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Cancer Biology and Translational Studies

Molecular Cancer Therapeutics

Nuclear Factor-κB Promotes Urothelial Tumorigenesis and Cancer Progression via Cooperation with Androgen Receptor Signaling

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Abstract

We investigated the role of NF-κB in the development and progression of urothelial cancer as well as cross-talk between NF-κB and androgen receptor (AR) signals in urothelial cells. Immunohistochemistry in surgical specimens showed that the expression levels of NF-κB/p65 ($P = 0.015$)/phospho-NF-κB/p65 ($P < 0.001$) were significantly elevated in bladder tumors, compared with those in nonneoplastic urothelial tissues. The rates of phospho-NF-κB/p65 positivity were also significantly higher in high-grade ($P = 0.015$)/muscle-invasive ($P = 0.033$) tumors than in lower grade/non–muscle-invasive tumors. Additionally, patients with phospho-NF-κB/p65-positive muscle-invasive bladder cancer had significantly higher risks of disease progression ($P < 0.001$) and cancer-specific mortality ($P = 0.002$). In immortalized human normal urothelial SVHUC cells stably expressing AR, NF-κB activators and inhibitors accelerated and prevented, respectively, their neoplastic transformation induced by a chemical carcinogen 3-methylcholanthrene. Bladder tumors were identified in 56% (mock), 89% (betulinic acid), and 22% (parthenolide) of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-treated male C57BL/6 mice at 22 weeks of age. NF-κB activators and inhibitors also significantly induced and reduced, respectively, cell proliferation/migration/invasion of AR-positive bladder cancer lines, but not AR-knockdown or AR-negative lines, and their growth in xenograft-bearing mice. In both nonneoplastic and neoplastic urothelial cells, NF-κB activators/inhibitors upregulated/downregulated, respectively, AR expression, whereas AR overexpression was associated with increases in the expression levels of NF-κB/p65 and phospho-NF-κB/p65. Thus, NF-κB appeared to be activated in bladder cancer, which was associated with tumor progression. NF-κB activators/inhibitors were also found to modulate tumorigenesis and tumor outgrowth in AR-activated urothelial cells. Accordingly, NF-κB inhibition, together with AR inactivation, has the potential of being an effective chemopreventive and/or therapeutic approach for urothelial carcinoma. *Mol Cancer Ther; 17(6); 1303–14. ©2018 AACR.*

Introduction

Urinary bladder cancer, mostly urothelial carcinoma, is one of most frequently diagnosed malignancies predominantly affecting males throughout the world (1). There are two distinct forms of bladder cancers, including non–muscle-invasive and muscle-invasive diseases. Patients with non–muscle-invasive disease undergoing transurethral tumor resection and currently available intravesical pharmacotherapy still carry a lifelong risk of disease recurrence, while those with muscle-invasive disease are at a high risk of disease progression even after more aggressive treatment such as radical cystectomy with neoadjuvant/adjuvant chemotherapy. Consequently, identification of key molecules or pathways responsible for the development and/or growth of urothelial tumor may offer novel targeted therapy that improves patient outcomes.

The imbalance in the risk of bladder cancer between men and women prompted us to investigate the role of sex hormone receptor signaling in urothelial tumorigenesis. Indeed, increasing evidence suggests a critical role of androgen-mediated androgen receptor (AR) signals in the development and progression of bladder cancer (2). Thus, AR inactivation has been shown to correlate with bladder cancer regression using preclinical models. Nonetheless, precise mechanisms for the functions of AR and related signals in urothelial cells remain poorly understood.

Nuclear factor (NF)-κB is a protein complex of transcription factors, consisting of RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52), which has been implicated in a wide variety of physiological and pathological processes, such as immunity, inflammation, and cell proliferation/differentiation (3). Activation of NF-κB, which is enhanced by its phosphorylation, has also been linked to cancer initiation and outgrowth. Indeed, the NF-κB pathway has been shown to be constitutively

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activated in some cell lines and tissue specimens of, for instance, prostate cancer, which further induces tumor progression (4–6). Remarkably, in prostate cancer cells, functional interplay between NF-κB and AR signals has been suggested (7–10). Specifically, AR activation has been shown to correlate with inhibition of NF-κB/p65 expression and transcriptional activity (7), whereas NF-κB/p65 activation leads to induction of the expression/nuclear translocation of AR and its transcriptional activity (8, 9).

We recently demonstrated that androgens could reduce the cytotoxic activity of a chemotherapeutic agent cisplatin in AR-positive bladder cancer cells presumably via inducing the expression of NF-κB/p65 and its nuclear translocation (11). In the current study, we aimed to determine the role of NF-κB in urothelial tumorigenesis and tumor progression in relation to AR signaling. We found that NF-κB/p65 could modulate neoplastic transformation of AR-positive urothelial cells and the growth of AR-positive bladder cancer cells, but not those of AR-negative cells.

Materials and Methods

Antibodies and chemicals

Anti-AR (N-20), anti-NF-κB/p65 (sc-109), anti-GAPDH (6c5), and anti-Lamin B1 (B-10) antibodies and an anti-p-NF-κB/p65 antibody (Ser 536) were purchased from Santa Cruz Biotechnology and Cell Signaling Technology, respectively. An anti-Ki-67 antibody (clone MIB-1) was from Dako. We obtained betulinic acid (BA; ref. 12), parthenolide (PAR; ref. 13), tumor necrosis factor (TNF)-α, dihydrotestosterone (DHT), and hydroxyflutamide (HF; ref. 14) from Sigma-Aldrich. Dehydroxymethylepoxyquinomicin (DHMEQ; ref. 15) was from MedChem Express.

Tissue microarray (TMA) and immunohistochemistry (IHC)

TMAs consisting of retrieved bladder tissue specimens obtained by transurethral resection performed at the University of Rochester Medical Center or the Johns Hopkins Hospital were constructed with waiver of patient consent, as we described previously (16). Appropriate approval from the Institutional Review Board was obtained at each institution before construction and use of the TMAs. The Institutional Review Boards approved the request to waive the documentation of informed consent and we were thus not required to obtain the signatures. In addition, the study was conducted in compliance with the Declaration of Helsinki, Belmont Report, and U.S. Common Rule. These consisted of 149 cases of urothelial neoplasm from 114 men and 35 women with a mean age of 65.9 years (range, 26–89). All 65 patients with muscle-invasive tumor ultimately underwent radical cystectomy. None of the patients had received therapy with radiation or anticancer drugs prior to the collection of the tissues.

IHC was performed on the 5-μm sections, using a primary antibody to NF-κB/p65 (dilution 1:200) or p-NF-κB/p65 (dilution 1:50), as we described previously (11). All stains were manually quantified by a single pathologist (H. Miyamoto) blinded to sample identity. The German immunoreactive scores calculated by multiplying the percentage (0% = 0; 1%–10% = 1; 11%–50% = 2; 51%–80% = 3; 81%–100% = 4) of immunoreactive cells in their cytoplasms (NF-κB) or nuclei (p-NF-κB) by staining intensity (negative = 0; weak = 1; moderate = 2; strong = 3) were considered negative (0; 0–1), weakly positive (1+; 2–4), moderately positive (2+; 6–8), and strongly positive (3+; 9–12).

Cell lines

An immortalized human normal urothelial cell line (SVHUC) and a human urothelial carcinoma cell line (UMUC3) were originally obtained from the American Type Culture Collection. Another human urothelial carcinoma cell line (647V) was used in our previous studies (11, 17–21). All these lines were recently authenticated, using GenePrint 10 System (Promega), and routinely tested for *Mycoplasma* contamination, using the PCR Mycoplasma Detection Kit (Applied Biological Materials). Stable sublines, including SVHUC-vector/SVHUC-AR (22–24), UMUC3-control-short hairpin RNA (shRNA)/UMUC3-AR-shRNA (25), and 647V-vector/647V-AR (19), were established in our previous studies. Similarly, NF-κB/p65-shRNA lentiviral particles (sc-29411-V; Santa Cruz Biotechnology) were stably expressed in SVHUC-AR and UMUC3 cells. Multiple frozen aliquots were made upon the acquisition and all experiments were performed with cells undergoing fewer than 20 passages. SVHUC (or its sublines) and UMUC3/647V (or their sublines) were maintained in Kaighn's Modification of Ham's F-12K (Mediatech) and Dulbecco's modified Eagle's medium (Mediatech), respectively, supplemented with 10% fetal bovine serum (FBS) and cultured in phenol red-free medium supplemented with either 5% FBS or 5% charcoal-stripped FBS (for DHT treatment) at least 24 hours before experimental treatment.

In vitro transformation

We used a method for neoplastic transformation in SVHUC with exposure to a carcinogen 3-methylcholanthrene (MCA), as described in a previous study (26), with minor modifications. Briefly, cells (2×10^6/10-cm culture dish incubated for 24 hours) were cultured in serum-free F-12K containing 5 μg/mL MCA (Sigma-Aldrich). After the first 24 hours of MCA exposure, FBS (1%) was added to the medium. After additional 24 hours, the cells were cultured in medium containing 5% FBS without MCA until near confluence. Subcultured cells (1/3 split) were again cultured in the presence of MCA for two 48-hour exposure periods, using the above protocol. These cells were then subcultured for 6 weeks with or without NF-κB modulators.

MTT assay

Cells (0.5×10^3–1×10^3) seeded in 96-well plates were cultured for up to 7 days and then incubated with 0.5 mg/mL of MTT (Sigma-Aldrich) in 100 μL of medium for 3 hours at 37°C. MTT was dissolved by DMSO, and the absorbance was measured at a wavelength of 570 nm with background subtraction at 630 nm.

Plate colony formation assay

Cells (5×10^2) seeded in 12-well plates were allowed to grow until colonies in the control well were easily distinguishable. The cells were then fixed with methanol, stained with 0.1% crystal violet, and photographed. The numbers of colonies were quantitated, using ImageJ software (NIH).

Scratch wound-healing assay

Cells at a density of ≥90% confluence in 12-well plates were scratched manually with a sterile 200 μL plastic pipette tip, cultured for 24 hours, fixed with methanol, and stained with 0.1% crystal violet. The width of the wound area was quantitated, using the ImageJ.

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Cell invasion assay

Cells (1×10^5) in 500 μL of serum-free medium were added to the upper chamber of a Matrigel-coated Transwell chamber (8.0-μm pore size polycarbonate filter with 6.5-mm diameter; Corning), whereas 750 μL of medium containing 10% FBS was added to the lower chamber of the Transwell. After incubation for 16 hours, invaded cells were fixed, stained with 0.1% crystal violet, and counted.

Western blot

Equal amounts of proteins (30–50 μg) obtained from cell extracts were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane electronically, blocked, and incubated with a specific antibody and a secondary antibody (anti-mouse IgG HRP-linked antibody or anti-rabbit IgG HRP-linked antibody; Cell Signaling Technology) followed by scanning with an imaging system (ChemiDOC MP, Bio-Rad). We also used a nuclear and cytoplasmic extraction reagent kit (NE-PAR, Thermo Scientific) for obtaining separate nuclear/cytoplasmic fractions.

Reverse transcription (RT) and real-time PCR

Total RNA isolated from cultured cells by TRIzol (Invitrogen) was reverse transcribed, using oligo-dT primers and Ominiscript reverse transcriptase (Qiagen). Real-time PCR was then performed, using RT2 SYBR Green FAST Mastermix (Qiagen). The primer sequences are given in Supplementary Table S1.

Reporter gene assay

Cells at a density of 50% to 70% confluence in 24-well plates were cotransfected with 250 ng of an androgen response element (ARE)-reporter plasmid (MMTV-Luc; ref. 17) or a pNF-κB reporter plasmid DNA (LR-2001, Signosis) and 2.5 ng of a control reporter plasmid (pRL-CMV), using Lipofectamine 3000 transfection reagent (Life Technologies). After 18 hours of transfection, the cells were cultured in the presence or absence of NF-κB modulators, DHT, and/or HF for 24 hours. Cell lysates were then assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit (Promega).

Mouse models for bladder cancer

The animal protocols in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals were approved by the Institutional Animal Care and Use Committee.

Male C57BL/6 mice (Johns Hopkins University Research Animal Resources) were supplied ad libitum with tap water containing 0.1% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN; Sigma-Aldrich) at 6 weeks of age for 12 weeks and thereafter with tap water without BBN, as described previously (17, 27). These mice also received injections of BA or PAR. Starting at 18 weeks of age, urine was assessed for the presence of hematuria twice a week, using Chemostrip 5 OB (Roche) urine test strips. When more than trace amount of blood was detected in three consecutive assessments, the animal was euthanized for macroscopic/microscopic analyses of the bladder and other major organs.

UMUC3-derived cells (1×10^6) mixed with 100 μL Matrigel (BD Biosciences) were subcutaneously injected into the flank of 6-week-old male immunocompromised athymic NCr-nu/nu mice (National Cancer Institute). Tumor size estimated by serial caliper measurements of perpendicular diameters using the following formula [(short diameter)^2 × (longest diameter) × 0.5] was monitored in animals treated with mock, BA, or PAR.

Statistical analysis

The Fisher exact test and Student *t* test were used to assess statistical significance for categorized variables and those with ordered distribution, respectively. Correlations between variables were determined by the Spearman correlation coefficient (CC). Survival rates in patients and tumor development rates in animals were calculated by the Kaplan–Meier method and comparison was made by the log-rank test. The Cox proportional hazards model was used to determine statistical significance of predictors in a multivariate setting. *P* values less than 0.05 were considered statistically significant.

Results

Expression of NF-κB/p65 in bladder cancer specimens

We first stained immunohistochemically for NF-κB/p65 and its phosphorylated form (p-NF-κB/p65) in the bladder TMAs consisting of 149 urothelial neoplasms and corresponding 88 non-neoplastic bladder tissues. Positive signals of NF-κB (Fig. 1A/1C/1E) and p-NF-κB (Fig. 1B/1D/1F) were detected predominantly in the cytoplasm and nucleus, respectively, of benign/malignant urothelial cells.

Overall, NF-κB/p-NF-κB was positive in 100% (25% 1+, 40% 2+, 35% 3+)/47% (36% 1+, 10% 2+) of benign urothelial tissues and 100% (9% 1+, 38% 2+, 52% 3+)/69% (44% 1+, 24% 2+, 1% 3+) of tumors, respectively (Supplementary Table S2). Thus, the rates of moderate–strong (1+ vs. 2+/3+) or strong (1+/2+ vs. 3+) NF-κB expression as well as p-NF-κB positivity (0 vs. 1+/2+/3+) or moderate–strong p-NF-κB expression (0/1+ vs. 2+/3+) were significantly higher in tumors than in benign tissues. In tumors, the expression levels of NF-κB versus p-NF-κB were correlated (CC = 0.294, $P < 0.001$; Supplementary Fig. S1A). We next analyzed the correlations of NF-κB/p-NF-κB expression with the histopathologic profile available for our patient cohort (Supplementary Table S3). The positive rate of p-NF-κB expression was significantly higher in high-grade (76%) or muscle-invasive (78%) tumors than in lower-grade (56%) or non–muscle-invasive (62%) tumors, respectively. Moderate/strong positivity (2+/3+) of NF-κB ($P = 0.096$) and positivity (1+/2+/3+) of p-NF-κB ($P = 0.095$) also tended to be more often seen in male tumors than in female tumors. However, there were no significant correlations between NF-κB expression and tumor grade or pT stage as well as between NF-κB or p-NF-κB expression and the status of lymph node involvement. We then performed Kaplan–Meier analysis coupled with the log-rank test to assess possible associations between NF-κB/p-NF-κB expression and patient outcomes. There were no significant correlations between the status of NF-κB or p-NF-κB expression in non–muscle-invasive tumors and recurrence-free or progression-free survival (PFS) rate ($P > 0.1$). However, patients with p-NF-κB–positive muscle-invasive tumor had significantly higher risks for disease progression ($P < 0.001$; Fig. 1G) and cancer-specific mortality ($P = 0.002$, Fig. 1H). By contrast, no significant associations between NF-κB levels (1+/2+ vs. 3+) in

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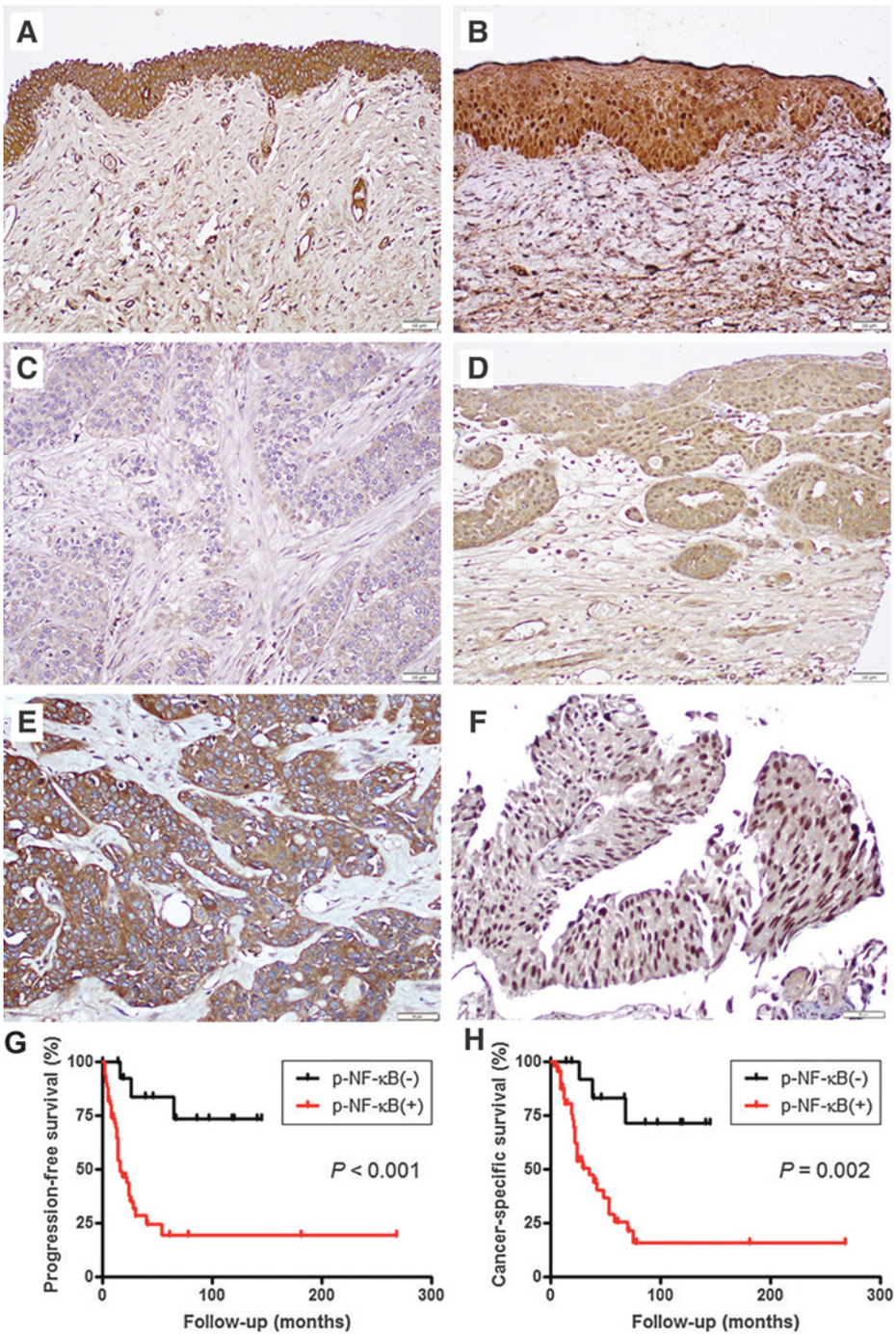

Figure 1.
IHC of NF-κB/p65 (**A, C, E**; cytoplasmic signals) and p-NF-κB/p65 (**B, D, F**; nuclear signals) in normal urothelial tissues (**A**, **B**) and bladder cancer specimens (**C–F**). The staining is scored by a combination of the intensity [i.e., strong (**A, B, E, F**) and weak (**C, D**)] and distribution (i.e., percent immunoreactive cells). Original magnification, ×200. Kaplan–Meier analyses for PFS (**G**) and CSS (**H**) in patients with muscle-invasive tumor, according to p-NF-κB/p65 positivity (0 vs. 1+/2+/3+).

muscle-invasive tumors and patient outcomes were found [PFS: $P = 0.485$; cancer-specific survival (CSS): $P = 0.192$]. To determine whether p-NF-κB expression was an independent prognosticator, multivariate analysis was performed with Cox model (Supplementary Table S4). In muscle-invasive tumors, p-NF-κB positivity was strongly associated with a lower PFS (HR = 6.424, $P = 0.003$) or CSS (HR = 4.718, $P = 0.012$) rate. In addition, correlations between the expression levels of NF-κB or p-NF-κB versus AR for which we previously stained in our bladder TMAs including the 149 tumors (16) were assessed. There were significant associations between moderate/strong NF-κB expression and AR positivity in all 149 tumors (CC = 0.230, $P = 0.005$; Supplementary Fig. S1B) as well as 32 high-grade non–muscle-invasive tumors (CC = 0.504, $P = 0.003$), but not between p-NF-κB and AR expression in these 149 cases (CC = 0.098, $P = 0.235$; Supplementary Fig. S1C).

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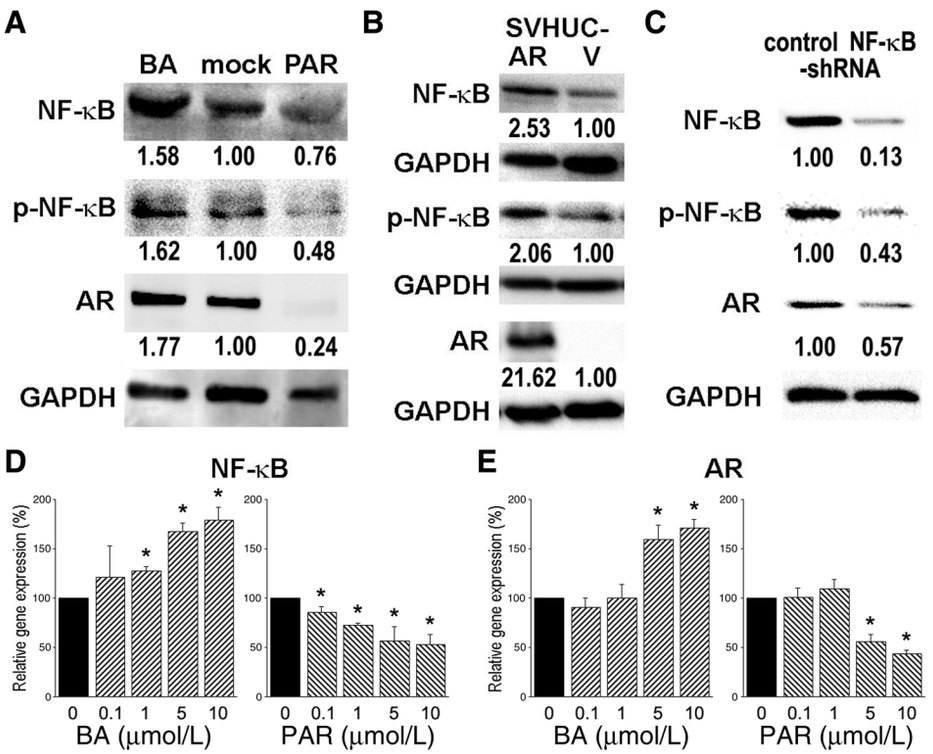

Figure 2.
Relationship between NF-κB and AR expression in urothelial cells. Western blotting of NF-κB/p65, p-NF-κB/p65, and AR, using proteins extracted from SVHUC-AR cells cultured with ethanol (mock), BA (10 μmol/L), or PAR (10 μmol/L) for 24 hours (**A**), SVHUC-AR vs. SVHUC-vector cells (**B**), or SVHUC-AR-control-shRNA vs. SVHUC-AR-NF-B-shRNA cells (**C**). GAPDH served as a loading control. Densitometry values for NF-κB/p-NF-κB/AR standardized by GAPDH that are relative to those of mock treatment or control cells are included below the lanes. Quantitative real-time RT-PCR of *NF-κB/p65* (**D**) and *AR* (**E**) in SVHUC-AR cells cultured with ethanol (mock), BA (0.1–10 μmol/L), or PAR (0.1–10 μmol/L) for 6 hours. Expression of each gene was normalized to that of *GAPDH*. Transcription amount is presented relative to that of mock-treated cells. Each value represents the mean (+SD) from three independent experiments. *, $P < 0.05$ (vs. mock treatment).

Interplay between NF-κB and AR signals in nonneoplastic urothelial cells

To investigate potential cross-talk between NF-κB and AR signals, Western blot was performed in immortalized human normal urothelial SVHUC cells. In SVHUC-AR cells, a NF-κB activator BA and a NF-κB inhibitor PAR increased and decreased, respectively, the expression of not only NF-κB/p65 and p-NF-κB/p65 but also AR (Fig. 2A). Similar results in their expression levels were obtained by the treatment with another activator (TNF-α) or inhibitor (DHMEQ), except p-NF-B/p65 expression with DHMEQ (Supplementary Fig. S2A). Consistent with previous observations (28), DHMEQ overall increased the level of p-NF-B/p65 by inducing its cytoplasmic expression (but inhibiting its nuclear expression; Supplementary Fig. S2B). In addition, the levels of NF-κB/p65 and p-NF-κB/p65 were considerably higher in SVHUC-AR cells than in AR-negative SVHUC-vector cells (Fig. 2B). Furthermore, AR expression was downregulated in NF-B knockdown cells, compared with SVHUC-AR control cells (Fig. 2C). A quantitative RT-PCR analysis was then performed to confirm the modulation of NF-κB/p65 and AR expression at a mRNA level by NF-κB activator/inhibitor. The expression levels of *NF-κB* (Fig. 2D) and *AR* (Fig. 2E) genes were significantly elevated and reduced when 5 to 10 μmol/L BA and 5 to 10 μmol/L PAR, respectively, were treated in SVHUC-AR cells.

Effects of NF-κB modulators on urothelial tumorigenesis

To assess the effects of NF-κB activators/inhibitors on urothelial tumorigenesis, we first used an *in vitro* transformation model where nonneoplastic SHVUC cells could undergo stepwise transformation upon exposure to a chemical carcinogen MCA (26). MCA-exposed SVHUC cells were treated with NF-κB modulators for 6 weeks during the process of neoplastic transformation. Oncogenic activity was then monitored by cell viability (MTT assay; Fig. 3A; Supplementary Fig. S2C) and colony formation (clonogenic assay; Fig. 3B) without further drug treatment that could directly affect cell proliferation or colony formation. Thus, we compared the degree of neoplastic transformation in urothelial cells exposed to a carcinogen and subsequently cultured with NF-κB modulators but did not intend to simply assess their effects on the growth of transformed cells. In accordance with our previous *in vitro* transformation data (23, 24), AR overexpression resulted in induction of neoplastic transformation of SVHUC cells (i.e., >2-fold increase in both assays), which was prevented by an antiandrogen HF (Fig. 3A and B). Additionally, BA/TNF-α and PAR/DHMEQ could strongly promote and inhibit, respectively, neoplastic transformation of SVHUC-AR cells, but not that of SVHUC cells. NF-B knockdown in SVHUC-AR cells without further NF-B modulator treatment also resulted in significant inhibition of their neoplastic transformation (Supplementary Fig. S2D).

By using a quantitative RT-PCR method, we compared the expression levels of an oncogene, *c-myc*, as well as various molecules that are known to play a protective role in bladder tumorigenesis, such as *p21*, *p53*, and *UDP-glucuronosyltransferase-1A* (*UGT1A*), in urothelial cells undergoing neoplastic transformation. In SVHUC-AR cells with MCA exposure, 6-week BA/PAR treatment resulted in upregulation/downregulation, respectively, of *c-myc* expression (Fig. 3C). Similarly, in these cells, PAR significantly upregulated the expression of *p21*, *p53*, and *UGT1A* (Fig. 3D).

We also utilized a chemical carcinogen BBN known to reliably induce the development of bladder tumor in male rodents to further assess the effects of NF-κB modulators on urothelial carcinogenesis. To detect bladder tumorigenesis at an early stage,

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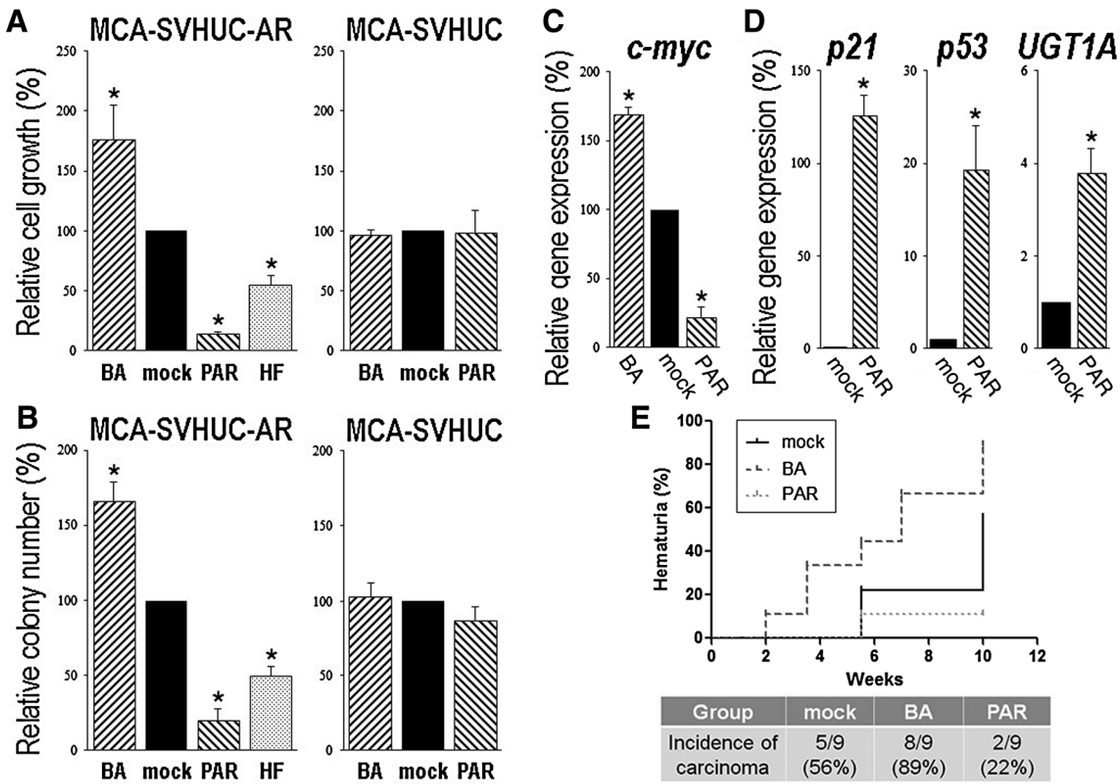

Group	mock	BA	PAR
Incidence of carcinoma	5/9 (56%)	8/9 (89%)	2/9 (22%)

Figure 3.

Effects of NF-κB activator/inhibitor on neoplastic transformation of urothelial cells. SVHUC-AR/SVHUC cells exposed to MCA and subsequently treated with ethanol (mock), BA (10 μmol/L), PAR (10 μmol/L), or HF (1 μmol/L) for 6 weeks were seeded for MTT assay (**A**; cultured for 7 days) or clonogenic assay (**B**; cultured for 2 weeks) without BA/PAR/HF treatment. Cell viability or colony number (≥20 cells) is presented relative to that in mock-treated cells. SVHUC-AR cells exposed to MCA and subsequently treated with ethanol (mock), BA (10 μmol/L), or PAR (10 μmol/L) for 6 weeks were subjected to RNA extraction and real-time RT-PCR. Expression of *c-myc* (**C**), as well as *p21*, *p53*, or *UGT1A* (**D**), was normalized to that of *GAPDH*. Transcription amount is presented relative to that of mock-treated cells. Each value represents the mean (+SD) from three independent experiments. *, $P < 0.05$ (vs. mock treatment). **E,** Male C57BL/6 mice ($n = 9$/group) were treated with BBN in drinking water (for 12 weeks) as well as ethanol (mock; 1/1,000 in 0.2 mL sterile distilled water), BA (10 mg/kg), or PAR (20 mg/kg) via subcutaneous injections 3 times a week, starting at 6 weeks of age. Kaplan–Meier analysis for the detection of hematuria via three consecutive positive urine tests (top). Then, the development of bladder cancer was histologically confirmed (bottom).

hematuria was monitored via urine test strips. There was a significant or marginal difference in the detection of hematuria between mock versus BA ($P = 0.032$) or PAR ($P = 0.066$), respectively (Fig. 3E). Bladder tumors as high-grade urothelial carcinomas were microscopically confirmed in all mice with three consecutive positive urine tests as well as another undergoing PAR treatment without three positive tests by 22 weeks. Thus, a higher/lower incidence of bladder cancer was seen in the BA (89%)/PAR (22%) group, respectively, than in the control group (56%). None of the mice developed metastatic tumors.

Interplay between NF-κB and AR signals in bladder cancer cells

We further investigated potential cross-talk between NF-κB and AR signals in bladder cancer cells. Western blot showed considerably higher levels of NF-κB/p65 and p-NF-κB/p65 expression in AR-positive lines than in AR knockdown (UMUC3-AR-shRNA) or AR-negative (647V-vector) lines (Fig. 4A). Similar to the findings in SVHUC-AR cells, BA and PAR increased and decreased, respectively, the expression of not only NF-κB/p65 and p-NF-κB/p65 but also AR in UMUC3 and 647V-AR cells (Fig. 4B). Similarly, TNF-α induced NF-B/p-NF-B/AR expression in UMUC3 cells (Supplementary Fig. S3A). By contrast, DHMEQ reduced the levels of NF-B/AR as well as nuclear p-NF-B, while inducing cytoplasmic p-NF-B expression (Supplementary Fig. S3A and S3B). Additionally, in UMUC3 cells, NF-B/p65 knockdown resulted in a considerable decrease in AR expression (Fig. 4C). As expected, BA/PAR increased/decreased, respectively, the expression of NF-B downstream genes, including *c-myc*, *cyclin D1*, and *MMP-9* (Supplementary Fig. S3C). AR-mediated transcriptional activity was then determined in the cell extracts with transfection of an ARE luciferase reporter plasmid and subsequent treatment with BA or PAR. BA significantly augmented AR luciferase activity in UMUC3 and 647V-AR cells, whereas PAR significantly reduced it (Fig. 4D). Meanwhile, DHT was able to induce NF-κB transcriptional activity in AR-positive lines, which was restored by HF (Fig. 4E).

Effects of NF-κB modulators on bladder cancer growth

We then assessed the effects of NF-κB activator/inhibitor on cell proliferation, migration, and invasion of AR-positive and AR-negative bladder cancer lines. First, we compared cell viability via

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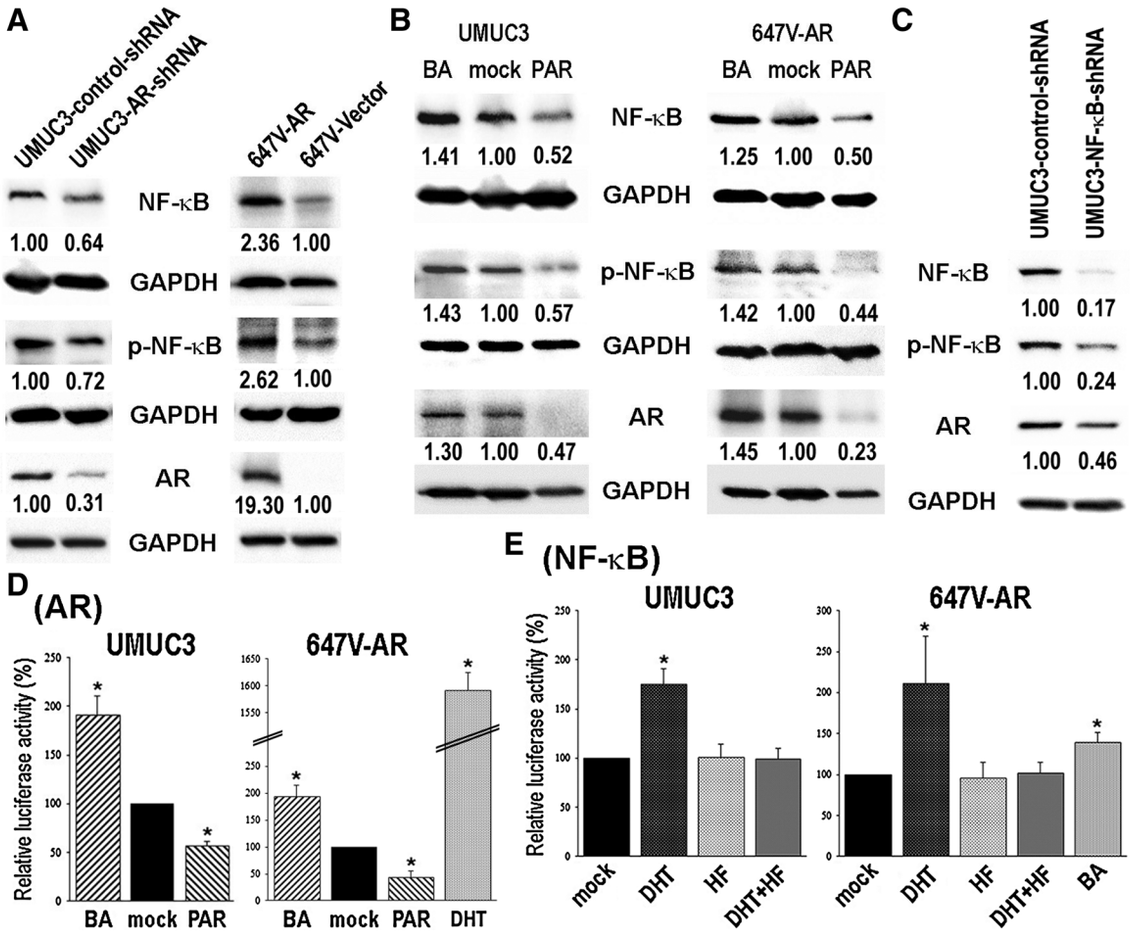

Figure 4.

Effects of AR activation and NF-κB activator/inhibitor on their expression and transcriptional activity in bladder cancer cells. Western blotting of NF-κB/p65, p-NF-κB/p65, and AR, using proteins extracted from UMUC3-control-shRNA vs. UMUC3-AR-shRNA or 647V-AR vs. 647V-vector (**A**), UMUC3/647V-AR cultured with ethanol (mock), BA (10 μmol/L), or PAR (10 μmol/L) for 12 hours (**B**), or UMUC3-control-shRNA vs. UMUC3-NF-κB-shRNA (**C**). GAPDH served as a loading control. Densitometry values for NF-κB/p-NF-κB/AR standardized by GAPDH that are relative to those of control cells or mock treatment are included below the lanes. The luciferase reporter activity of AR (**D**) and NF-κB (**E**) was determined in UMUC3/647V-AR cells cultured with ethanol (mock), BA (10 μmol/L), PAR (10 μmol/L), or DHT (10 nmol/L) in media containing normal FBS and ethanol (mock), DHT (10 nmol/L), HF (5 μmol/L), or BA (10 μmol/L) in media containing charcoal-stripped FBS, respectively, for 24 hours. Luciferase activity is presented relative to that of mock treatment in each line. Each value represents the mean (+SD) from three independent experiments. *, $P < 0.05$ (vs. mock treatment).

MTT assay in those cultured with NF-κB modulators. After 48-hour treatment, BA significantly increased the viability of AR-positive cells, compared with mock treatment, whereas PAR significantly inhibited it (Fig. 5A). By contrast, both BA and PAR did not significantly affect the viability of AR-negative cells. Second, we compared colony formation via clonogenic assay in cells cultured with NF-κB modulators for 2 weeks. Compared with mock treatment, BA/PAR treatment resulted in significant increases/decreases, respectively, in the number of colonies in AR-positive cells, but not in AR-negative cells (Fig. 5B). Third, we performed a scratch wound-healing assay to compare cell migration in those cultured with NF-κB modulators. BA significantly induced wound closure of AR-positive cells 24 hours after wound generation, whereas PAR significantly inhibited it (Fig. 5C). By contrast, BA and PAR did not show significant effects on the migration of AR-negative cells. Fourth, we performed a Transwell invasion assay. Similar to the above findings, BA or PAR treatment demonstrated marked increases or decreases, respectively, in the invasion ability of AR-positive cells, compared with mock treatment, but not in that of AR-negative cells (Fig. 5D).

We repeated MTT and wound-healing assays in an androgen-depleted condition. The effects of BA/PAR on cell viability (Supplementary Fig. S4A) and migration (Supplementary Fig. S4B) were marginal, while they were similar to those cultured with normal FBS when DHT was supplemented (Supplementary Fig. S4C and S4D). Meanwhile, in UMUC3 cells cultured with normal FBS, TNF-α treatment augmented and DHMEQ treatment or NF-B knockdown reduced their viability/migration (Supplementary Fig. S4E–S4H).

Finally, we used mouse xenograft models to assess the effects of NF-κB modulators on bladder tumor outgrowth *in vivo*. UMUC3-control-shRNA/UMUC3-AR-shRNA cells were implanted subcutaneously into the flank of immunocompromised mice, and mock/BA/PAR treatment was commenced

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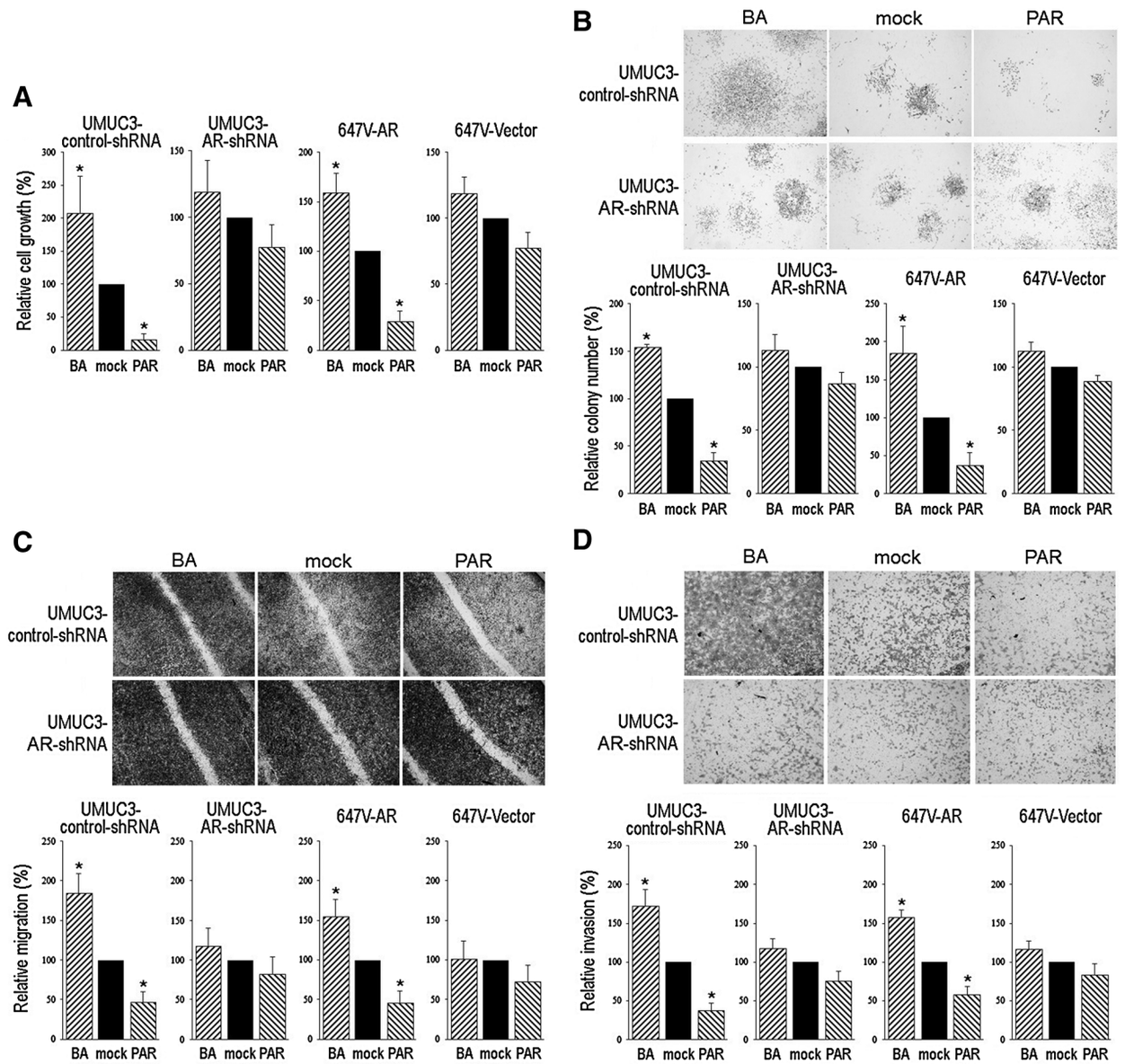

Figure 5.

Effects of NF-κB activator/inhibitor on bladder cancer cell growth. **A,** MTT assay in UMUC3-control-shRNA/AR-shRNA and 647V-AR/vector cells cultured with ethanol (mock), BA (10 μmol/L), or PAR (10 μmol/L) for 48 hours. Cell viability is presented relative to that of each line with mock treatment. **B,** Clonogenic assay in UMUC3-control-shRNA/AR-shRNA and 647V-AR/vector cells cultured with ethanol (mock), BA (10 μmol/L), or PAR (10 μmol/L) for 2 weeks. The number of colonies (consisting of ≥20 cells) is presented relative to that of each line with mock treatment. **C,** Wound-healing assay in UMUC3-control-shRNA/AR-shRNA and 647V-AR/vector cells cultured with ethanol (mock), BA (10 μmol/L), or PAR (10 μmol/L). The cells grown to confluence were gently scratched, and the wound area was measured after 24-hour culture. The migration determined by the rate of cells filling the wound area is presented relative to that of each line with mock treatment. **D,** Transwell invasion assay in UMUC3-control-shRNA/AR-shRNA and 647V-AR/vector cells cultured with ethanol (mock), BA (10 μmol/L), or PAR (10 μmol/L) in the Matrigel-coated Transwell chamber for 16 hours. The number of invaded cells present in the lower chamber was counted under a light microscope (100× objective in five random fields). Cell invasion is presented relative to that of each line with mock treatment. Each value represents the mean (+SD) from three independent experiments. *, $P < 0.05$ (vs. mock treatment).

after tumor was formed. Consistent with our previous observations showing growth inhibition by AR inactivation (17, 20, 21), AR knockdown without drug treatment resulted in striking delay in the formation of xenograft tumors ($P = 0.002$), as well as significant retardation of tumor growth ($P < 0.05$ at days 8–14; Fig. 6A), compared with the AR-positive control. More importantly, BA and PAR significantly induced and reduced, respectively, the growth of inoculated control-shRNA tumors, whereas they failed to significantly affect that of AR-shRNA xenografts. Correspondingly, BA/PAR increased/decreased, respectively, cell proliferation index in AR-positive xenografts determined by Ki-67 IHC (Fig. 6B). In addition, BA and PAR

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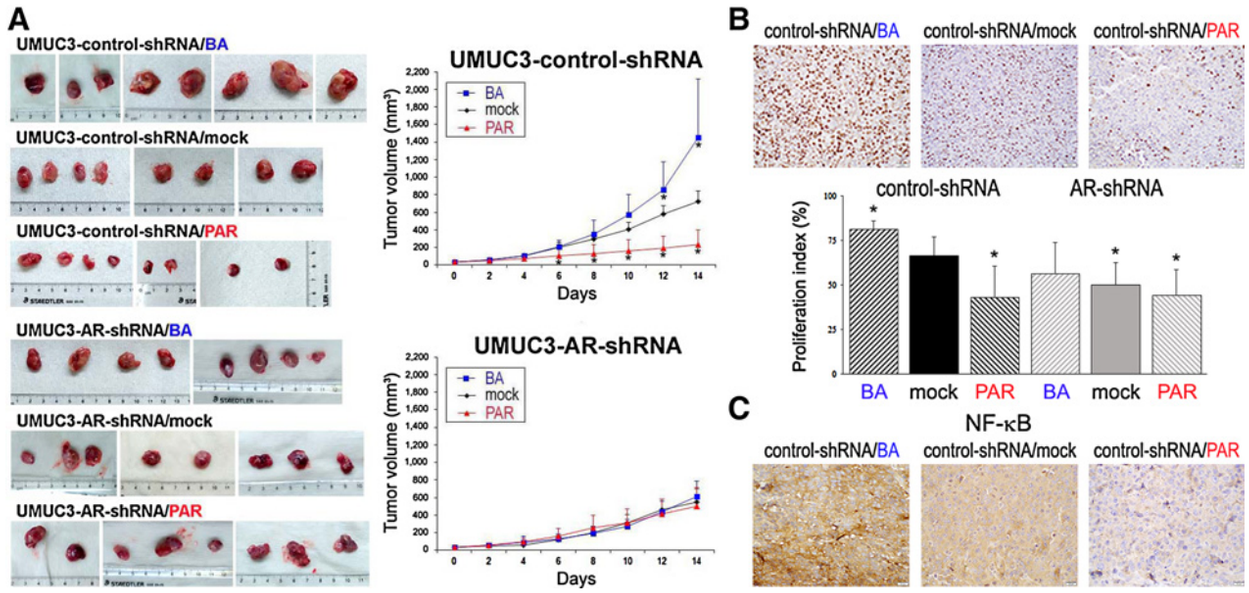

Figure 6.

Effects of NF-κB activator/inhibitor on tumor growth in mouse xenograft models. **A,** UMUC3-control-shRNA/UMUC3-AR-shRNA cells were implanted subcutaneously into the flank of athymic NCr-nu/nu mice. Once estimated volume of each tumor exceeded 30 mm^3 (day 0), the animals ($n = 8$/group) received intraperitoneal injections of DMSO (mock; 1/1000 in 0.1 mL sterile distilled water), BA (20 mg/kg), or PAR (4 mg/kg) every other day. Each value represents the mean (+ SD). *, $P < 0.05$ (vs. mock treatment at each day). **B,** IHC of Ki-67 in xenograft tumors. Each value represents the mean (+SD) of the proliferation index in three tumors/group. *, $P < 0.05$ (vs. mock treatment in UMUC3-control-shRNA). **C,** IHC of NF-κB/p65 in xenograft tumors. Representative images show an increase and a decrease in NF-κB/p65 expression in UMUC3-control-shRNA xenografts by BA and PAR treatments, respectively.

were confirmed to induce and reduce, respectively, NF-B/p65 expression in control-shRNA tumors (Fig. 6C).

Discussion

Several immunohistochemical studies have determined the expression levels of NF-κB in urothelial tumor specimens. In 116 cases of bladder cancer, NF-κB/p65 expression was associated with higher tumor grade/stage and worse overall survival (29). Similarly, nuclear NF-κB/p65 expression was shown to be elevated in higher grade (30) or stage (31) bladder cancers. In addition, we (11) and others (32) previously demonstrated associations between p-NF-κB/p65 expression and resistance to neoadjuvant chemotherapy and between NF-κB/p65 expression and resistance to neoadjuvant chemoradiation, respectively, in patients with muscle-invasive bladder cancer. NF-κB/p65 overexpression was also seen in upper urinary tract urothelial tumors, compared with normal urothelial tissues, which was an independent prognosticator for CSS (33). We confirmed some of these findings and further showed the elevated expression of p-NF-κB/p65 in bladder tumors, compared with nonneoplastic urothelial tissues, as well as high-grade/muscle-invasive tumors, compared with lower grade/non–muscle-invasive tumors. While there was no significant association between p-NF-κB/p65 expression and recurrence/progression of non–muscle-invasive tumors, p-NF-κB/p65 positivity in muscle-invasive tumors, as an independent factor, was found to precisely predict worse patient outcomes. Current immunohistochemical findings in 149 bladder tumors thus support our *in vitro*/*in vivo* data suggesting that activation of NF-κB/p65 signals contributes to urothelial tumorigenesis and tumor progression. Of note, there were positive correlations between the expression levels of NF-κB/p65 versus p-NF-κB/p65 and AR versus NF-κB/p65 in bladder cancer specimens, although the correlation between AR and p-NF-κB/p65 expression was not significant. Meanwhile, in our previous immunohistochemical analysis involving 188 patients, AR expression was downregulated in high-grade and muscle-invasive bladder cancers, yet it was associated with the risk of disease progression of muscle-invasive bladder cancer ($P = 0.07$; ref. 16). Our meta-analysis involving 2,049 patients also demonstrated an association between AR expression and better recurrence-free survival in those with non–muscle-invasive tumor (34). Thus, there appear to be some discrepancies between our preclinical findings in cell line/animal models and current/previous IHC data in bladder cancer specimens. These seen in surgical tissues include: (i) downregulation of AR expression in high-grade/muscle-invasive tumors where at least p-NF-κB/p65 expression is likely upregulated; (ii) no significant associations between NF-κB/p65 expression and tumor grade/stage or prognosis; and (iii) no significant associations between the levels of p-NF-κB/p65 and AR expression, whereas NF-κB/p-NF-κB and AR signals are correlated each other in preclinical models and cooperatively promote tumor growth. Accordingly, it remains unclear if phosphorylation of NF-κB/p65, as a measure of its activation and an independent prognosticator for bladder cancer, is induced by AR-mediated signals in tissue specimens. A potential reason for some of these discrepancies may include the influence of molecule(s)/factor(s), which are unrelated to AR signals yet induce or prevent nuclear translocation of p-NF-κB, derived from noncancer cells that are not present in cell line

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models. Further immunohistochemical studies for NF-κB/p-NF-κB/AR are required to validate or revise the conflicting results and ultimately to establish a working model for cross-talk between NF-κB and AR signals in bladder cancer cells.

NF-κB is well known to play a critical role in inflammation (3). Meanwhile, prolonged chronic inflammation promotes tumorigenesis via several mechanisms, such as induction of gene mutations or epigenetic alterations and the modulation of cell responses in the tissue microenvironment. Other functions of NF-κB, without the involvement of inflammation, are also thought to associate with the promotion of tumorigenesis. These include stimulation of cell proliferation, prevention of apoptosis, regulation of angiogenesis, and immortalization via regulating the expression/function of oncogenes, antiapoptotic genes, angiogenic factors, and telomerases, respectively (35). In urothelial cancer, associations between polymorphisms in the *NF-κB1/p50* gene, which could form a heterodimer with p65 as the NF-κB activator, and the risk of tumor development or recurrence have been reported (36, 37). In SVHUC cells, an extract of cigarette smoke, a well-known bladder carcinogen, was also shown to increase p65 and p50 expression (38). We here demonstrated further evidence, using two preclinical models, indicating that NF-κB/p65 could promote urothelial tumorigenesis in the presence of AR. Nonetheless, it has been documented that NF-κB also functions as a tumor suppressor in several specific cases (35). For instance, induction of p53 resulted in increases in DNA-binding activity of NF-κB and apoptosis in an osteosarcoma cell line (39). It still needs to be determined how NF-κB signals, especially p65 activation in the classical NF-κB pathway, regulate urothelial cancer initiation.

Activation of the NF-κB pathway is also known to correlate with induction of cell proliferation/migration/invasion and epithelial-to-mesenchymal transition (EMT; ref. 35). Using bladder cancer lines, inactivation of NF-κB/p65 via treatment with specific inhibitors or transfection of small-interfering RNAs has been shown to inhibit cell proliferation and/or induce apoptosis (31, 40–42). Overexpression and silencing of NF-κB/p65 in bladder cancer cells also resulted in induction and inhibition, respectively, of EMT (43). Additionally, in mouse xenograft models, NF-κB inhibition was associated with retardation of tumor growth (40, 41, 44). We here demonstrated that NF-κB activators and inhibitors/knockdown significantly induced and reduced, respectively, cell proliferation, migration, and invasion, as well as the growth of inoculated xenografts in mice, of AR-positive bladder cancer lines.

More importantly, functional interplay between NF-κB/p65 and AR signals, as documented in prostate cancer cells (7–9), was confirmed in urothelial cells. In particular, AR knockdown or overexpression in bladder cancer cells resulted in similar changes in the expression of NF-κB/p65 (36% decrease or 2.36-fold increase) versus p-NF-κB/p65 (28% decrease or 2.62-fold increase), respectively (Fig. 4A). This implies that AR signals upregulate the expression (and transcriptional activity) of NF-κB but may not directly modulate its phosphorylation in urothelial cells. Additionally, in contrast to some of previous observations (31, 41) in AR-negative bladder cancer lines (e.g., 5637, HT1197, ref. 17), NF-κB modulators significantly regulated neoplastic transformation of urothelial cells, as well as cancer cell growth, only in the presence of AR. Thus, activated AR appears to be required for the modulation of urothelial tumorigenesis and tumor progression by NF-κB. Indeed, a previous study showed that an antimitotic drug 2-methoxyestradiol increased NF-κB/p65 activity in AR-positive prostate cancer LNCaP cells, but not in AR-negative DU145 or PC3 cells (45). Meanwhile, it should be determined whether androgen supplement in androgen-depleted conditions could rescue the effects of NF-κB modulators on xenograft tumor growth in animals.

It must be acknowledged that the pharmacologic activators/inhibitors we used could show "off-target" effects on various nonNF-B signaling pathways. Specifically, BA has been shown to increase DNA-binding activity of NF-κB as well as its nuclear translocation and thereby enhance NF-κB transcriptional activity (46). However, BA could induce apoptosis in neuroblastoma cells where NF-κB cannot be phosphorylated (46), suggesting the involvement of non–NF-κB mechanisms. PAR has been shown to inhibit NF-κB activity, presumably via specifically modulating degradation of its inhibitory proteins, such as IκBα and IκBβ (47). In nonneoplastic urothelial cells, PAR was also shown to inhibit nuclear translocation of NF-κB and phosphorylation of IκBα (48). However, PAR could have an impact on the activity of ERK and AP-1 (49). We therefore used additional NF-κB activator (TNF-α) and inhibitor (DHMEQ), as well as NF-κB/p65 knockdown, to validate the results with BA/PAR. Nonetheless, the effects of PAR or DHMEQ on neoplastic transformation of urothelial cells and viability/migration of bladder cancer cells were stronger than those of NF-κB/p65 knockdown, suggesting the involvement of additional cellular targets by these NF-κB inhibitors. Furthermore, there may be yet undiscovered effects of these NF-B modulators on intracellular signaling.

In conclusion, NF-κB/p65 appeared to be activated in bladder cancer, which was associated with tumor progression. In particular, p-NF-κB/p65 expression in muscle-invasive bladder cancers may serve as a reliable prognosticator. NF-κB modulators were also found to involve the regulation of urothelial tumorigenesis as well as tumor outgrowth in the presence of activated AR. Accordingly, NF-κB inhibition, together with AR inactivation especially in AR-positive cases, has the potential of being an effective chemopreventive and/or therapeutic approach for urothelial carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Inoue, H. Ide, H. Miyamoto
Development of methodology: S. Inoue, H. Ide
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Inoue, H. Ide, T. Mizushima, G. Jiang, G.J. Netto
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Inoue, H. Ide, T. Mizushima, G. Jiang, H. Miyamoto
Writing, review, and/or revision of the manuscript: S. Inoue, H. Ide, H. Miyamoto
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G.J. Netto, H. Miyamoto
Study supervision: M. Gotoh, H. Miyamoto

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Molecular Cancer Therapeutics

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Satoshi Inoue, Hiroki Ide, Taichi Mizushima, et al.

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