Am J Cancer Res 2014;4(5):461-473
www.ajcr.us /ISSN:2156-6976/ajcr0001702

Original Article

GATA3 in the urinary bladder: suppression of neoplastic transformation and down-regulation by androgens

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Received August 4, 2014; Accepted August 17, 2014; Epub September 6, 2014; Published September 15, 2014

Abstract: Recent evidence suggests the involvement of sex hormone receptors in bladder cancer initiation, while precise functions of androgens and estrogens in the carcinogenesis step remain poorly understood. We recently found down-regulation of GATA3, a zinc-finger transcription factor and a new urothelial marker, in bladder cancer, which also correlated with expression status of androgen receptor (AR) and estrogen receptors (ERs). We here assessed whether GATA3 acted as a suppressor of bladder tumorigenesis and sex hormones exerted an influence on GATA3 in non-neoplastic urothelial cells. Androgen (R1881, dihydrotestosterone) treatment in SVHUC immortalized normal urothelial cells stably expressing AR (SVHUC-AR) decreased GATA3 expression at both mRNA and protein levels, which was abolished by anti-androgens. Conversely, 17β-estradiol treatment increased it in SVHUC-control endogenously expressing ERβ. GATA3 levels were also found to be higher in intact female mouse bladders compared with intact males, and orchiectomy/ovariectomy augmented/reduced GATA3 expression, respectively, which was at least partially restored by dihydrotestosterone/17β-estradiol supplement. Additionally, GATA3 silencing via short hairpin RNA (shRNA) promoted cell proliferation of SVHUC with exposure to a chemical carcinogen 3-methylcholanthrene. *In vitro* transformation assay with 3-methylcholanthrene then showed a significantly higher number of colonies in SVHUC-AR/GATA3-shRNA, compared with control SVHUC, and R1881 further induced colony formation. GATA3 knockdown also resulted in down-regulation of the molecules that play a protective role in bladder tumorigenesis (*i.e.* UGT1A, PTEN, p53, p21) and up-regulation of oncogenic genes (*i.e.* c-myc, cyclin D1, cyclin D3, cyclin E, FGFR3). Thus, GATA3 likely prevented neoplastic transformation of urothelial cells. Furthermore, sex hormone signals contrary regulated GATA3 in the bladder. These findings may offer not only a molecular basis for the gender-specific difference in bladder cancer incidence but also great potential for androgen deprivation as a chemopreventive option for tumor recurrence.

Keywords: Androgen, bladder cancer, estrogen, GATA3, tumorigenesis

Introduction

Urinary bladder cancer is one of commonly diagnosed malignancies predominantly affecting males throughout the world [1]. In the United States, bladder cancer is the fourth most common cancer type in men, while in women it is not among the top 10 leading types for newly diagnosed cases [2]. Although two major causes, cigarette smoking and exposure to industrial chemicals, may have contributed to male dominance in bladder cancer, the male-to-female ratio of the tumor remains virtually unchanged for many years before and after controlling for these carcinogenic factors [1-3].

Recent advances in bladder cancer research have suggested the involvement of sex hormones and their receptor signals, in addition to lifestyle or environmental risk factors, in bladder carcinogenesis [4-6]. In particular, using a chemical carcinogen *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine in androgen receptor (AR) knockout animal models, we have demonstrated molecular evidence indicating that androgen-mediated AR signaling promotes bladder cancer initiation [7, 8]. We also showed that androgens down-regulated the expression of UDP-glucuronosyltransferase-1A (UGT1A) subtypes, major phase II drug metabolism enzymes that play a critical role in detoxifying bladder carcin-

Table 1. Sequences of PCR primers

Gene	Sense	Anti-sense	Product size (bp)
Human			
GATA3	5'-TCACAAAATGAACGGACAGAACC-3'	5'-CAGCCTTCGCTTGGGCTTAAT-3'	52
UGT1A	5'-TGATTGGTTTCCTCTTGGC-3'	5'-GGGTCTTGGATTTGTGGG-3'	126
PTEN	5'-CTTTGTTACAATTTCGGGCACCG-3'	5'-AACCCTCATTCAGACCTTCACATTCC-3'	151
p53	5'-GGAGGGGCGATAAATACC-3'	5'-AACTGTAACTCCTCAGGCAGGC-3'	132
p21	5'-AAGACCATGTGGACCTGTCACTGT-3'	5'-GAAGATCAGCCGGCGTTTG-3'	155
p27	5'-CGAGTGGCAAGAGGTGGAGA-3'	5'-GGAGCCCCAATTAAAGGCG-3'	144
c-myc	5'-ACCAGATCCCGGAGTTGGAA-3'	5'-CGTCGTTTCCGCAACAAGTC-3'	136
cyclin D1	5'-GTGCTGCGAAGTGGAAACC-3'	5'-ATCCAGGTGGCGACGATCT-3'	174
cyclin D3	5'-TGCTGGCTTACTGGATGCTG-3'	5'-GACGCAAGACAGGTAGCGA-3'	101
cyclin E	5'-GCCAGCCTTGGGACAATAATG-3'	5'-CTTGCACGTTGAGTTTGGGT-3'	104
FGFR3	5'-CCCAAATGGGAGCTGTCTCG-3'	5'-CCCGGTCCTTGTCAATGCC-3'	109
GAPDH	5'-AAGGTGAAGGTCGGAGTCAAC-3'	5'-GGGGTCATTGATGGCAACAATA-3'	102
Mouse			
GATA3	5'-CCCCATTACCACCATTCCGC-3'	5'-CCTCGACTTACATCCGAACCC-3'	106
β-actin	5'-AGTGTGACGTTGACATCCGTA-3'	5'-GCCAGAGCAGTAATCTCCTTC-3'	112

ogens, in normal urothelial cells [9]. These findings may at least partially explain the gender-specific difference in the incidence of bladder cancer. However, precise functions of androgens and underlying molecular mechanisms for AR-induced bladder tumorigenesis need to be further determined.

A member of the GATA family of zinc finger transcription factors, GATA3, was originally identified as a T cell lineage-specific factor [10, 11]. It was subsequently shown that GATA3 could function outside of the hematopoietic system. Specifically, GATA3, in relation to estrogen receptor (ER) signaling pathways, has been implicated in breast tumorigenesis [12, 13]. *In vitro* and *in vivo* studies as well as immunohistochemistry in clinical samples have also demonstrated that loss of GATA3 is associated with more aggressive behavior of breast cancer [14-17]. Thus, GATA3 is considered a breast tumor suppressor.

GATA3 has recently been recognized as a urothelial marker [18]. Indeed, in diagnostic surgical pathology GATA3 immunohistochemistry is currently one of the most useful methods that distinguish primary or metastatic urothelial carcinoma from its mimickers [18-20]. Our immunohistochemical study additionally showed that the expression of GATA3 was down-regulated in urothelial neoplasms, compared with non-neoplastic urothelial tissues, and in high-grade or muscle-invasive carcinomas, compared with low-grade or superficial tumors [21]. Furthermore, in our recent study in bladder cancer lines, loss of GATA3 correlated with promotion of cell migration and invasion [22]. These results suggest an inhibitory role of GATA3 in the development and progression of bladder cancer. In addition, in both non-neoplastic bladder and bladder tumor tissue specimens, there were significant correlations of GATA3 expression with AR/ERα/ERβ status [21]. In the present study, we aim to investigate whether GATA3 contributes to bladder carcinogenesis and sex hormones affect GATA3 in non-neoplastic urothelial cells.

Materials and methods

Cell culture and chemicals

An immortalized human normal urothelial cell line (SVHUC) and human urothelial carcinoma cell lines (TCC-SUP, 5637, and UMUC3) were originally obtained from the American Type Culture Collection. 647V cells were used in our previous study [7]. All these lines were recently authenticated, using GenePrint 10 System (Promega), by the institutional core facility. Stable cell lines expressing a full-length wild-type human AR (*e.g.* SVHUC-AR, 5637-AR) and a control line (*e.g.* SVHUC-V) were described in

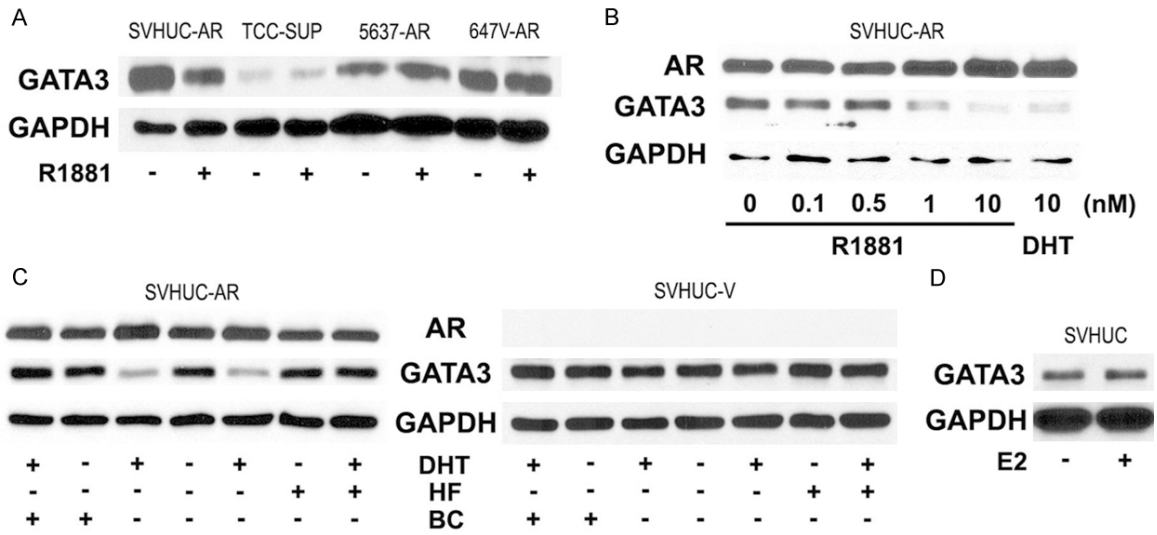

Figure 1. Effects of androgen and estrogen on GATA3 protein expression in urothelial cells. A. Cell extracts from SVHUC-AR, TCC-SUP, 5637-AR, and 647V-AR treated with ethanol or 10 nM R1881 for 24 hours were analyzed on western blotting, using an antibody to GATA3 (50 kDa). GAPDH (37 kDa) served as an internal control. B. Total proteins extracted from SVHUC-AR cells treated with ethanol, 0.1-10 nM R1881, or 10 nM DHT for 24 hours were immunoblotted for AR (110 kDa), GATA3, and GAPDH. C. Total proteins extracted from SVHUC-AR and SVHUC-V cells treated with ethanol, 10 nM DHT, 1 μM HF, and/or 1 μM BC for 24 hours were immunoblotted for AR, GATA3, and GAPDH. D. Total proteins extracted from SVHUC cells treated with ethanol or 1 nM E2 for 24 hours were immunoblotted for GATA3 and GAPDH.

our previous reports [9, 23, 24]. Similarly, stable cell lines expressing a GATA3-short hairpin RNA (shRNA) (*i.e.* SVHUC-AR/GATA3-shRNA, SVHUC-V/GATA3-shRNA) were established by transfecting a lentiviral vector (PLKO.1-puro; sh991, sh301, sh300, or sh105; Sigma) into 293T human embryonic kidney cell line cells, culturing target cells in the presence of the viral supernatant, and selecting stable clones with puromycin (Sigma), as described previously [22]. An ERβ-shRNA lentivirus plasmid (Sigma) was transiently expressed in parental SVHUC. These cells were maintained in appropriate medium (Mediatech; Kaighn's Modification of Ham's F-12K for SVHUC-derived lines and Dulbecco's modified eagle's medium for bladder cancer-derived lines) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. At least 18 hours prior to experimental treatment, cells were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS. We obtained methyltrienolone (R1881) from PerkinElmer; dihydrotestosterone (DHT), 17β-estradiol (E2), and tamoxifen (TAM) from Sigma; bicalutamide (BC) from Santa Cruz Biotechnology; and hydroxyflutamide (HF) from Schering.

Western blot

Whole cell protein extraction and western blot were performed, as described previously [22, 25], with minor modifications. Briefly, equal amounts of protein (30 μg) obtained from cell extracts were separated in 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore) by electroblotting, using a standard protocol. Specific antibody binding was detected, using an anti-GATA3 antibody (clone L50-823; diluted 1:1000; Biocare Medical), an anti-AR antibody (clone N20; diluted 1:2000; Santa Cruz Biotechnology), or an anti-GAPDH antibody (clone 6C5; diluted 1:1000; Santa Cruz Biotechnology), with horseradish peroxidase detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) and X-ray film.

Reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA (1.0 μg) isolated from cultured cells or mouse tissues, using TRIzol (Invitrogen), was reverse transcribed with 1 μmol/L oligo (dT) primers and 4 units of Ominiscript reverse transcriptase (Qiagen) in a total volume of 20 μl. Real-time PCR was then performed in 15 μl sys-

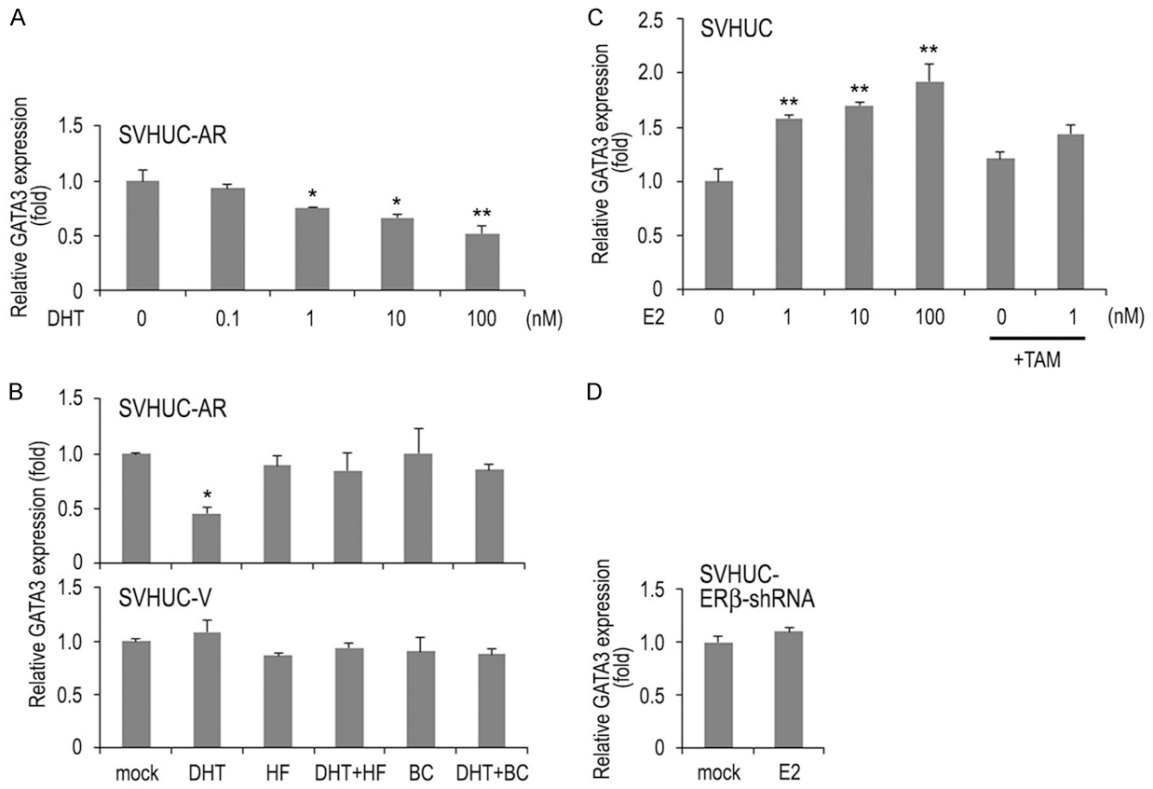

Figure 2. Effects of androgen and estrogen on *GATA3* mRNA expression in non-neoplastic urothelial cells. A quantitative RT-PCR measured the levels of *GATA3* mRNA expression in SVHUC-AR cultured with 0-100 nM DHT for 24 hours (A), SVHUC-AR and SVHUC-V cultured with ethanol (mock), 10 nM DHT, 1 μM HF, and/or 1 μM BC for 24 hours (B), SVHUC cultured with 0-100 nM E2 and/or 1 μM TAM for 24 hours (C), or SVHUC transfected with control-shRNA or ERβ-shRNA and cultured with ethanol (mock) or 1 nM E2 for 24 hours (D). Expression of *GATA3* was normalized to that of *GAPDH*. Transcription amount is presented relative to that with mock treatment in each cell line (set as one-fold). Each value represents the mean + standard deviation from at least three independent experiments. $*P < 0.05$ (*vs.* mock). $**P < 0.01$ (*vs.* mock).

tem by using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously [9, 22]. The primer sequences are given in **Table 1**.

Immunofluorescent staining

Immunofluorescent staining was performed, as described previously [25, 26], with minor modifications. Briefly, cells plated onto chamber slides (8-well Thermo Scientific Nunc Lab-Tek) were cultured in medium containing ethanol or R1881 for 24 hours, and the adherent cells were fixed with 4% paraformaldehyde. After being blocked, a primary antibody (GATA3, AR) was incubated at 4°C overnight, and Alexa 488- or 568-conjugated secondary antibody (diluted 1:200, Invitrogen) was added for one hour at 37°C. Fluorescence images were acquired with an Olympus FV1000 confocal microscope and nuclear expression of the proteins was quantified by a single observer who was unaware of the treatment group for the cells.

Orchiectomized/ovariectomized mice

CL57BL/6-129SV mice were housed according to the institutional guidelines and were allowed food and water ad libitum. At five weeks of age, male mice received bilateral orchiectomy or sham surgery and female mice received bilateral ovariectomy or sham surgery, as described previously [9, 27]. In castrated males and females, 0.1 ml of peanut oil with or without DHT (200 μg) and E2 (20 μg), respectively, was injected subcutaneously every other day. One week after the surgery, all the mice were sacrificed and urinary bladders were harvested. These specimens were rapidly frozen in liquid nitrogen and stored at -80°C for subsequent RNA analysis.

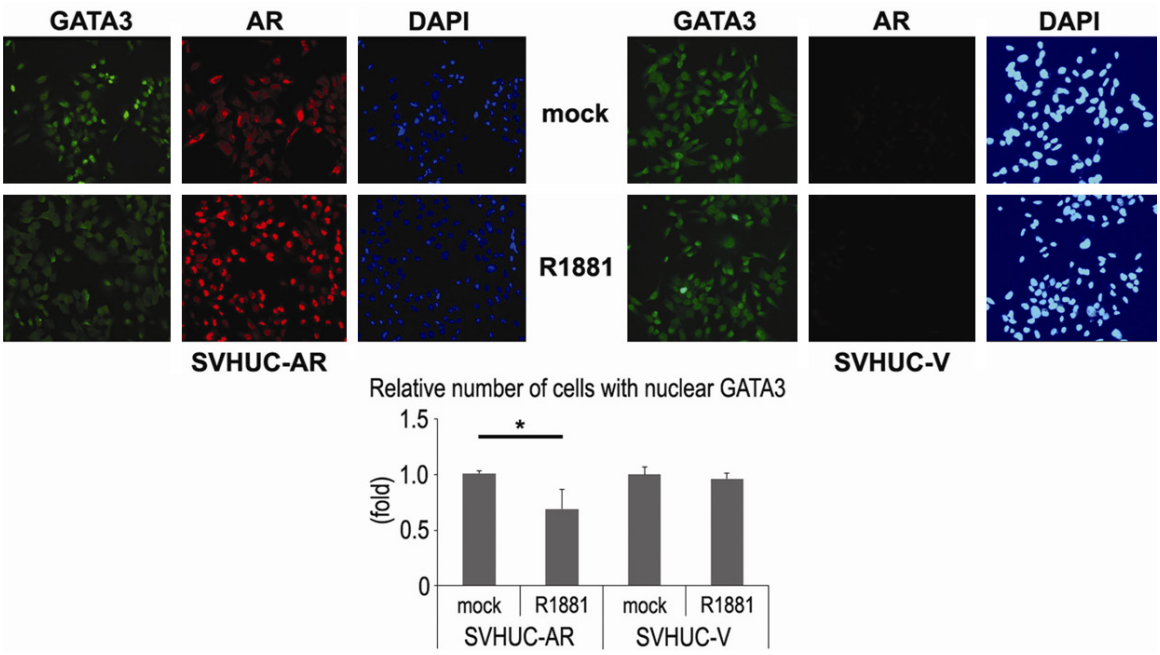

Figure 3. Effects of androgen on nuclear expression of GATA3 in non-neoplastic urothelial cells. SVHUC-AR and SVHUC-V cells cultured with ethanol (mock) or 10 nM R1881 for 24 hours were analyzed on immunofluorescence, using an antibody to GATA3 or AR. DAPI was used to visualize nuclei. The number of nuclear staining per visual field was quantified in five randomly selected visual fields per chamber (total 1,000 cells). The amount is presented relative to that with mock treatment in each cell line (set as one-fold). Each value represents the mean + standard deviation. $*P < 0.05$.

In vitro transformation

We used a method for neoplastic transformation of SVHUC by exposure to a carcinogen, 3-methylcholanthrene (MCA), described in a previous study [28] with minor modifications. Briefly, cells (2×10^6) seeded in 10-cm culture dish were cultured for 48 hours, and the culture medium was replaced with serum-free F-12K containing 5 μg/ml MCA (Sigma) with or without ligands (R1881, HF). After the first 24 hours of MCA exposure, FBS (1%) was added to the medium. After additional 24 hours, the cells were cultured in F-12K with 1% FBS and the ligands until near confluence. Subcultured cells (1/3 split) were again cultured in the presence of 5 μg/ml MCA for a 48-hour exposure period, using the above protocol. MCA exposure was repeated once more. These cells were then subcultured for six weeks in the presence or absence of the ligands and 6 μg/ml puromycin (for stable lines), and thereafter used for further assays.

Cell proliferation

We used the MTT (thiazolyl blue) assay to assess cell viability. SVHUC lines (3×10^3 cells/ligand) in which neoplastic transformation with three periods of MCA exposure was completed were seeded in 96-well tissue culture plates. These cells were cultured with ligands for 24-96 hours. Before measuring, 10 μl of MTT (Sigma) stock solution (5 mg/ml) was added to each well with 0.1 ml of medium for four hours at 37°C. Then, we replaced the medium with 100 μl of DMSO, incubated for five minutes at room temperature, and measured the absorbance at a wavelength of 570 nm with background subtraction at 630 nm.

Plate colony formation and soft agar colony formation assays

The resultant SVHUC sublines exposed to MCA were used for colony formation assays. For plate colony formation assay, 300 cells per well in 6-well plates were seeded and cultured for two weeks with 6 μg/ml puromycin (for stable lines). Fresh medium was replaced every other day. Then, the cells were stained with 0.1% crystal violet and photographed, and the number of colonies was counted in each well. For soft agar colony formation assay, after being dispersed to single cells, they were suspended

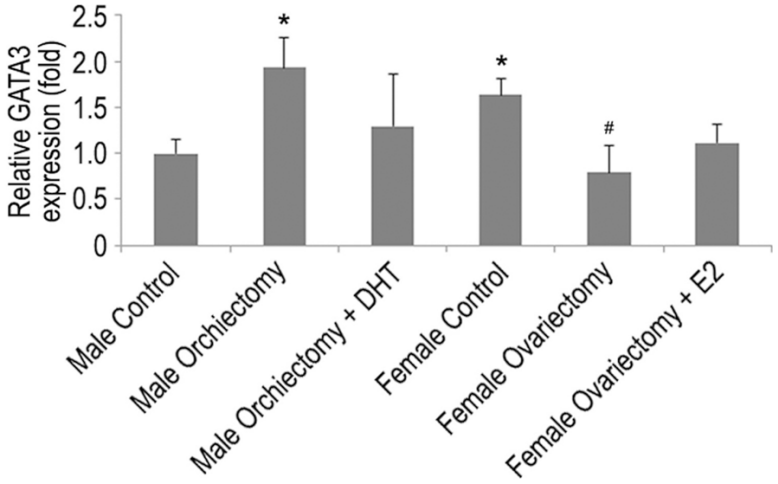

Figure 4. Effects of castration on GATA3 expression in mouse bladder tissues. Wild-type male mice underwent sham surgery (n = 6), orchiectomy only (n = 6), or orchiectomy followed by DHT supplement (n = 3). Wild-type female mice underwent sham surgery (n = 6), ovariectomy only (n = 6), or ovariectomy followed by E2 supplement (n = 3). One week after surgery, urinary bladders were harvested and analyzed on real-time RT-PCR for GATA3. Expression of *GATA3* was normalized to that of *β-actin*. Transcription amount is presented relative to that in control males with sham surgery (set as one-fold). Each value represents the mean + standard error. $*P < 0.05$ (*vs.* male control). $^{\#}P < 0.05$ (*vs.* female control).

at 2 × 10^4 cells per well in 6-well plates in a solution of 0.3% agarose and F-12K with 1% FBS. The cell suspension was layered into wells with a solidified 0.5% agarose and 1% FBS-containing medium sublayer. F-12K medium containing 10% FBS and ligands (R1881, HF) was then added and changed every three days. The cells were cultured until colonies were visible, stained with 0.05% crystal violet, and photographed. Colonies (≥ 20 cells) per well were counted by adjusting the depth of the field and changing the focus under the dissecting microscope. Dishes stained 24 hours after seeding were examined for cell clumping and served as zero-growth controls.

Statistical analyses

Differences in variables with a continuous distribution were analyzed by Student's *t*-test. *P*-values less than 0.05 were considered to be statistically significant.

Results

Androgens decrease GATA3 expression in normal urothelial cells

We recently showed relatively strong expression of GATA3 in immortalized normal urothelial SVHUC cells and its weaker expression in bladder cancer lines (undetectable in UMUC3) [22]. We first determined changes in GATA3 levels upon treatment with androgen in these lines harboring a functional AR endogenously (*i.e.* TCC-SUP, UMUC3) or exogenously [7, 9, 23-25] by western blot. R1881 treatment considerably reduced GATA3 expression in SVHUC-AR, whereas marginal changes or at least no decreases in GATA3 were seen in TCC-SUP, 5637-AR, and 647V-AR (**Figure 1A**). GATA3 remained undetectable in UMUC3 with or without R1881 (data not shown). Thus, androgen appeared to down-regulate GATA3 expression only in non-neoplastic urothelial cells. Western blots in SVHUC-AR further showed similar decreases in GATA3 expression by R1881 (in a dose-dependent manner) and DHT (**Figure 1B**) as well as antagonized effects of anti-androgens HF and BC on GATA3 (**Figure 1C**). In SVHUC-V, no significant changes of GATA3 induced by DHT and anti-androgens were seen (**Figure 1C**). Thus, down-regulation of GATA3 expression by androgens in urothelial cells appeared to be mediated via the AR pathway. In contrast, in parental SVHUC cells expressing endogenous ERβ but not ERα [27], an estrogen E2 slightly increased the level of GATA3 protein (**Figure 1D**).

We then assessed the effects of androgen and estrogen on *GATA3* mRNA expression in SVHUC-derived lines, using a quantitative RT-PCR. Similar to its protein expression, DHT reduced GATA3 levels in a dose-dependent manner in SVHUC-AR (**Figure 2A**), which could be antagonized by HF or BC, but showed marginal effects in SVHUC-V (**Figure 2B**). Moreover, E2 induced GATA3 expression in a dose-dependent manner, and an anti-estrogen TAM partially abolished the effect of E2 (**Figure 2C**). In ERβ-silencing SVHUC, no significant effect of E2 on GATA3 expression was seen (**Figure 2D**).

Immunofluorescent staining was also performed to compare nuclear expression of GATA3

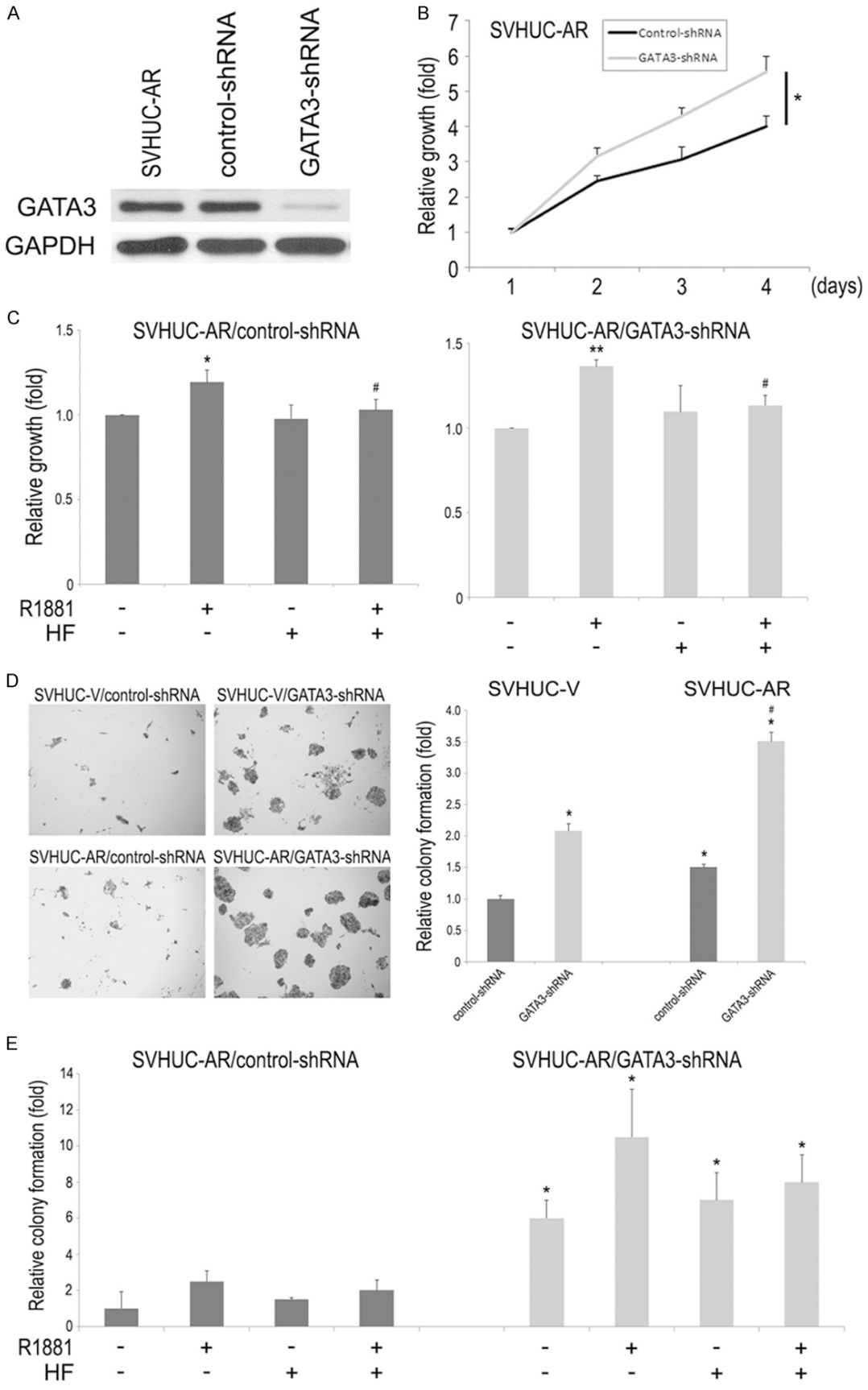
A
SVHUC-AR
control-shRNA
GATA3-shRNA
GATA3
GAPDH
B
SVHUC-AR
Control-shRNA
GATA3-shRNA
Relative growth (fold)
(days)
C
SVHUC-AR/control-shRNA
SVHUC-AR/GATA3-shRNA
Relative growth (fold)
R1881
HF
D
SVHUC-V/control-shRNA
SVHUC-V/GATA3-shRNA
SVHUC-AR/control-shRNA
SVHUC-AR/GATA3-shRNA
SVHUC-V
SVHUC-AR
Relative colony formation (fold)
control-shRNA
GATA3-shRNA
E
SVHUC-AR/control-shRNA
SVHUC-AR/GATA3-shRNA
Relative colony formation (fold)
R1881
HF

Figure 5. Effects of GATA3 knockdown and androgen treatment on neoplastic transformation of urothelial cells. A. GATA3 protein (50 kDa) expression was determined in SVHUC-AR, SVHUC-AR/control-shRNA, and SVHUC-AR/GATA3-shRNA by western blotting. GAPDH (37 kDa) served as an internal control. B. SVHUC-AR/control-shRNA and SVHUC-AR/GATA3-shRNA exposed to MCA three times and subsequently cultured for six weeks were further incubated for 24-96 hours. Cell viability was assayed with MTT, and growth induction is presented relative to cell number of control line at 24 hours (set as one-fold). Each value represents the mean + standard deviation from at least three independent experiments. $*P < 0.01$. C. SVHUC-AR/control-shRNA and SVHUC-AR/GATA3-shRNA exposed to MCA three times, subsequently cultured for six weeks, and further incubated for 96 hours in the presence or absence of 10 nM R1881 and/or 1 μM HF were assayed with MTT. Growth induction is presented relative to cell number of each line with mock treatment (set as one-fold). Each value represents the mean + standard deviation from at least three independent experiments. $*P < 0.05$ (*vs.* mock treatment). $**P < 0.01$ (*vs.* mock treatment). $^{\#}P < 0.05$ (*vs.* R1881 treatment only in each cell line). D. SVHUC sublines exposed to MCA three times and subsequently cultured for six weeks were seeded for plate colony formation assay. The cells were stained with 0.1% crystal violet to count the colonies. The amount is presented relative to that in SVHUC-V/control-shRNA (set as one-fold). Each value represents the mean + standard deviation from at least three independent experiments. $*P < 0.01$ (*vs.* SVHUC-V/control-shRNA). $^{\#}P < 0.01$ (*vs.* SVHUC-AR/control-shRNA). E. SVHUC-AR/control-shRNA and SVHUC-AR/GATA3-shRNA exposed to MCA three times and subsequently cultured for six weeks in the presence or absence of 10 nM R1881 and/or 1 μM HF were seeded for soft agar colony formation assay and cultured for three weeks. The amount is presented relative to that in SVHUC-AR/control-shRNA with mock treatment (set as one-fold). Each value represents the mean + standard deviation from at least three independent experiments. $*P < 0.05$ (*vs.* SVHUC-AR/control-shRNA with mock treatment).

among SVHUC-derived lines with and without androgen treatment (**Figure 3**). As shown in bladder cancer lines [25], non-ligand-bound AR predominantly present in the cytoplasm was translocated into the nucleus of SVHUC-AR cells in the presence of R1881. On the other hand, GATA3 predominantly expressed in the nucleus was significantly reduced by R1881 treatment in SHVUC-AR, but not in SVHUC-V.

Androgen ablation increases GATA3 expression in male mouse bladders

To confirm the effects of androgens and estrogens on GATA3 expression *in vivo*, its mRNA levels were analyzed in the bladders from mice with physiological levels of AR and ERs undergoing castration or sham surgery followed by hormone or mock treatment (**Figure 4**). In male mice GATA3 was up-regulated by orchiectomy, while in female mice it was down-regulated by ovariectomy. Supplement with DHT and E2 at least partially restored the effects of orchiectomy and ovariectomy, respectively. Of note were significantly higher levels of GATA3 in control female mice (1.6-fold over control male mice).

GATA3 silencing and/or androgen treatment induce neoplastic transformation of urothelial cells

We assessed the functional role of GATA3 in bladder tumorigenesis via an *in vitro* transformation assay, using SVHUC cells where, upon exposure to MCA, stepwise neoplastic transformation can be observed [28]. SVHUC sublines stably transfected with GATA3-shRNA [sh991 (**Figure 5A**); other shRNAs were less effectively silenced] were subjected to neoplastic transformation with MCA. The viability of SVHUC-AR cells completed for exposures to MCA was first compared between GATA3-positive control versus its knockdown lines in the presence or absence of androgen/anti-androgen by MTT assay. GATA3 knockdown resulted in significant increases in cell growth after 4-day culture (**Figure 5B**). In SVHUC-AR/control-shRNA, R1881 augmented the cell growth to 19% ($P = 0.042$), compared with mock treatment, and HF antagonized the R1881 effect (**Figure 5C**). Growth induction by R1881 was also found to be more significant (36% increase, $P = 0.003$) in SVHUC-AR/GATA3-shRNA.

Neoplastic transformation was further assessed, using plate and soft agar colony formation assays that determine the ability of anchorage-dependent and anchorage-independent growth, respectively. In the former assay (**Figure 5D**), GATA3 knockdown cells had significantly higher colony formation efficiencies (2.1-fold increase in SVHUC-V and 2.3-fold increase in SVHUC-AR over respective controls). Overexpression of AR also increased the efficiencies (1.5-fold increase in control-shRNA lines and 1.7-fold increase in GATA3-shRNA lines). Similarly, in the latter assay (**Figure 5E**), GATA3 silencing resulted in significant increases in colony number (*e.g.* 6.0-fold increase with mock treatment). In addition, R1881 treatment further induced colony formation (2.5-fold increase in control-shRNA lines and 1.8-fold

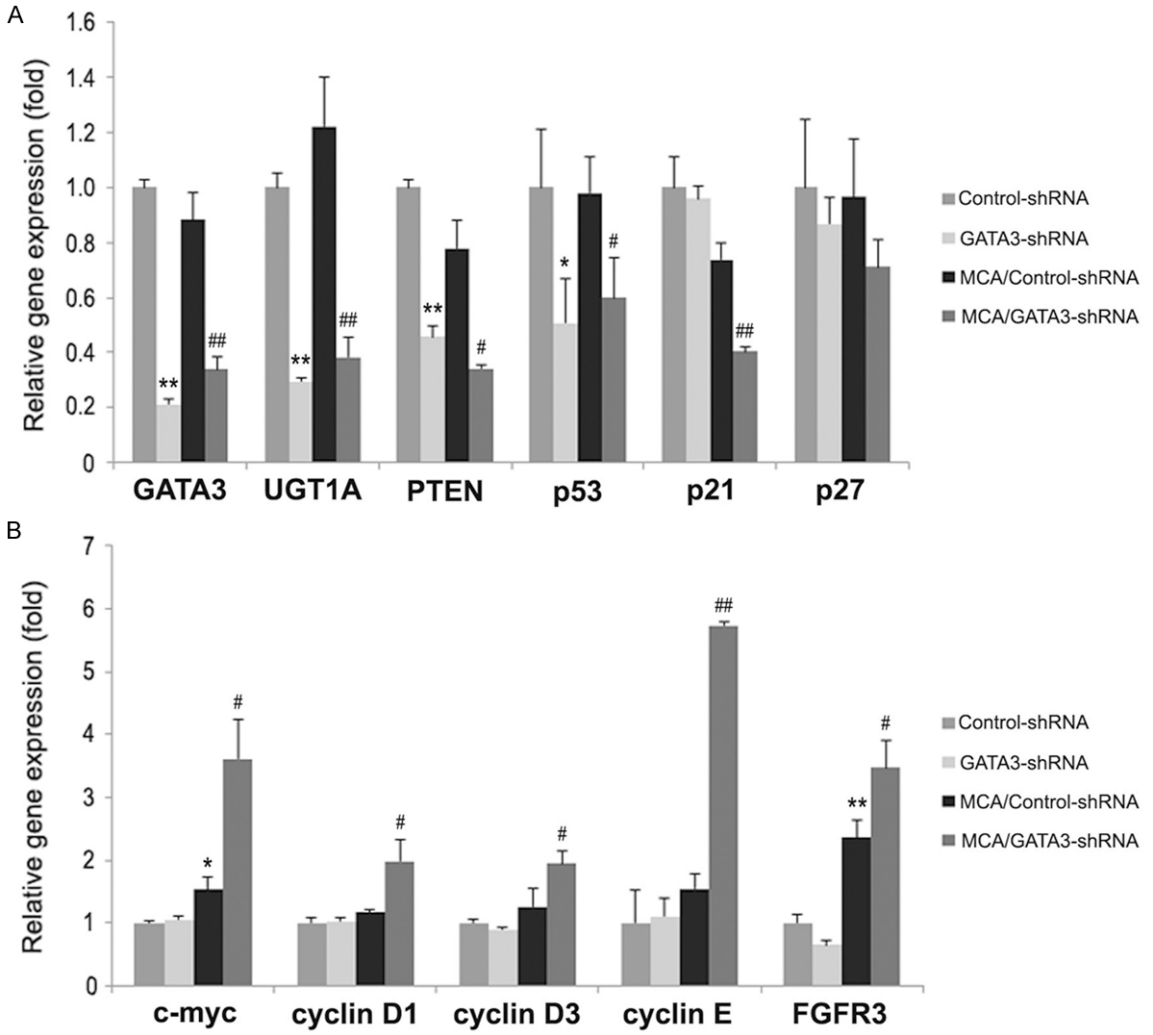

Figure 6. Effects of GATA3 knockdown on the expression of tumor suppressors and oncogenes in non-neoplastic urothelial cells. SVHUC-AR/control-shRNA and SVHUC-AR/GATA3-shRNA with or without neoplastic transformation induced by MCA were subjected to a quantitative RT-PCR for GATA3, UGT1A, PTEN, p53, p21, and p27 (A) as well as c-myc, cyclin D1, cyclin D3, cyclin E, and FGFR3 (B). Expression of each gene was normalized to that of *GAPDH*. Transcription amount is presented relative to that in SVHUC-AR/control-shRNA without MCA exposure (set as one-fold). Each value represents the mean + standard deviation from at least three independent experiments. $^{*}P < 0.05$ (*vs.* SVHUC-AR/control-shRNA without MCA exposure). $^{**}P < 0.01$ (*vs.* SVHUC-AR/control-shRNA without MCA exposure). $^{\#}P < 0.05$ (*vs.* SVHUC-AR/control-shRNA with MCA exposure). $^{\#\#}P < 0.01$ (*vs.* SVHUC-AR/control-shRNA with MCA exposure).

increase in GATA3-shRNA lines), which was at least partially blocked by HF. These results indicate that GATA3 prevents neoplastic transformation of urothelial cells while androgens promote it.

Loss of GATA3 up/down-regulates the expression of oncogenes/tumor suppressor genes

By using a quantitative RT-PCR method, we finally compared the expression levels of various molecules that play a protective role in bladder tumorigenesis, such as UGT1A, phosphatase and tensin homolog (PTEN), p53, p21, and p27 (**Figure 6A**), and oncogenic molecules, including c-myc, cyclin D1, cyclin D3, cyclin E, and fibroblast growth factor receptor 3 (FGFR3) (**Figure 6B**) in SVHUC-AR lines with/without GATA3-shRNA and with/without MCA exposure. GATA3 silencing resulted in down-regulation of UGT1A, PTEN, and p53 (both in cells with and without MCA) as well as p21 (only in cells with MCA). MCA considerably (c-myc, FGFR3) or marginally (cyclin D1, cyclin D3, cyclin E) increased

the expression levels of oncogenic genes in control cells, while GATA3 knockdown strongly induced the expression of all these five genes in cells with MCA exposure, but not in those without neoplastic transformation.

Discussion

In accordance with its expression in a variety of tissues, a wide range of biological functions of GATA3 has been recognized. These include the normal development of tissues, such as regulation of Th1/Th2 differentiation and luminal cell differentiation/proliferation in the mammary glands, as well as tumor differentiation and progression, mainly studied in breast cancer [10-17, 29-31]. Complementary DNA microarrays and subsequent immunohistochemical analyses have also revealed that GATA3 acts as a marker of urothelial differentiation [18, 32]. Nonetheless, the role of GATA3 in urothelial tumorigenesis and its involvement in sex hormone receptor pathways in urothelial cells remained uncertain. We are presenting new data suggesting that GATA3 inhibits the development of bladder cancer. GATA3 is also found to be regulated by sex hormones, especially androgen-mediated AR signals that down-regulate GATA3 in non-neoplastic urothelial cells.

As aforementioned, GATA3 is known to be expressed in cells with urothelial differentiation [18-22, 32]. Our immunohistochemical data showed that GATA3 was positive in 98% of non-neoplastic urothelial tissues versus 86% of urothelial neoplasms as well as in 98% of lower grade (papillary urothelial neoplasm of low malignant potential + low-grade urothelial carcinoma) and/or superficial (pTa + pT1) tumors versus 72-80% of high-grade and/or muscle-invasive (pT2-4) urothelial carcinomas [21]. Thus, GATA3 was down-regulated particularly in high-grade invasive bladder tumors. In addition, our *in vitro* study showed that enforced expression and silencing of GATA3 in bladder cancer lines resulted in inhibition and promotion, respectively, of cell migration and invasion, while they had marginal effects on cell proliferation [22]. Accordingly, although it is contradictory that strong expression of GATA3 was associated with poor prognosis in muscle-invasive tumors [21], GATA3 is suggested to have a preventive role in bladder tumorigenesis and cancer progression. Using *in vitro* transformation and colony formation assays with a chemical carcinogen MCA, we confirmed that GATA3 prevented a process of neoplastic transformation of urothelial cells. Moreover, in urothelial cells undergoing neoplastic transformation, loss of GATA3 down-regulated the expression of "tumor suppressors", such as UGT1A, PTEN, p53, and p21, and up-regulated that of "oncogenic" genes, such as c-myc, cyclin D1, cyclin D3, cyclin E, and FGFR3. The significance of these findings is enhanced by molecular evidence indicating, for instance, that UGT1A subtypes are key enzymes involved in detoxification of major bladder carcinogens, such as aromatic amines and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol derived from tobacco and its smoke [33], and that aberrant activation of FGFR3 via mutation or overexpression is frequently detected in bladder tumors and promotes their outgrowth [34, 35]. However, precise mechanisms of how GATA3 inhibited urothelial carcinogenesis need to be further determined.

GATA3 and ER signals have been highly correlated in breast cancer cell lines and tissue specimens [12, 13, 17, 36]. In a meta-analysis of multiple cancer studies, predominantly focused on breast cancer, GATA3 was suggested to be integrally involved in the ER pathway [37] whose activation is thought to be critical for breast tumorigenesis. Additionally, although no convincing evidence has verified direct regulation between the GATA3 and ER pathways, the forkhead transcription factor FOXA1, a downstream effector of GATA3, has been suggested to be cross-linked in breast cancer cells [30, 37, 38]. It is noteworthy that, like GATA3, FOXA1 is known as an urothelial differentiation marker and its loss is often seen in high-grade invasive bladder tumors [39, 40]. In non-neoplastic bladder and bladder tumor tissue specimens, we also found significant correlations of GATA3 expression with overexpression of AR or ERα or loss of ERβ [21]. Therefore, we anticipated the involvement of sex hormone receptor signals in regulation of GATA3 in urothelial cells. In the current study, we demonstrated that androgen-AR and estrogen-ERβ could down-regulate and up-regulate, respectively, the expression of GATA3 at both mRNA and protein levels in non-neoplastic urothelial cells. Androgens were also suggested to facilitate their neoplastic transformation. Emerging evidence indicates a critical role of sex hormones, espe-

cially androgen-mediated AR signals, in bladder carcinogenesis [4, 5, 7, 8], and GATA3 could thus be a key molecule regulating the carcinogenesis step(s) involving the AR pathway. However, because induction of neoplastic transformation, although less significant, was also seen in GATA3-silencing cells, the effect of AR activation on bladder cancer initiation might not be totally dependent on the regulation of GATA3 via the AR pathway. Further investigation is required to determine whether GATA3 is a direct target of the AR and ER signals in urothelial cells.

The current results showing regulation of GATA3, which likely prevents tumorigenesis, by sex hormones may be very useful for clarifying underlying mechanisms for male dominance in bladder cancer incidence. We previously demonstrated only marginal differences in GATA3 expression between male versus female bladders [*i.e.* 2.3% higher immunohistochemical score in female normal urothelial tissues (P = 0.732), 5.7% lower immunohistochemical score in female tumors (P = 0.549)] [21], while "normal" bladder tissues were obtained from patients with bladder tumor. Indeed, no studies have identified a significant gender-specific difference in the expression status of even AR or ERs in human bladder tumors [4, 5, 41]. Therefore, we suggested the importance of not only the predominance or functional activity of sex hormone receptors but also the levels of circulating sex hormones and the systemic hormonal milieu in biological functions of GATA3 [21]. Unlike GATA3 protein expression in human tissues, its mRNA levels in mouse bladders were found to be significantly higher in females than in males, further supporting the higher incidence of bladder cancer in men. Moreover, in animals with physiological levels of AR and ERs, androgen/estrogen ablation resulted in up/down-regulation, respectively, of GATA3 in their bladders, supporting our findings in SVHUC cells.

Most of bladder tumors are superficial at presentation and can typically be managed in a conservative manner. However, patients with superficial tumor carry a lifelong risk of frequent recurrence after complete resection of the tumor via a transurethral approach, even though some of them may benefit from available forms of intravesical therapy. Therefore, new options that effectively prevent bladder tumor recurrence are urgently needed. Previous studies have indicated the potential application of androgen deprivation therapy often used for patients with advanced prostate cancer to those with bladder cancer as a preventive approach [4, 5, 7-9]. Our current findings may significantly enhance the feasibility of hormonal treatment in patients with superficial tumor who have undergone transurethral resection. Furthermore, GATA3 may represent a new molecular target for the prevention of tumor recurrence.

In conclusion, GATA3 appears to play an important role in inhibiting urothelial tumorigenesis. It is also likely that androgen/estrogen decreases/increases the expression of GATA3 in normal urothelial cells presumably through the AR/ERβ pathways, respectively. These findings may therefore not only offer a molecular basis for male dominance in the incidence of bladder cancer but also contribute to the development of novel chemopreventive options targeting GATA3 or sex hormones, especially androgen-mediated AR signaling.

Disclosure of conflict of interest

None to declare.

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