

Original Article

ELK1 promotes urothelial tumorigenesis in the presence of an activated androgen receptor

Satoshi Inoue^{1,2,4,5}, Hiroki Ide^{4,5}, Taichi Mizushima^{1,2,4,5}, Guiyang Jiang^{1,2}, Takashi Kawahara^{1,4,5}, Hiroshi Miyamoto^{1,2,3,4,5}

¹Department of Pathology and Laboratory Medicine, ²James P. Wilmot Cancer Institute, ³Department of Urology, University of Rochester Medical Center, Rochester, NY, USA; ⁴Department of Pathology, ⁵James Buchanan Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Received August 6, 2018; Accepted August 14, 2018; Epub November 1, 2018; Published November 15, 2018

Abstract: We have recently demonstrated that ELK1, a transcription factor that triggers downstream targets including *c-Fos* proto-oncogene, promotes the growth of bladder cancer cells possessing a functional androgen receptor (AR). We here assessed the function of ELK1, as well as the efficacy of a selective α 1A-adrenergic blocker silodosin that has been shown to inhibit ELK1 activity in bladder cancer cells, in urothelial tumorigenesis. The level of ELK1 expression in an immortalized normal urothelial cell line SVHUC stably expressing wild-type AR (SVHUC-AR) was considerably higher than that in AR-negative SVHUC-vector cells, which was induced further or reduced by dihydrotestosterone or silodosin treatment, respectively. In SVHUC-AR cells exposed to a chemical carcinogen 3-methylcholanthrene, silodosin significantly reduced the expression levels of oncogenes (e.g. *c-Fos*, *Jun*, *Myc*), as well as phospho-p38 MAPK and phospho-ERK proteins, and increased those of tumor suppressor genes (e.g. *p53*, *PTEN*, *UGT1A*). ELK1 suppression via ELK1-short hairpin RNA virus infection or silodosin treatment also resulted in significant inhibition in 3-methylcholanthrene-induced neoplastic transformation of SVHUC-AR cells, but not that of SVHUC-vector cells. In *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-treated male C57BL/6 mice, the incidence rate of bladder tumors was significantly ($P = 0.007$) lower in the silodosin group than in the control group. ELK1 thus appears to play a critical role in urothelial tumorigenesis, and silodosin prevents it presumably via down-regulation of ELK1. Moreover, ELK1 may require an activated AR for inducing neoplastic transformation of urothelial cells. Our findings may therefore offer a novel chemopreventive approach, via ELK1 inactivation using silodosin treatment, for bladder cancer.

Keywords: Androgen receptor, ELK1, malignant transformation, silodosin, tumorigenesis, urothelial cancer

Introduction

Urinary bladder cancer, generally urothelial carcinoma, has been one of most frequently diagnosed malignancies predominantly affecting males throughout the world [1]. Non-muscle-invasive bladder tumors account for approximately three-fourth of all newly diagnosed cases and are typically managed with a conservative approach. However, patients with non-muscle-invasive bladder tumor following transurethral resection and currently available intravesical pharmacotherapy still carry a life-long risk of tumor recurrence occasionally with more invasive disease for which aggressive treatment modalities, such as radical cystectomy with or without neoadjuvant/adjuvant che-

motherapy, are often required. Consequently, identifying key molecules/pathways responsible for urothelial tumorigenesis may offer novel targeted therapy that more effectively prevents the recurrence of superficial bladder cancer.

Emerging preclinical evidence indicates an essential role of androgen-mediated androgen receptor (AR) signaling in the development and progression of bladder cancer, while precise mechanisms for the functions of AR and related signals in urothelial cells remain poorly understood (reviewed in [2]). We have demonstrated that androgens induce the expression and activity of ELK1, a transcription factor whose phosphorylation via the MAPK/ERK pathways is known to lead to activation of downstream tar-

gets, such as a proto-oncogene *c-Fos* [3, 4], in AR-positive bladder cancer cells [5]. In prostate cancer cells, AR was also found to function as a co-activator of ELK1 [6]. We have additionally demonstrated that silodosin, a selective α 1A-adrenergic receptor antagonist that has been prescribed for urinary symptomatic relief from benign prostatic hyperplasia [7] and has also been shown to reduce the expression of ELK1 in human prostate smooth muscle cells [8] as well as that of *c-Fos* in the rat spinal cord [9], inhibits ELK1 activity in bladder [10] and prostate [11] cancer cells.

By contrast, little is known about the role of ELK1 signaling in urothelial tumorigenesis. In the current study, we aimed to determine it, in relation to AR signaling, and found that ELK1 knockdown or silodosin treatment resulted in inhibition of neoplastic transformation of AR-positive urothelial cells, but not AR-negative cells.

Materials and methods

Antibodies and chemicals

We purchased anti-ELK1 (I-20), anti-AR (N-20), and anti-GAPDH (6c5) antibodies, and anti-phospho-p38 MAPK (Thr180/Tyr182) (p-p38) and anti-phospho-p44/42 MAPK (Thr202/Tyr204) (p-ERK) antibodies from Santa Cruz Biotechnology and Cell Signaling Technology, respectively. We obtained dihydrotestosterone (DHT) and hydroxyflutamide (HF) from Sigma-Aldrich. Silodosin was from Cayman Chemical.

Cell lines

An immortalized human normal urothelial cell line (SVHUC) was originally obtained from the American Type Culture Collection and recently authenticated, using GenePrint 10 System (Promega), by the institutional core facility. Stable sublines, including SVHUC-vector and SVHUC-AR expressing a full-length wild-type human AR, were established in our previous studies [12-14]. Similarly, a short hairpin RNA (shRNA) plasmid targeting human ELK1 (sc-35290-SH, Santa Cruz Biotechnology) or a non-silencing control shRNA plasmid (sc-108060, Santa Cruz Biotechnology) was stably expressed in SVHUC-vector or SVHUC-AR cells, as we described previously [5, 10, 11]. All these parental cells and stable sublines were main-

tained in Ham's F-12K (Kaighn's) medium (Mediatech) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ at 37°C and routinely tested for Mycoplasma contamination, using PCR Mycoplasma Detection Kit (Applied Biological Materials). Cells were then cultured in phenol red-free medium supplemented with either 5% regular FBS or 5% charcoal-stripped FBS (for DHT treatment) at least 24 hours before experimental treatment.

In vitro transformation

An *in vitro* neoplastic/malignant transformation system was employed, using SVHUC line upon exposure to a carcinogen 3-methylcholanthrene (MCA), as established in a previous study [15], with minor modifications. In brief, cells (2×10^6 /10-cm culture dish incubated for 24 hours) were cultured in FBS-free F-12K containing 5 μ g/ml MCA (Sigma-Aldrich). After the first 24 hours of MCA exposure, 1% FBS was added to the medium. After additional 24 hours of MCA exposure, the cells were cultured in medium containing 5% FBS (without MCA) until near confluence. Subcultured cells (1:3 split ratio) were again incubated with MCA for two 48-hour exposure periods, using the above protocol. These MCA-exposed cells were subcultured for 6 weeks in the presence or absence of silodosin (without MCA) and then utilized for subsequent assays.

Cell proliferation assay

We used the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay to assess cell viability. Cells (500-1000/well) seeded in 96-well tissue culture plates were cultured for up to 96 hours, and then incubated with 0.5 mg/mL of MTT (Sigma-Aldrich) in 100 μ L 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 (500/well) seeded in 12-well tissue culture plates were allowed to grow until colonies in the control well were certainly detectable. The cells were then fixed with methanol and stained with 0.1% crystal violet. The number of colonies in photographed pictures was qu-

ELK1 and urothelial tumorigenesis

Table 1. Sequences of PCR primers

Gene	Sense	Anti-sense
<i>ELK1</i>	5'-CAGCCAGAGGTGTCTGTACC-3'	5'-GAGCGCATGTACTCGTTCC-3'
<i>c-Fos</i>	5'-CGAGATGGAGATCGGTATGGT-3'	5'-GGGTCTTCTTACCCGGCTTG-3'
<i>JUN</i>	5'-TGTACCGACTGAGAGTTCTTGA-3'	5'-ACAGAGCGAGTGAAAATGTGTAT-3'
<i>Myc</i>	5'-ACCAGATCCCGGAGTTGGAA-3'	5'-CGTCGTTTCCGCAACAAGTC-3'
<i>P53</i>	5'-GGAGGGGCGATAAATACC-3'	5'-AACTGTAACCTCTCAGGCAGGC-3'
<i>PTEN</i>	5'-GTTTACCGGCAGCATCAAAT-3'	5'-CCCCACTTTAGTGCACAGT-3'
<i>UGT1A</i>	5'-TGGGTGGAGTTTGTGATGAGGC-3'	5'-CAATGGTCTTGGATTGTGGG-3'
<i>GAPDH</i>	5'-AAGGTGAAGTCCGGAGTCAAC-3'	5'-GGGTTCATTGATGGCAACAATA-3'

antitated, using ImageJ software (National Institutes of Health).

Reverse transcription (RT) and real-time polymerase chain reaction (PCR)

Total RNA isolated from cultured cells by TRIzol (Invitrogen) was subject to RT, using oligo-dT primers and Ominiscript reverse transcriptase (Qiagen). Real-time PCR was then conducted, using RT2 SYBR Green FAST Mastermix (Qiagen). The primer sequences are given in **Table 1**.

Western blot

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 an appropriate dilution of each specific antibody and then a secondary antibody (anti-mouse IgG HRP-linked antibody or anti-rabbit IgG HRP-linked antibody; Cell Signaling Technology), which was followed by scanning with an imaging system (ChemiDOC™ MP, Bio-Rad).

Reporter gene assay

Cells at a density of 50-70% confluence in 24-well tissue culture plates were co-transfected with 250 ng of pELK-Luc reporter plasmid DNA (LR-2061, Signosis) and 2.5 ng of a control reporter plasmid (pRL-CMV), using Lipofectamine® 3000 transfection reagent (Life Technologies). After transfection, the cells were cultured in the presence or absence of silodosin for 24 hours. Cell lysates were then assayed for luciferase activity measured using a Dual-Luciferase Reporter Assay kit (Promega).

Mouse models

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. Six-week-old male NOD-SCID mice and 5-week-

old male C57BL/6 mice were obtained from Johns Hopkins University Research Animal Resources.

SVHUC-derived cells (1×10^6) were suspended, mixed with 100 µL Matrigel (BD Biosciences), and subcutaneously injected into the flank of the NOD-SCID mice, as we described previously [5, 14]. Tumor formation was monitored every other day.

The C57BL/6 mice at age of 6 weeks were supplied ad libitum with tap water containing 0.1% *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) (Sigma-Aldrich) for 12 weeks and thereafter with tap water without BBN, as we described previously [16, 17]. These mice also received daily subcutaneous injections of vehicle (1/1000 ethanol in 0.2 mL sterile distilled water) or silodosin (40 mg/Kg). At 27 weeks of their age, all the animals were euthanized for macroscopic and microscopic analyses of the bladder and other major organs.

Statistical analysis

Fisher's exact test and Student's *t*-test were used to assess statistical significance for categorized variables and those with ordered distribution, respectively. The rates of tumor development in animals were calculated by the Kaplan-Meier method, and comparison was made by log-rank test. *P* values less than 0.05 were considered statistically significant.

Results

Expression of *ELK1* in AR-positive versus AR-negative urothelial cells

We first compared the expression levels of *ELK1* in normal urothelial cells with versus with-

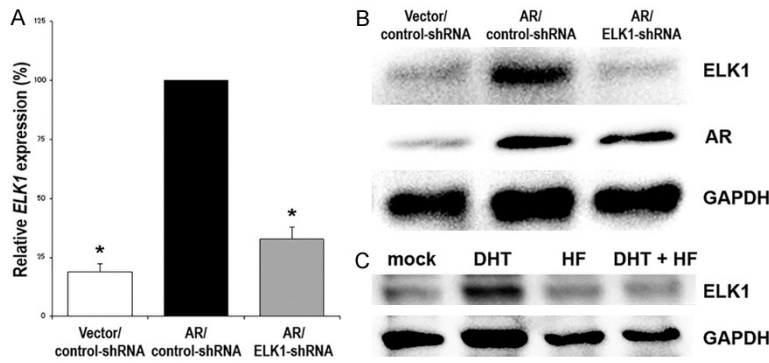


Figure 1. Relationship between ELK1 and AR expression in urothelial cells. A. Quantitative real-time RT-PCR for *ELK1* in SVHUC-vector/control-shRNA, SVHUC-AR/control-shRNA, and SVHUC-AR/ELK1-shRNA cells. A house-keeping gene *GAPDH* was used to normalize *ELK1* gene expression that is presented relative to that in SVHUC-AR/control-shRNA (mean + standard deviation of three independent experiments). * $P < 0.05$ (vs. SVHUC-AR/control-shRNA). B. Western blot analysis of ELK1 and AR, using protein extracts from SVHUC-vector/control-shRNA, SVHUC-AR/control-shRNA, and SVHUC-AR/ELK1-shRNA. C. Western blot analysis of ELK1, using protein extracts from SVHUC-AR cells treated with ethanol (mock), DHT (10 nM), and/or HF (5 μ M) for 24 hours.

out AR overexpression. A quantitative RT-PCR (Figure 1A) and western blotting (Figure 1B) showed considerable up-regulation of ELK1 expression in SVHUC-AR cells, compared with AR-negative control SVHUC cells. To investigate the functional role of ELK1 in urothelial tumorigenesis, an ELK1-shRNA was stably expressed in SVHUC-AR cells. As expected, the levels of ELK1 mRNA (Figure 1A) and protein (Figure 1B) were substantially lower in SVHUC-AR/ELK1-shRNA than in SVHUC-AR/control-shRNA. However, AR protein expression was only marginally down-regulated in SVHUC-AR/ELK1-shRNA, compared with SVHUC-AR/control-shRNA.

We then assessed the effect of androgen on ELK1 expression in normal urothelial cells. As we demonstrated in bladder cancer cells [5], DHT treatment increased the expression of ELK1 in SVHUC-AR cells, which was antagonized by an anti-androgen HF (Figure 1C).

Suppression of neoplastic/malignant transformation of urothelial cells by ELK1 knockdown

To determine the impact of ELK1 expression on urothelial tumorigenesis, we made use of an *in vitro* transformation model. Following exposure to chemical carcinogens, such as MCA, non-neoplastic SVHUC cells could undergo stepwise transformation during subsequent 6-week

culture [15]. The degree of neoplastic transformation in SVHUC-vector/control-shRNA, SVHUC-AR/control-shRNA, and SVHUC-AR/ELK1-shRNA cells with the carcinogen challenge was then assessed by cell viability (via MTT assay; Figure 2A) and colony formation (via clonogenic assay; Figure 2B). In accordance with our previous observations [12, 13], AR overexpression induced neoplastic transformation of SVHUC cells. ELK1 knockdown in SVHUC-AR cells demonstrated significant inhibition of their neoplastic transformation to the levels similar to those in SVHUC-vector/control-shRNA cells.

By using a quantitative RT-PCR method, we compared the expression levels of oncogenic molecules, including *c-Fos*, *Jun*, and *Myc* (Figure 2C), as well as other molecules having suppressive functions in bladder tumorigenesis, such as *p53*, *PTEN*, and *UGT1A* (Figure 2D), in SVHUC-derived sublines with the carcinogen challenge. Consistent with data on cell viability and colony formation, AR overexpression resulted in considerable increases and decreases in the expression levels of oncogenes and tumor suppressors, respectively. Similarly, ELK1 knockdown in SVHUC-AR demonstrated considerable decreases and increases in their expression levels, respectively.

Inactivation of ELK1 by silodosin in non-neoplastic urothelial cells

We assessed the efficacy of silodosin in ELK1 expression, using a quantitative PCR and western blotting, in SVHUC-derived sublines. Silodosin at 1-10 μ M significantly reduced the levels of *ELK1* gene expression in SVHUC-AR/control-shRNA cells, but not in SVHUC-vector/control-shRNA and SVHUC-AR/ELK1-shRNA cells (Figure 3A). Similarly, silodosin diminished ELK1 protein expression in SVHUC-AR cells (Figure 3B). We also determined ELK1-mediated transcriptional activity in the extracts of cells transfected with an ELK1 luciferase reporter plasmid and subsequently treated

ELK1 and urothelial tumorigenesis

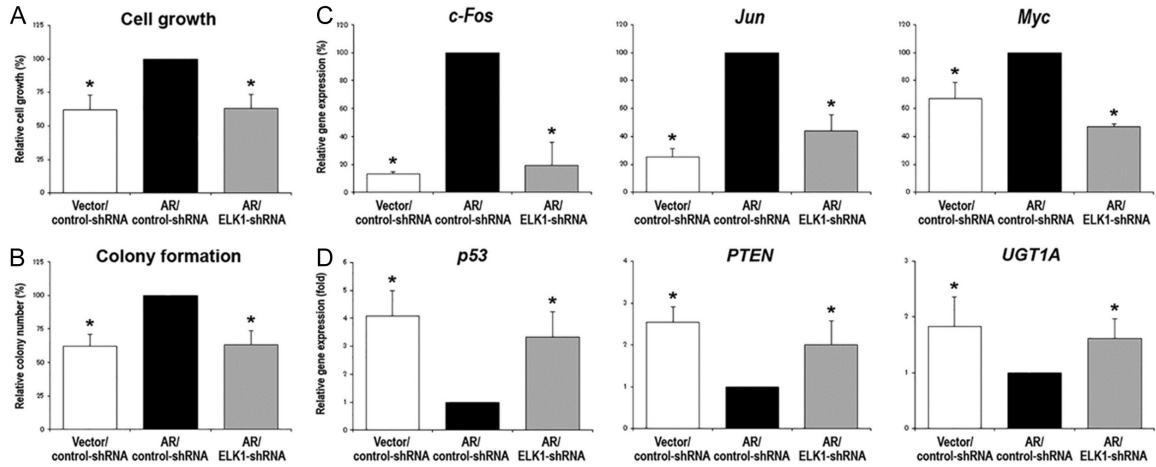


Figure 2. Effects of ELK1 knockdown on neoplastic transformation of urothelial cells. SVHUC-vector/control-shRNA, SVHUC-AR/control-shRNA, and SVHUC-AR/ELK1-shRNA cells first exposed to MCA and subsequently cultured for 6 weeks were seeded for MTT assay (A; additional 96-hour culture) or clonogenic assay (B; additional 2-week culture). The cell viability or colony number (≥ 20 cells) is presented relative to that in SVHUC-AR/control-shRNA (mean + standard deviation of three independent experiments). SVHUC-vector/control-shRNA, SVHUC-AR/control-shRNA, and SVHUC-AR/ELK1-shRNA cells with MCA expose and subsequent 6-week culture were subjected to RNA extraction, RT, and real-time PCR. *GAPDH* was used to normalize the expression of *c-Fos*, *Jun*, or *Myc* (C), as well as *p53*, *PTEN*, or *UGT1A* (D), which is presented relative to that in SVHUC-AR/control-shRNA (mean + standard deviation of three independent experiments). * $P < 0.05$ (vs. SVHUC-AR/control-shRNA).

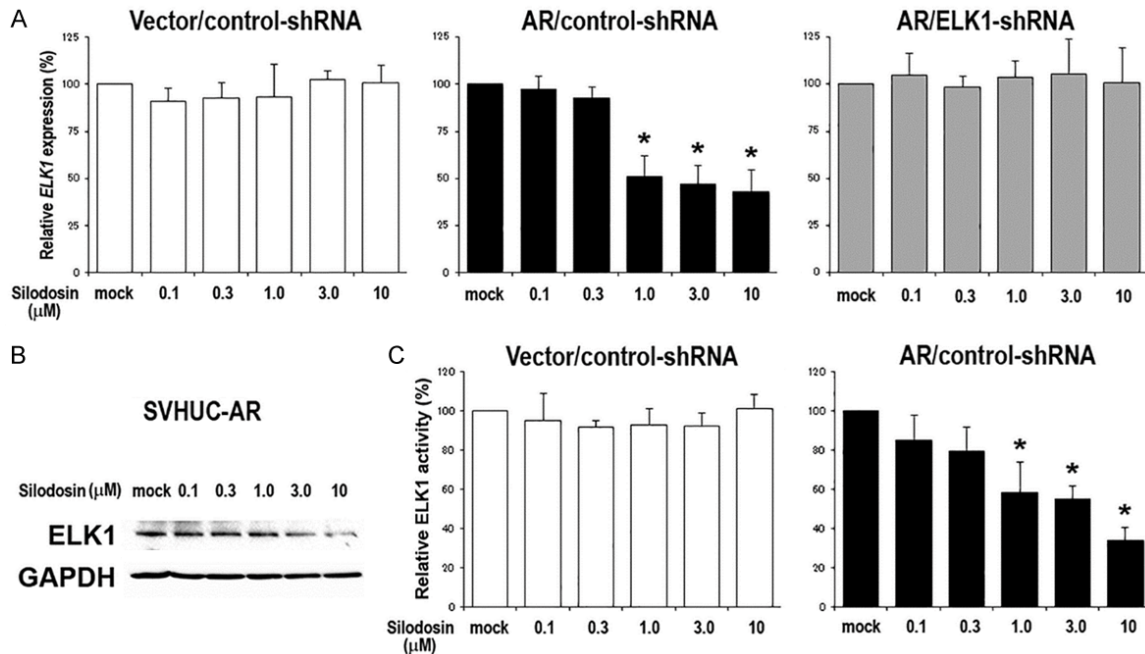


Figure 3. Effects of silodosin on the expression and transcriptional activity of ELK1 in urothelial cells. A. Quantitative real-time RT-PCR for *ELK1* in SVHUC-vector/control-shRNA, SVHUC-AR/control-shRNA, and SVHUC-AR/ELK1-shRNA cells treated with 0-10 μM silodosin for 24 hours. *GAPDH* was used to normalize the expression of each gene that is presented relative to that of mock-treated cells in each subline (mean + standard deviation of three independent experiments). * $P < 0.05$ (vs. mock treatment). B. Western blot analysis of ELK1, using protein extracts from SVHUC-AR cells cultured with 0-10 μM silodosin for 24 hours. C. The luciferase reporter activity of ELK1 was determined in SVHUC-vector and SVHUC-AR cells cultured with 0-10 μM silodosin for 24 hours. The luciferase activity is presented relative to that of mock treatment in each subline (mean + standard deviation of three independent experiments). * $P < 0.05$ (vs. mock treatment).

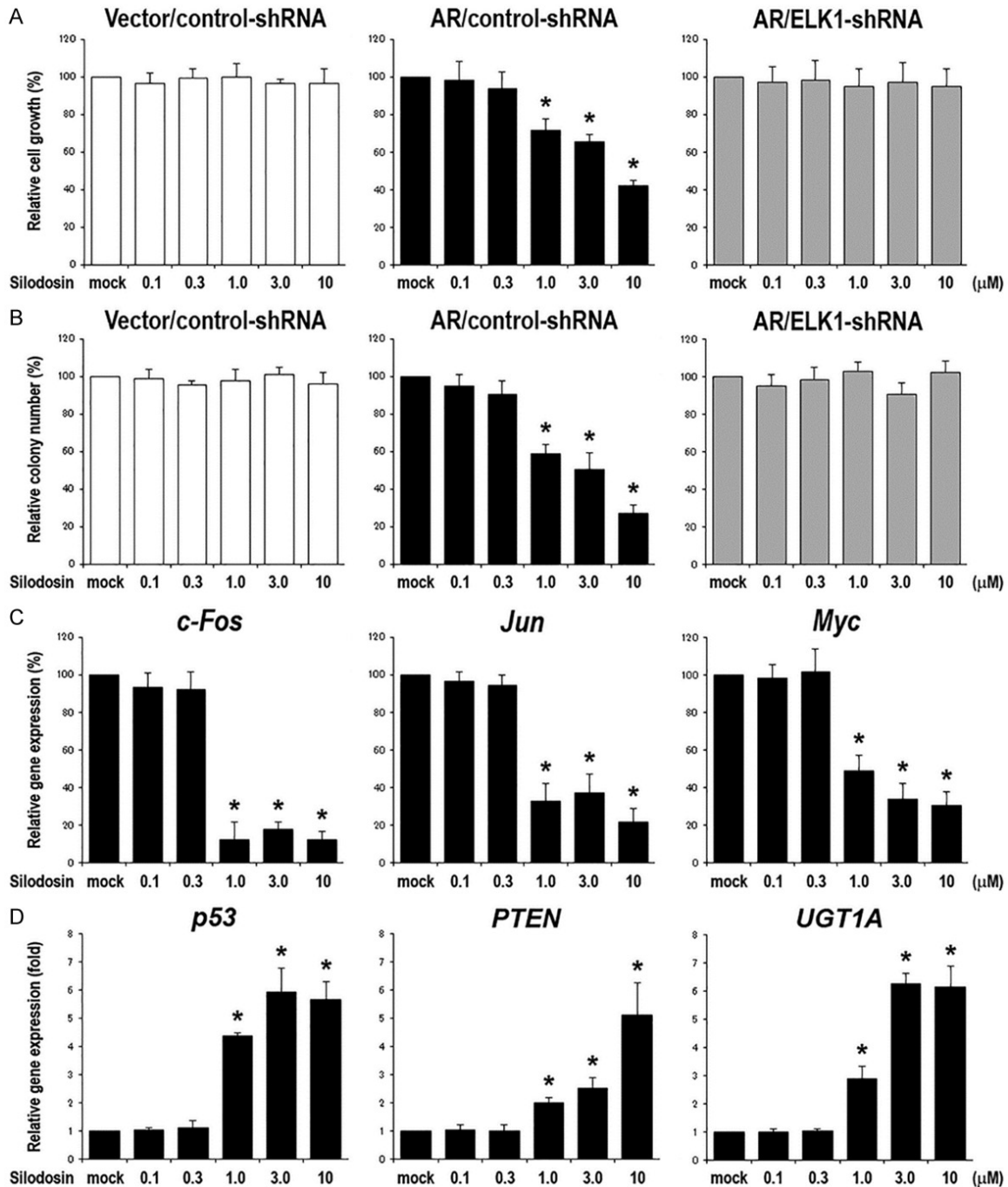


Figure 4. Effects of silodosin on neoplastic transformation of urothelial cells. SVHUC-vector/control-shRNA, SVHUC-AR/control-shRNA, and SVHUC-AR/ELK1-shRNA cells first exposed to MCA and subsequently cultured with 0-10 μM silodosin for 6 weeks were seeded for MTT assay (A; additional 96-hour culture) or clonogenic assay (B; additional 2-week culture) without silodosin treatment during actual assays. The viability or colony number (≥ 20 cells) is presented relative to that of mock-treated cells in each subline (mean + standard deviation of three independent experiments). SVHUC-AR/control-shRNA cells with MCA expose and subsequent 6-week culture were subjected to RNA extraction, RT, and real-time PCR. *GAPDH* was used to normalize the expression of *c-Fos*, *Jun*, or *Myc* (C), as well as *p53*, *PTEN*, or *UGT1A* (D), which is presented relative to that in mock-treated cells (mean + standard deviation of three independent experiments). **P* < 0.05 (vs. mock treatment).

with silodosin. Compared with mock treatment, silodosin significantly reduced the luciferase

activity of ELK1 in SVHUC-AR cells, but not in AR-negative control SVHUC cells (Figure 3C).

ELK1 and urothelial tumorigenesis

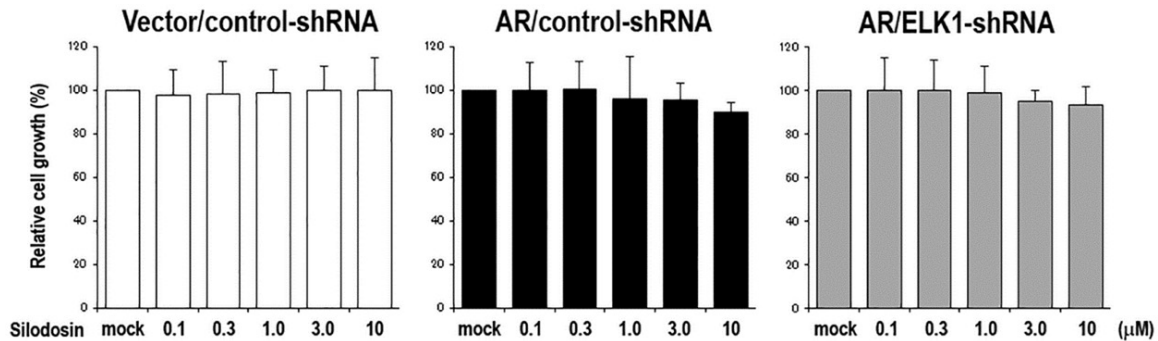


Figure 5. Effects of silodosin on the growth of urothelial cells. MTT assay in SVHUC-vector/control-shRNA, SVHUC-AR/control-shRNA, and SVHUC-AR/ELK1-shRNA cells cultured with 0-10 μ M silodosin. Cell viability is presented relative to that of each subline with mock treatment (mean + standard deviation of three independent experiments).

Suppression of neoplastic/malignant transformation of urothelial cells by silodosin

We next assessed the efficacy of long-term treatment with silodosin in urothelial tumorigenesis. SVHUC-derived sublines exposed to MCA were cultured in the presence of various concentrations of silodosin for 6 weeks during the process of neoplastic transformation. Oncogenic activity in transformed cells was again monitored by cell viability (**Figure 4A**) and colony formation (**Figure 4B**) with no further drug treatment that could directly have an impact on cell proliferation or colony formation, although short-term (96 hours) treatment with silodosin did not significantly inhibit the growth of SVHUC-vector/control-shRNA, SVHUC-AR/control-shRNA, and SVHUC-AR/ELK1-shRNA cells (**Figure 5**). Thus, we compared the progress of carcinogen-induced neoplastic transformation in urothelial cells, which might be inhibited by silodosin treatment, but did not intend to simply assess the effects of silodosin on the growth of transformed cells. Similar to *in vitro* transformation data in MCA-exposed SVHUC sublines with or without ELK1 knockdown described above, silodosin treatment at 1-10 μ M resulted in significant inhibition of neoplastic transformation of SVHUC-AR/control-shRNA cells in both assays, but not that of SVHUC-vector/control-shRNA or SVHUC-AR/ELK1-shRNA cells.

A quantitative RT-PCR method was again performed to compare the expression levels of oncogenes and tumor suppressors in urothelial cells undergoing malignant transformation in the presence of various concentrations of silodosin during its process. In SVHUC-AR/control-

shRNA cells with MCA exposure, 6-week silodosin treatment (1-10 μ M) resulted in down- or up-regulation of the expression of *c-Fos/Jun/My*c (**Figure 4C**) or *p53/PTEN/UGT1A* (**Figure 4D**), respectively. By contrast, silodosin even at 10 μ M did not significantly alter the expression levels of these 6 genes in SVHUC-vector/control-shRNA (**Figure 6A** and **6B**) and SVHUC-AR/ELK1-shRNA (**Figure 6C** and **6D**).

The inhibitory effects of ELK1 knockdown and silodosin treatment on urothelial tumorigenesis were confirmed in mouse xenograft models. SVHUC-AR/control-shRNA or SVHUC-AR/ELK1-shRNA cells with the carcinogen challenge and subsequent 6-week culture with or without silodosin were inoculated subcutaneously into immunocompromised mice that received no additional treatment, and tumor formation was monitored as an endpoint. Compared with control-shRNA/mock, silodosin treatment ($P = 0.002$), ELK1 knockdown ($P < 0.001$), or both ($P < 0.001$) remarkably delayed or even prevented the development of SVHUC-AR xenograft tumors (**Figure 7**). There was no statistically significant difference in tumor formation between control-shRNA/silodosin vs. ELK1-shRNA/mock ($P = 0.596$), control-shRNA/silodosin vs. ELK1-shRNA/silodosin ($P = 0.922$), or ELK1-shRNA/mock vs. ELK1-shRNA/silodosin ($P = 0.634$).

Expression of upstream proteins of ELK1 in urothelial cells treated with silodosin

To determine if silodosin treatment has an impact on the expression of upstream proteins of ELK1, western blotting for p-p38 and p-ERK was performed in SVHUC-AR sublines. In SV-

ELK1 and urothelial tumorigenesis

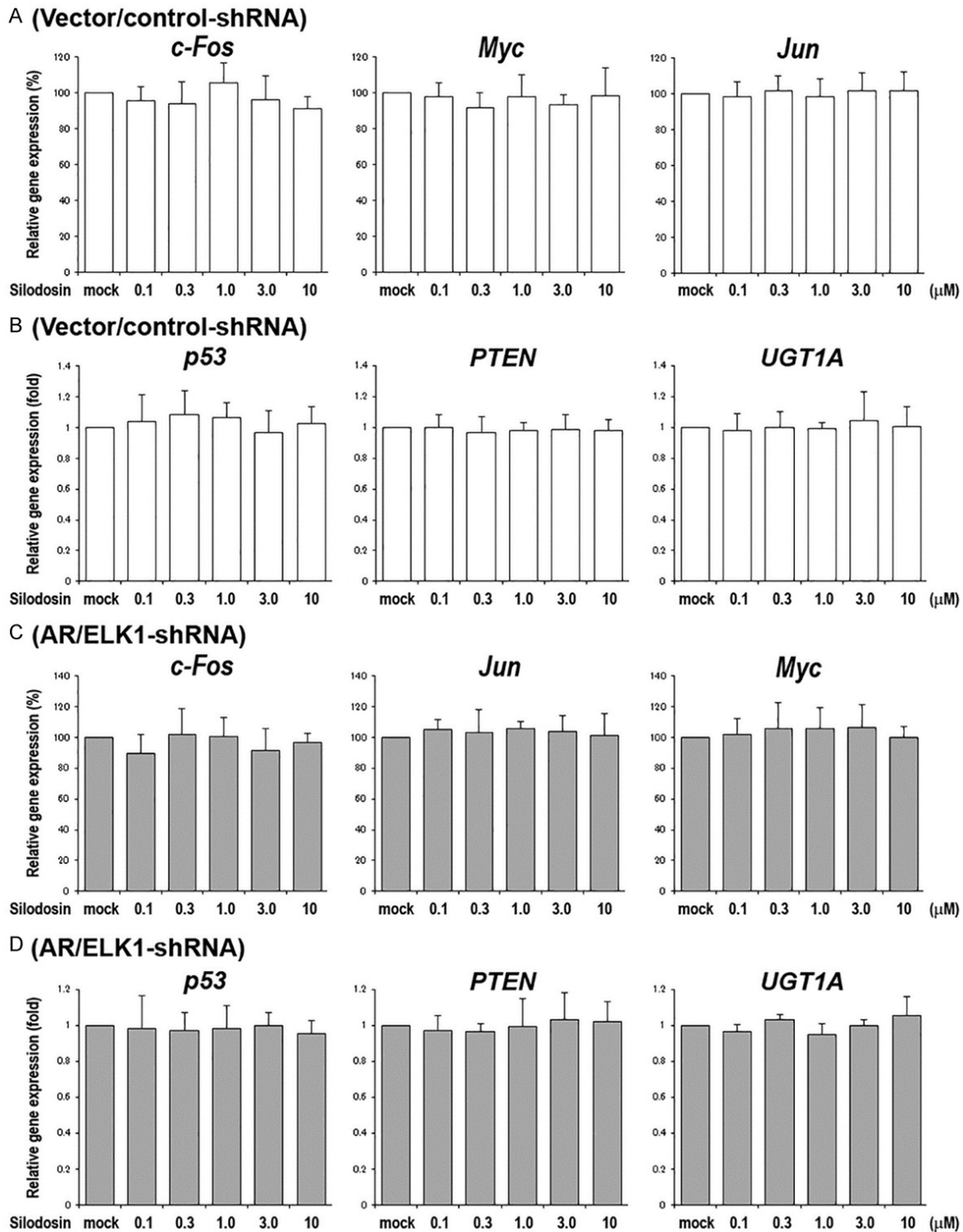


Figure 6. Effects of silodosin on the expression of oncogenes and tumor suppressor genes in urothelial cells undergoing neoplastic transformation. SVHUC-vector/control-shRNA (A, B) and SVHUC-AR/ELK1-shRNA (C, D) cells exposed to MCA and subsequently cultured with 0-10 μ M silodosin for 6 weeks were subjected to RNA extraction, RT, and real-time PCR. *GAPDH* was used to normalize the expression of *c-Fos*, *Jun*, or *Myc* (A, C), as well as *p53*, *PTEN*, or *UGT1A* (B, D), which is presented relative to that in mock-treated cells (mean + standard deviation of three independent experiments).

HUC-AR/control-shRNA and SVHUC/ELK1-shRNA cells without the carcinogen challenge, only

modest decreases in the expression of p-p38 and p-ERK by short-term (24 hours) silodosin

ELK1 and urothelial tumorigenesis

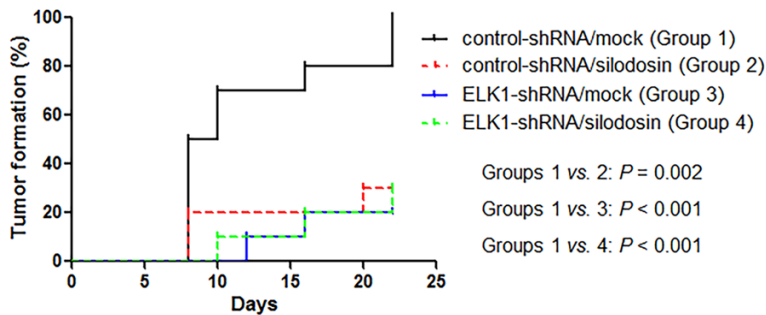


Figure 7. Effects of ELK1 knockdown or silodosin treatment on urothelial tumor formation in mouse xenograft models. SVHUC-AR/control-shRNA or SVHUC-AR/ELK1-shRNA cells exposed to MCA and subsequently cultured for 6 weeks in the presence of ethanol (mock) or silodosin (1 μ M) was suspended and subcutaneously implanted into the flank of 6-week-old male NOD-SCID mice (n = 10 in each group). The endpoint for this study was tumor formation (exceeding 30 mm³ in its estimated volume [by the following formula: (short diameter)² \times (longest diameter) \times 0.5] or 5 mm in greatest dimension).

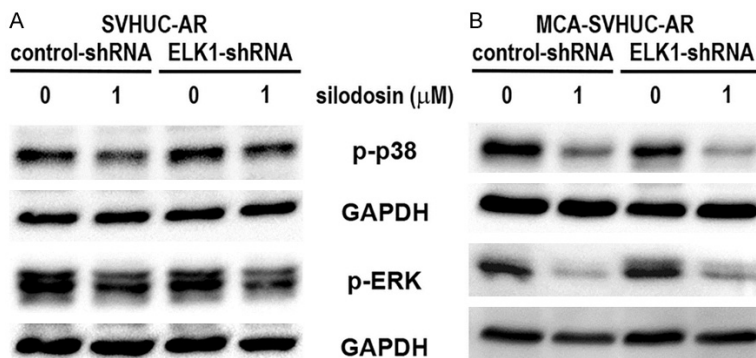


Figure 8. Effects of silodosin on the expression of upstream proteins of ELK1 in urothelial cells. Western blotting of p-p38 and p-ERK, using proteins extracted from SVHUC-AR cells treated with ethanol (mock) or silodosin (1 μ M) for 24 hours (A) or SVHUC-AR cells first exposed to MCA and subsequently cultured with ethanol (mock) or silodosin (1 μ M) for 6 weeks (B).

treatment were seen (**Figure 8A**). By contrast, in SVHUC-AR/control-shRNA and SVHUC-AR/ELK1-shRNA cells undergoing neoplastic transformation induced by MCA exposure, 6-week silodosin treatment during the course of neoplastic transformation resulted in substantial decreases in their expression (**Figure 8B**).

Prevention of the development of BBN-induced bladder cancer by silodosin

We finally utilized a chemical carcinogen BBN that was known to reliably induce the development of bladder tumor in male rodents to further assess the effects of silodosin on urothelial carcinogenesis. Male C57BL/6 mice were

treated with BBN as well as either mock or silodosin for 12 weeks. These animals were sacrificed at 27 weeks of age to detect urothelial tumors macroscopically and microscopically (**Table 2**). Bladder tumors were grossly identified in 5 mock-treated and 2 silodosin-treated mice and were histologically confirmed as high-grade carcinomas. In addition, in situ lesion was histologically seen in 3 mock-treated mice with no gross tumor. Thus, there was a significant difference in the incidence of bladder tumors between mock [8 (100%) of 8] versus silodosin [2 (25%) of 8] treatment ($P = 0.007$). None of the mice in either group developed upper urinary tract or metastatic tumors.

Discussion

Cancer initiation/malignant transformation and tumor growth/progression are distinct steps. In the present study, we have focused on investigating the functional role of ELK1, as well as the efficacy of silodosin, in the former. Indeed, we previously demon-

strated that ELK1 silencing resulted in significant delay ($P = 0.013$) in the formation of xenograft tumors when human bladder cancer UMUC3 cells stably expressing either a control-shRNA or an ELK1-shRNA were implanted into immunocompromised mice [5]. In addition, our immunohistochemistry data in surgical specimens showed significant up-regulation of the expression of phospho-ELK1 (p-ELK1), an active form of ELK1, in bladder [5] and upper urinary tract [18] urothelial cancers, compared with normal urothelial tissues, and a strong association of p-ELK1 positivity in non-muscle-invasive bladder tumors with the risk of recurrence after transurethral resection. These findings implied the involvement of ELK1 signals in the development of urothelial cancer.

Table 2. Effects of silodosin on bladder cancer incidence in a BBN-induced mouse model

	Mock	Silodosin
Gross tumor	5	2
In situ lesion	3	0
All malignancy	8	2
No malignancy	0	6

We have demonstrated preclinical data indicating that ELK1, as a transcriptional activator involving the MAPK/ERK pathway, induces the proliferation and/or migration/invasion of bladder [5] and prostate [11] cancer cells as well as resistance to the cytotoxic effects of a chemotherapeutic agent cisplatin in bladder cancer cells [10]. We have additionally shown that silodosin inhibits the expression and activity of ELK1 and thereby exhibits the suppressive effects similar to those of ELK1 knockdown in both types of cells, especially those possessing a functional AR [10, 11]. Thus, the function of ELK1 in bladder cancer progression, but not tumor development, has been well documented.

As aforementioned, ELK1 is known to be a regulator of c-Fos [3, 4]. c-Fos has been further shown to form a heterodimer with Jun, leading to the formation of the AP-1 complex and regulation of target gene expression, and thereby involve tumorigenesis [19]. Indeed, ELK1 has been implicated in colon carcinogenesis [20]. Direct regulation of BRCA1, variations of which are linked to increased risks of breast and ovarian cancers, by ELK1/c-Fos/Jun has also been documented [21, 22]. We here demonstrated further evidence, using an *in vitro* transformation model, suggesting that ELK1 could promote urothelial tumorigenesis. ELK1 knockdown in MCA-exposed SVHUC-AR cells resulted in significant decreases in the expression of oncogenes, including *c-Fos*, *Jun*, and *Myc*, and significant increases in that of tumor suppressors, such as *p53*, *PTEN*, and *UGT1A*. Nonetheless, further studies are necessary to determine precisely how ELK1 signals regulate urothelial cancer initiation.

As we previously showed in bladder [10] and prostate [11] cancer cells, silodosin was found to inhibit the expression of ELK1 and its transcriptional activity in non-neoplastic urothelial cells. We could then show the preventive

effects of silodosin on MCA-induced neoplastic transformation of urothelial cells similar to those of ELK1 knockdown. Importantly, silodosin was most likely to function via the ELK1 pathway, because no significant additional inhibitory effects of silodosin were seen in ELK1 knockdown cells. Our western blotting further showed that long-term treatment of silodosin considerably reduced the expression of upstream proteins of ELK1, p-p38 and p-ERK, in both control and ELK1-silenced urothelial cells undergoing malignant transformation, whereas its short-term (*i.e.* 24 hours) treatment only modestly reduced it. In addition, the prevention of tumor development by silodosin treatment was confirmed in a BBN-induced bladder cancer model. Silodosin was thus found to inhibit urothelial tumorigenesis via inactivating ELK1 and its upstream signals, suggesting the possibility of clinical application of silodosin therapy to the patients with non-muscle-invasive bladder tumor following transurethral surgery to efficiently prevent tumor recurrence. Indeed, selective α 1A-adrenergic receptor antagonists, including silodosin, have been widely used for the symptomatic treatment in men with benign prostatic hyperplasia [7]. Meanwhile, α 1-adrenergic receptors have been shown to activate p38 and ERK in the human prostate [23, 24]. Of note, silodosin at 1-10 μ M showing the significant effects in our *in vitro* studies is tolerable, as its plasma levels, without acute toxicity or marked histological changes in the major organs in rodents [25], while plasma levels of silodosin in healthy men after receiving pharmacological doses (*e.g.* oral dose of 8 mg) are up to approximately 0.1 μ M [26]. Mice that received BBN in our bladder cancer model were also tolerable to a higher dose (*i.e.* 40 mg/Kg) of daily subcutaneous injections of silodosin, aiming for a C_{max} of 1 μ M, for 12 weeks.

Functional interplay between ELK1 and AR signals has been documented in prostate cancer cells [6]. We also previously demonstrated that the expression levels of ELK1 or p-ELK1 versus AR in bladder cancer tissue specimens were significantly associated and that androgens activated ELK1 in AR-positive bladder cancer cells [5]. Interestingly, ELK1 knockdown or silodosin treatment could inhibit the cell growth of bladder or prostate cancer lines only in the presence of an activated AR (and ELK1) but the

cell migration of both AR-positive and AR-negative lines [5, 10, 11]. We here found that AR overexpression or DHT treatment augmented ELK1 expression in non-neoplastic urothelial cells. Silodosin was also found to inactivate ELK1 signals only in AR-positive urothelial cells. Moreover, the significant inhibitory effects of ELK1 knockdown and silodosin treatment on MCA-induced neoplastic transformation were seen in AR-positive urothelial cells, but not in AR-negative cells. These findings suggest that ELK1 requires AR for inducing urothelial tumorigenesis. While androgens and AR are known to play a vital role in the development of urothelial cancer [2, 12, 14, 16, 17], it needs to be determined if an activated AR is necessary for the involvement of ELK1 in malignant transformation of non-urothelial cells especially where AR-related signals are unlikely to be of importance for their tumorigenesis.

In conclusion, our results indicate that ELK1 contributes to inducing urothelial tumorigenesis via cooperation with a functional AR. Accordingly, ELK1 inactivation via treatment with tolerable doses of silodosin has the potential of being an effective chemopreventive approach against bladder cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hiroshi Miyamoto, Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, 601 Elmwood Avenue, Box 626, Rochester, NY 14642, USA. Tel: 585-285-8748; E-mail: hiroshi_miyamoto@urmc.rochester.edu

References

- [1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; 65: 87-108.
- [2] Inoue S, Mizushima T, Miyamoto H. Role of the androgen receptor in urothelial cancer. *Mol Cell Endocrinol* 2018; 465: 73-81.
- [3] Hipskind RA, Rao VN, Mueller CG, Reddy ES, Nordheim A. Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF. *Nature* 1991; 354: 531-534.
- [4] Gille H, Kortenjann M, Thomae O, Moomaw C, Slaughter C, Cobb MH, Shaw PE. ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J* 1995; 14: 951-962.
- [5] Kawahara T, Shareef HK, Aljarah AK, Ide H, Li Y, Kashiwagi E, Netto GJ, Zheng Y, Miyamoto H. ELK1 is up-regulated by androgen in bladder cancer cells and promotes tumor progression. *Oncotarget* 2015; 6: 29860-29876.
- [6] Patki M, Chari V, Sivakumaran S, Gonit M, Trumbly R, Ratnam M. The ETS domain transcription factor ELK1 directs a critical component of growth signaling by the androgen receptor in prostate cancer cells. *J Biol Chem* 2013; 288: 11047-11065.
- [7] McVary KT, Roehrborn CG, Avins AL, Barry MJ, Bruskewitz RC, Donnell RF, Foster HE Jr, Gonzalez CM, Kaplan SA, Penson DF, Ulchaker JC, Wei JT. Update on AUA guideline on the management of benign prostatic hyperplasia. *J Urol* 2011; 185: 1793-1803.
- [8] Hennenberg M, Strittmatter F, Beckmann C, Rutz B, Füllhase C, Waidelich R, Montorsi F, Hedlund P, Andersson KE, Stief CG, Gratzke C. Silodosin inhibits noradrenaline-activated transcription factors Elk1 and SRF in human prostate smooth muscle. *PLoS One* 2012; 7: e50904.
- [9] Yazaki J, Aikawa K, Shishido K, Yanagida T, Nomiya M, Ishibashi K, Haga N, Yamaguchi O. Alpha1-adrenoceptor antagonists improve bladder storage function through reduction of afferent activity in rats with bladder outlet obstruction. *Neurourol Urodyn* 2011; 30: 461-467.
- [10] Kawahara T, Ide H, Kashiwagi E, Patterson JD, Inoue S, Shareef HK, Aljarah AK, Zheng Y, Baras AS, Miyamoto H. Silodosin inhibits the growth of bladder cancer cells and enhances the cytotoxic activity of cisplatin via ELK1 inactivation. *Am J Cancer Res* 2015; 5: 2959-2968.
- [11] Kawahara T, Aljarah AK, Shareef HK, Inoue S, Ide H, Patterson JD, Kashiwagi E, Han B, Li Y, Zheng Y, Miyamoto H. Silodosin inhibits prostate cancer cell growth via ELK1 inactivation and enhances the cytotoxic activity of gemcitabine. *Prostate* 2016; 76: 744-756.
- [12] Izumi K, Zheng Y, Hsu JW, Chang C, Miyamoto H. Androgen receptor signals regulate UDP-glucuronosyltransferases in the urinary bladder: a potential mechanism of androgen-induced bladder carcinogenesis. *Mol Carcinog* 2013; 52: 94-102.
- [13] Li Y, Ishiguro H, Kawahara T, Miyamoto Y, Izumi K, Miyamoto H. GATA3 in the urinary bladder: suppression of neoplastic transformation and down-regulation by androgens. *Am J Cancer Res* 2014; 4: 461-473.
- [14] Kawahara T, Inoue S, Kashiwagi E, Chen J, Ide H, Mizushima T, Li Y, Zheng Y, Miyamoto H. Enzalutamide as an androgen receptor inhibitor prevents urothelial tumorigenesis. *Am J Cancer Res* 2017; 7: 2041-2050.

ELK1 and urothelial tumorigenesis

- [15] Reznikoff CA, Loretz LJ, Christian BJ, Wu SQ, Meisner LF. Neoplastic transformation of SV40-immortalized human urinary tract epithelial cells by in vitro exposure to 3-methylcholanthrene. *Carcinogenesis* 1988; 9: 1427-1436.
- [16] Miyamoto H, Yang Z, Chen YT, Ishiguro H, Uemura H, Kubota Y, Nagashima Y, Chang YJ, Hu YC, Tsai MY, Yeh S, Messing EM, Chang C. Promotion of bladder cancer development and progression by androgen receptor signals. *J Natl Cancer Inst* 2007; 99: 558-568.
- [17] Hsu JW, Hsu IW, Xu D, Miyamoto H, Liang L, Wu XR, Shyr CR, Chang C. Decreased tumorigenesis and mortality from bladder cancer in mice lacking urothelial androgen receptor. *Am J Pathol* 2013; 182: 1811-1820.
- [18] Inoue S, Ide H, Fujita K, Mizushima T, Jiang G, Kawahara T, Yamaguchi S, Fushimi H, Nonomura N, Miyamoto H. Expression of phospho-ELK1 and its prognostic significance in urothelial carcinoma of the upper urinary tract. *Int J Mol Sci* 2018; 19: E777.
- [19] Vessely PW, Staber PB, Hoefler G, Kenner L. Translational regulation mechanisms of AP-1 proteins. *Mutat Res* 2009; 682: 7-12.
- [20] Hollander D, Donyo M, Atias N, Mekahel K, Melamed Z, Yannai S, Lev-Maor G, Shilo A, Schwartz S, Barshack I, Sharan R, Ast G. A network-based analysis of colon cancer splicing changes reveals a tumorigenesis-favoring regulatory pathway emanating from ELK1. *Genome Res* 2016; 26: 541-553.
- [21] Chai Y, Chipitsyna G, Cui J, Liao B, Liu S, Aysola K, Yezdani M, Reddy ES, Rao VN. c-Fos oncogene regulator Elk-1 interacts with BRCA1 splice variants BRCA1a/1b and enhances BRCA1a/1b-mediated growth suppression in breast cancer. *Oncogene* 2001; 20: 1357-1367.
- [22] Zhou L, Graves M, MacDonald G, Cipollone J, Mueller CR, Roskelley CD. Microenvironmental regulation of BRCA1 gene expression by c-Jun and Fra2 in premalignant human ovarian surface epithelial cells. *Mol Cancer Res* 2013; 34: 1853-1864.
- [23] Bauer RM, Strittmatter F, Gratzke C, Göttinger J, Schlenker B, Reich O, Stief CG, Hedlund P, Andersson KE, Hennenberg M. Coupling of α 1-adrenoceptors to ERK1/2 in the human prostate. *Urol Int* 2011; 86: 427-433.
- [24] Strittmatter F, Gratzke C, Walther S, Göttinger J, Beckmann C, Roosen A, Schlenker B, Reich O, Stief CG, Hennenberg M. Alpha1-adrenoceptor signaling in the human prostate involves regulation of p38 mitogen-activated protein kinase. *Urology* 2011; 78: 969, e7-13.
- [25] U.S. Food and Drug Administration via http://www.accessdata.fda.gov/drugsatfda_docs/nda/2008/022206s000TOC.cfm.
- [26] Zhou Y, Sun PH, Liu YW, Zhao X, Meng L, Cui YM. Safety and pharmacokinetic studies of silodosin, a new α_{1A} -adrenoceptor selective antagonist, in healthy Chinese male subjects. *Biol Pharm Bull* 2011; 34: 1240-1245.