diagnosis. Benign bladder tissues were selected for further examination of DNA damage markers γ-H2AX than did Uro-AR−/y urothelium, indicating more DNA damage in Uro-AR+/y urothelium (positive signal indicated by arrowheads). Together, results from in vivo mouse models that lack active p53 (Figure 4, A–C) and the in vitro neoplastic transformation assay containing p53 inhibitor (Figure 4D) suggest that the presence of p53 is necessary for Uro-AR to promote bladder tumorigenesis.

Uro-AR Promotes BBN-Induced BCa Development through Modulation of p53-p21

To further study the roles of Uro-AR in BCa development after tumorigenesis, we evaluated BCa development in Uro-AR+/y and Uro-AR−/y mice at age 30 weeks. Bladder tumors (at stages pTa-pT1) were grossly identified in all Uro-AR+/y and Uro-AR−/y mice. IHC staining showed a higher index of proliferation marker PCNA in bladder tumors developed in Uro-AR+/y than those found in Uro-AR−/y mice (Figure 5A), which suggests that Uro-AR promotes cell proliferation in bladder tumors. We further detected higher expression of p21, a cell cycle modulator that controls cell proliferation and is up-regulated by p53, in BCa developed in Uro-AR−/y mice as compared with Uro-AR+/y mice (Figure 5B). We also examined cell apoptosis in the tumors via TUNEL assay and found a higher apoptotic index in Uro-AR+/y mice compared with AR−/y mice (Figure 5C). This cell apoptotic result is similar to that of an earlier study that found that p53 expression level is positively associated with apoptosis in urothelial cells.

The results of Uro-AR−/y mice at age 20 weeks. The results showed little difference in either PCNA

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The results of Uro-AR−/y mice at age 20 weeks. The results showed little difference in either PCNA
expression (Figure 5D) or apoptotic index (Figure 5E) between tumors of Uro-SV40T-AR^+/y and Uro-SV40T-AR^-/y mice. Together, we provided in vivo evidence that functional p53 activity is required for Uro-AR to regulate BCa development.

Increased Survival Rate in BBN-Treated Mice Lacking Uro-AR

The consequence of Uro-AR promoting BBN-induced BCa development was demonstrated by the decreased survival rate in Uro-AR^+/y mice as compared with Uro-AR^-/y mice (Figure 6). Considered together, our data indicate that Uro-

Figure 4  AR fails to regulate bladder tumorigenesis by inactivating p53. A: BCa incidence in Uro-SV40T-AR^+/y and Uro-SV40T-AR^-/y mice at various ages. There was no difference in BCa incidence at age 30 (n = 6), 20 (n = 5), and 15 (n = 3) weeks. In all Uro-SV40T-AR^+/y and Uro-SV40T-AR^-/y mice, the incidence of BCa was 100% of BCa at the three time points. B: Morphologic features of BCa in Uro-SV40T-AR^+/y and Uro-SV40T-AR^-/y mice at age 20 weeks. Both genotypes of mice revealed carcinoma in situ. C: Detection of p53 expression in urothelium of Uro-SV40T-AR^+/y and Uro-SV40T-AR^-/y mice at age 20 weeks. Tumors in both genotypes of mice expressed similar levels of p53. D: Colony formation assay of SV-HUC-V and SV-HUC-AR stable cells with PFT treatment during neoplastic transformation. The results showed no statistical difference in colony numbers between these two stable cells by adding p53 inhibitor. Results are expressed as the means ± SEM of three experiments. Original magnification, ×400 (B and C). Scale bars: 50 μm (B and C).

Figure 5  Uro-AR regulates BCa tumor growth through p53. A: Detection of PCNA in bladder tumors of Uro-AR^+/y and Uro-AR^-/y mice. PCNA is a cellular proliferation marker. The results showed that Uro-AR^-/y bladder tumors express more PCNA (P = 0.015; n = 4). B: Detection of p21 in bladder tumors of Uro-AR^+/y and Uro-AR^-/y mice. Uro-AR^-/y tumors revealed significantly more p21 (arrowheads) than did Uro-AR^-/y (P = 0.005; n = 4). p21 can be up-regulated by p53, promote cell cycle arrest, and serve as a tumor-suppressor gene. C: TUNEL assay for apoptosis in Uro-AR^-/y and Uro-AR^-/y bladder tumors. Uro-AR^-/y tumors revealed significantly less apoptosis (P = 0.004; n = 4). Green spots represent the apoptosis signal. D: Detection of the cell proliferation marker PCNA in Uro-SV40T-AR^+/y and Uro-SV40T-AR^-/y bladder tumors during IHC staining, with no statistical difference in PCNA expression between Uro-SV40T-AR^+/y and Uro-SV40T-AR^-/y tumors (P = 0.924; n = 5). E: TUNEL assay for apoptosis in Uro-SV40T-AR^+/y and Uro-SV40T-AR^-/y tumors. No statistical difference in apoptosis was found between Uro-SV40T-AR^+/y and Uro-SV40T-AR^-/y tumors (P = 0.240; n = 5). For statistical analysis, differences in mean values between the two groups were analyzed using the Student’s t-test. Original magnification: ×400 (A–E). Scale bars: 50 μm (A–E).
AR promoted bladder tumorigenesis and that cancer progression resulted in higher mortality in the BBN-induced BCa mouse model.

Approach to Suppress BCa Development via AR Degradation Enhancer ASC-J9

Our data revealed that Uro-AR−/y mice exhibited a lower incidence of carcinogen-induced BCa than did their WT littermates, which suggests that Uro-AR promotes bladder tumorigenesis. Therefore, targeting Uro-AR using anti-AR molecules could be a potential approach to suppress BCa development. To mimic the clinical conditions of BCa, we first administered ASC-J9 in mice via daily i.p. injection of BBN-induced BCa (75 mg/kg body weight) at the first sign of hematuria and continued until the mice were aged 24 weeks. The results revealed that ASC-J9–treated mice demonstrated a significantly lower incidence of BCa than did vehicle control–treated mice (20% versus 80%) (Figure 7A). We also observed that ASC-J9 treatment had little effect on serum testosterone concentration (data not shown) and body weight as compared with vehicle control treatment (Figure 7B). These results suggested that targeting AR via ASC-J9 could be a novel approach to treat BCa at an early stage of cancer progression.

Discussion

Multiple cells are involved in the BCa microenvironment, including urothelial cells, fibroblast cells, smooth muscle cells, and infiltrating immune cells. More than 90% of bladder tumors are derived from urothelium. Finding the role of Uro-AR in promoting bladder tumorigenesis and targeting AR with ASC-J9, leading to suppression of BCa at an early developing stage, provide new insights that should help reduce BCa progression and increase survival rates. However, Uro-AR promotes bladder tumorigenesis and later progression through modulation of p53, and an early report has suggested that more than 50% of high-grade invasive BCa tumors contain dysfunctional p53. Therefore, such a therapeutic approach targeting Uro-AR may yield more profound effects if administered in the stages of non–muscle invasive BCa, which contains more functional p53 expression. Furthermore, as early studies have suggested that recurrence of BCa might be associated with expression of Uro-AR, it will be beneficial if additional clinical studies prove targeting Uro-AR via ASC-J9 leads to suppression of BCa recurrence.

Our results suggest that the dominance of Uro-AR in BCa is due to modulation of p53 activity. Loss of p53 function has been associated with bladder tumorigenesis and BCa progression. For bladder tumorigenesis, Ozaki et al have reported that p53-positive and p53-negative knockout mice have a higher incidence of BBN-induced BCa than do WT mice. In addition, Gao et al have suggested that p53 deficiency synergizes with activated Ha-ras in promoting bladder tumorigenesis. For BCa progression, both grade and
stage of BCa have been associated with the mutation of p53.29,30 These results also explain why AR has a consistent role in promoting bladder tumorigenesis and BCa progression, because AR can regulate p53 activities in both normal and neoplastic urothelium.

The mechanism by which Uro-AR negatively regulates p53 activity was not examined in our study. However, earlier reports provide two mechanisms that may support our conclusion. Nantermet et al31 indicated that p53 activity is negatively regulated by AR activation without changing p53 RNA level. Experiments from prostate cancer (PCa) LNCaP cells indicate that AR suppresses accumulation of p53 in the nucleus, which suggests that AR may negatively regulate p53 activity through a posttranscriptional mechanism. Beitel et al32 indicated that the AR N-terminal transactivation domain can interact with PIRH2 (p53-induced RING-H2 protein), a p53-induced ubiquitin-protein, which promotes p53 degradation.33 Lin et al34 also indicated that suppressing AR via siRNA induces p53 protein expression through PIRH2 and further promotes downstream p21 expression and results in increased apoptosis, which provides the other mechanism in which AR suppresses p53 activity through ubiquitin-induced protein degradation.

CD24, a glycosyl phosphatidylinositol-linked sialoglycoprotein, has been associated with urothelial carcinoma and contributes to BCa progression.35,36 That the mechanism by which CD24 mediates BCa development may be through androgen/AR signaling was further suggested by Overdevest et al.37 who used the BBN-induced cancer model with CD24-deficient male mice to demonstrate that such mice have a lower incidence of bladder tumor and delayed bladder malignancy, including metastasis and death. In the molecular mechanical dissection, they found that CD24 mRNA and protein expression are androgen dependent and that AR activation can induce CD24 promoter activity in human BCa cell lines. The in vivo results of Overdevest et al37 are consistent with our finding in Uro-ARKO mice, both of which suggested that AR is involved in bladder tumorigenesis, cancer progression, and mortality. This conclusion provides a solid rationale for BCa treatment that targets AR.

Uro-AR had positive roles in promoting both bladder tumorigenesis and tumor progression, which was in apposition to early findings that showed dual yet opposing roles of AR in PCa38,39 and hepatocellular carcinoma.40 In PCa, AR functions as a proliferator in PCa stroma cells, a survivor in PCa luminal epithelial cells, and a suppressor in PCa basal epithelial cells.38,39 In hepatocellular carcinoma, AR was found to promote tumorigenesis,40 yet functioned as a suppressor for hepatocellular carcinoma metastasis,41 which could be due to different intracellular signals in the same hepatocytes at various stages.41 In the present study, we found that Uro-AR had a consistent role in promoting BCa progression at tumorigenesis and tumor progression, which could be due to suppression of p53 expression or function.

In a previous study,12 we showed that the urothelium of general ARKO mice implanted with dihydrotestosterone pellets has significantly higher apoptotic signals than does that of WT mice after BBN treatment. This result provides evidence that lacking AR in the urothelium can promote BBN-induced cell apoptosis (Figure 5C). It has been reported that p53 activity is negatively regulated by AR activation31 and that p53 can induce BAX activation, which promotes cell apoptosis.42 Therefore, we speculate that the possible mechanism behind the data in Figure 5C is that lost or decreasing AR expression in urothelium induces p53 activation, which promotes urothelial apoptosis through p53 downstream target genes such as Bax. An earlier study also suggested that apoptosis may be closely associated with carcinogenesis and tumor invasion of the rat bladder induced by BBN,43 which supports the data in Figure 5C.

Umbrella cells are the first lining layer of urothelial cells, which directly contacts with urine in the bladder lumen. Therefore, umbrella cells have a higher risk of being exposed to urinary carcinogens. Indeed, urinary carcinogens are associated with the risk of BCa.44,45 In the present study, the major urinary metabolite of BBN was BCPN.48 After α-hydroxylation, BCPN chemically induced DNA alkylation on urothelial cells49 and resulted in phosphorylation of H2AX.50 Therefore, our data (Figure 3) suggest that γ-H2AX signals shown in umbrella cells may be due to direct exposure to BCPN in the urine. Furthermore, not all bladder tumors derive from basal cells. Cytokeratin 20, an umbrella cell marker, has been reliably observed in the primary tumor and its matched lymph node metastasis.51 p63 is a member of the p53 family, which encodes multiple products with transactivating, death-inducing, and dominant-negative activities,52 and Guo et al53 have suggested that p63 could induce senescence and suppress tumorigenesis in vivo. Moreover, a clinical report has also indicated that altered expression of p63 is associated with
human BCa. We have used IHC staining to examine the p63 protein expression on the normal urothelium of wild-type and Uro-ARKO mice at age 20 weeks after BBN treatment. The results showed that p63 protein expression level is significantly higher in Uro-ARKO mouse urothelium than in WT mouse urothelium (Supplemental Figure S3), whereas Uro-ARKO mice have a lower rate of bladder cancer than do WT mice (Figure 1B). Therefore, our results suggest that elevated p63 expression in Uro-ARKO urothelium is associated with a lower incidence of BCa. This conclusion is consistent with the references described previously.

In summary, using multiple in vitro and in vivo approaches, our results demonstrate that Uro-AR consistently promotes bladder tumorigenesis, tumor progression, and animal death. We further dissected the mechanisms by which Uro-AR regulates BCa development through p53 (Figure 8). The present study revealed that AR degradation enhancer ASC-J9 could substantially suppress bladder tumorigenesis in a carcinogen-induced BCa mouse model, which suggests that Uro-AR is a potential target against BCa.

Supplemental Data

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References


