

## Original Article

# Androgen receptor (AR) promotes male bladder cancer cell proliferation and migration via regulating CD24 and VEGF

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**Abstract:** Increasing evidence has proved the pivotal roles of androgen receptor in various diseases, including prostate cancer and bladder cancer. The CD24 has been proved to be correlated to bladder cancer metastasis and tumorigenesis in recent study. This study was aimed to investigate the roles of AR in bladder cell proliferation and metastasis and to explore its potential mechanism. Expressions of AR in two kinds of bladder tumor cells (T24 and UM-UC-3) were analyzed using the CRISPR Activation Plasmid transfection or siRNA-mediated gene. The effects of AR on tumor cell proliferation and migration were also analyzed. Moreover, the effects of CD24 and influence of AR on cell proliferation and metastasis-related protein were also analyzed. The results showed that AR was significantly down-regulated in T24 cells but was significantly overexpressed in UM-UC-3 cells. The up-regulated T24 promotes cell proliferation, but this enhance effect was blocked by silencing CD24. Additionally, the AR overexpression significantly increased the VEGF and CD24 expression. Besides, the migrated bladder cells was increased by the up-regulated AR, but was decreased by silencing CD24 or silencing VEGF. Taken together, our study suggested that the up-regulated AR enhances the male bladder tumor cell proliferation and metastasis via modulating the CD24 and VEGF. This study may provide theoretical basis for the possibility of AR to be a therapeutic target for bladder cancer.

**Keywords:** Bladder cancer, androgen receptor, cell proliferation, cell migration, CD24 and VEGF

## Introduction

Bladder cancer is the most common urinary system malignancy that originates from the bladder mucosa [1]. Statistics has shown that morbidity for bladder cancer is high among the urinary system malignancies [2]. Previous evidence refers that bladder cancer occurs at any age both in male and in female, but the incidence is three to four times greater in male than in female [3, 4]. There is a popular belief that sex hormone and sex hormone receptor-related signal pathway may be the most possible reasons for expanding gender difference of bladder cancer morbidity [5, 6]. However, the gender difference of morbidity for bladder cancer still remains unknown. Therefore, it will be of great significance to explore the potential mechanism of sex hormone receptor in gender difference for bladder cancer.

Androgen receptor (AR) is associated with AR-related diseases including prostate cancer,

bladder cancer, and hypospadias, as well as several signal pathways [7, 8]. AR can be activated by the combination of androgen in cytoplasm, which results in the dissociation of HSP-AR complex, and then the unbounded AR come into the nucleus [9]. A variety of evidences have reported the correlation between AR and bladder cancer, for example, the AR-mediated bladder cancer invasion and progression can be promoted by epidermal growth factor [10], and the activated AR promotes bladder cancer metastasis via slug mediated epithelial-mesenchymal transition [11].

CD24, a glycosyl phosphatidylinositol-linked sialoglycoprotein, is associated with modulating cell growth and differentiation signals and with poor outcome in urinary system tumors [12]. Many articles have shown that CD24 is associated with cancer development [13, 14], as well as in bladder cancer tumorigenesis, metastasis, and invasion [15]. Recently, it has been reported that AR may be a target for CD24

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in the male bladder cancer tumorigenesis and metastasis [16]. Although several studies have reported the role and mechanism of AR in bladder cancer, few have investigated the correlation and mechanism of AR in the development and metastasis of male bladder cancer.

In this study, we investigated the potential effects of AR expression on human bladder cell proliferation and migration using T24 and UM-UC-3 cells and siRNA-mediated gene silencing. Comprehensive experimental methods were used to assess the potential effects of AR expression on cell proliferation-related protein expression. This study aimed to elucidate the basis evidence to explain the greater incidence of bladder cancer in male. Our study may provide theoretical basis for the therapeutic possibility of targeting AR in bladder cancer treatment.

### Materials and methods

#### *Cell culture*

Human bladder cancer T24 and UM-UV-3 cell lines were cultured in DMEM medium containing 10% fetal bovine serum (FBS) in an atmosphere of 5% CO<sub>2</sub> at 37°C.

#### *MTT assay*

Cell proliferation was assessed by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay according to previous described [17]. Cells were plated onto 96-well plates at  $1 \times 10^3$ - $5 \times 10^3$  cells/well in 0.1 mL of DMEM medium. After 24 h of incubation, cells were transfected with siRNA-AR, siRNA-CD24, siRNA-control or pcDNA-AR for another 24 to 96 h of incubation. Then, 20  $\mu$ L of MTT were added to each well and incubated at 37°C for 4 h. Finally, 150  $\mu$ L of dimethylsulfoxide (DMSO) was mixed with the cells for 10 min to stop reactions. Absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan).

#### *Cell migration assay*

Cells in each group were cultured in 6-well plates at the density of  $5 \times 10^5$  per well using Matrigel method [18]. The upper layer of Transwell was enveloped with serum-free DMEM medium containing 50 mg/L of Matrigel and then air-dried at 4°C. After being sucked

out the medium, 50  $\mu$ L of fresh serum-free medium containing 10 g/L of BSA was added and then cultured at 37°C for 30 min. After that, Transwell was put into the 24-well plates and cultured with DMEM (Dulbecco's Modified Eagle Medium) medium (Sigma, USA) mixed with 10% FBS. Then cells in Transwell were suspended with serum-free DMEM medium. After 48 h of incubation, Transwell in each group was washed with PBS buffer to remove the upper cells on microporous membrane, followed with fixed in ice-cold alcohol. Finally, Transwell from each group was stained with 0.1% crystal violet for 30 min, and then decolorated with 33% acetic acid. The absorbance of eluents was observed at OD 570 nm using a microplate reader (Biotech, USA). Transwell in control group was treated without Matrigel.

#### *Colony formation assay*

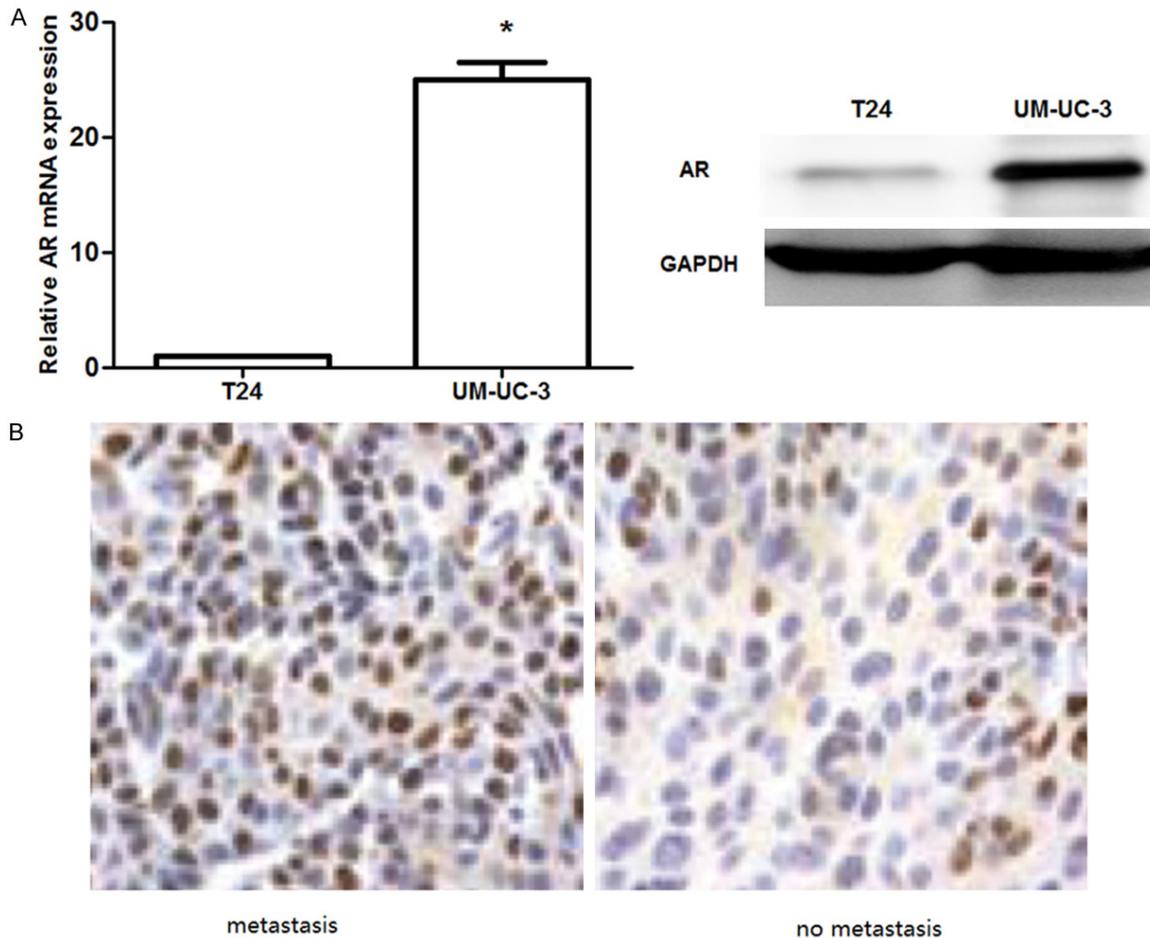
Clonogenic assay was performed with a modification of previously published method [19]. Briefly, after 24 h of siRNA transfection, the cells were plated into 60-mm dishes at a cell density of 100 cells/dish. Cells were then allowed to grow in DMEM medium supplemented with 10% FBS for 14 days. Cells were then fixed and stained with Diff-Quick. After air dry, colonies were counted under microscope and cell number each colony was at least 20 cells.

#### *Plasmid and cell transfection*

Clustered regularly interspaced short palindromic repeats (CRISPR) Activation Plasmid transfection is the fewest method for plasmid transfection worldwide, which can integrate the plasmid onto the genome sequence, and resulting in the permanently expression for target genes [20]. For plasmid transfection, the AR CRISPR Activation Plasmid (h2) was chosen as an alternative activation plasmid designed to specifically up-regulate AR gene expression [21]. The sgRNA (MS2) plasmid in AR CRISPR Activation Plasmid (h2) encodes a target-specific 20-nt guide RNA that differs from the guide RNA in AR CRISPR Activation Plasmid (h). Cells were plated onto the 6-well plates for 24 h incubation, and then 0.4  $\mu$ g of plasmid was added into cells for another 48 h of incubation.

For siRNA transfection, the cells were plated onto the 60-mm dish, and after 24 h of incubation, cells were treated with Lipofectamine

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**Figure 1.** Expression of androgen receptor (AR) in bladder cancer cell lines and tissues. A: Protein and mRNA levels of AR in T24 and UM-UC-3 cells, AR was highly expressed in UM-UC-3 cells but was lowly expressed in T24 cells; B: Immunohistochemistry analysis of AR in metastasis and non-metastasis bladder cancer tissues, the positive rate of AR in metastasis bladder cancer tissues was higher than that in non-metastasis cancer tissues. \*:  $P < 0.05$  compared to the control.

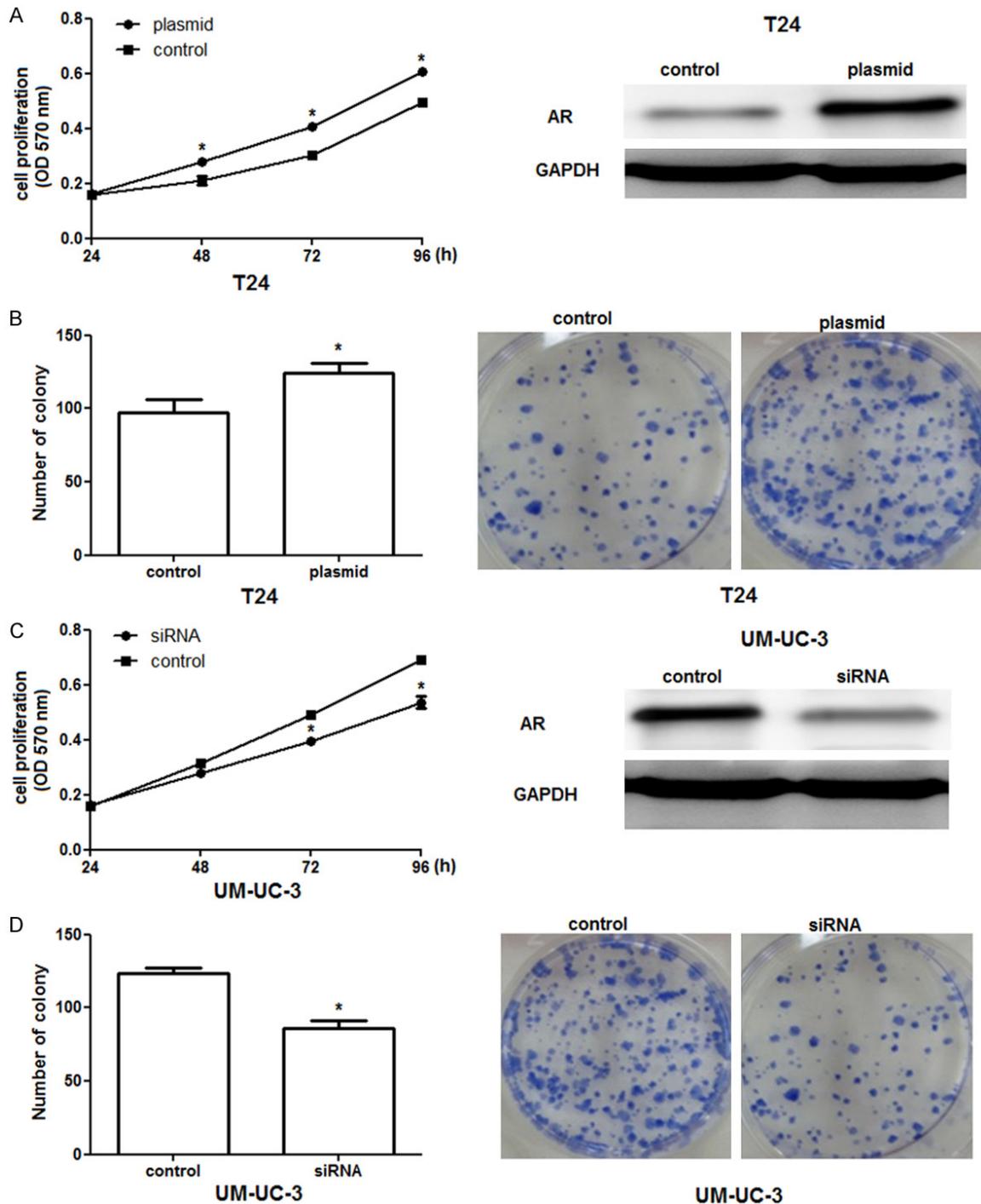
2000 transfection reagent (Life Technologies, USA). Mature miR-141 (mirVana™ miRNA mimic; Ambion, Foster City, CA, USA) and miR-141 inhibitor siRNA-CD24 or siRNA-control vectors was transfected into cells. After 48 h of transfection, cells were prepared for further analysis.

### Western blotting

Cells cultured for 48 h were lysed with RIPA buffer (Radioimmunoprecipitation; Sangon Biotech, China) containing PMSF (phenylmethanesulfonyl fluoride; Sigma, USA), and the lysates were then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were collected, and the protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Protein samples (30 µg per lane) were sepa-

rated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto polyvinylidenedifluoride (PVDF) membranes (Mippro). Then the PVDF membranes were blocked in Tris-Buffered Saline/Tween (TBST) containing 5% non-fat milk for 1 h at room temperature. Membranes were incubated with rabbit anti-human antibodies (CD24, VEGF, MMP9, and AR; 1:100 dilution, Invitrogen) and overnight at 4°C. Consequently, membrane was incubated with horseradish peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the PVDF membranes were washed 3 times with 1× TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control.

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**Figure 2.** Influence of AR expression on bladder cancer cell proliferation. A, B: pcDNA-AR transfection promotes the T24 cell proliferation and colony formation compared to that in controls; C, D: siRNA-AR transfection inhibited the UM-UC-3 cell proliferation and colony formation compared to the controls. \*:  $P < 0.05$  compared to the control.

### Immunohistochemistry

Human tissue samples were fixed in formalin, paraffin embedded, and sectioned (6  $\mu$ m) with a Microm HM 355S microtome (Thermo Fisher Scientific, Waltham, USA). Antigens were

retrieved using antigen unmasking solution (Vector Laboratory, Burlingame, CA). After antibody was incubated for 30 min at 37°C, reaction was blocked, and then slides were incubated overnight at 4°C with an anti-AR antibody (ab74272, abcam). Staining of the tissue was

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performed with the SignalStain® DAB Substrate Kit followed by hematoxylin counterstain.

### *Real time PCR*

Total RNA was isolated from cells after 48 h of transfection using TRIzol Reagent (Invitrogen) as previously described [22]. Isolated RNA was treated with RNase-free DNase I (Promega Biotech, USA), and the concentration and purity of the isolated RNA were determined using SMA 400 UV-VIS (Merinton, Shanghai, China). cDNA synthesis was conducted using the reverse transcriptase PrimeScript 1<sup>st</sup> Strand cDNA Synthesis Kit (Invitrogen, USA). The expression levels of miRNAs were detected using the SYBR ExScript RT-qPCR Kit (Takara, China). Phosphoglyceraldehyde dehydrogenase (GAPDH) was chosen as the internal control. Primers used for target amplification are AR sense: 5'-AAGACGCTTCTACCAGCYCACCAA-3', and anti-sense: 5'-TCCCAGAAAGGATCTGGGCACTT-3'; GAPDH sense: 5'-TATGATGATATCAAGAGGGTAGT-3', and GAPDH anti-sense: 5'-TGTATCCAACTCATTGTCATAC-3'.

### *Statistically analysis*

All experiments were conducted independently 3 times. Data are presented as the mean ± SD. Statistical analyses were performed using Graph Prism 5.0 software (GraphPad Prism, San Diego, CA). Significant differences in the data between two groups were analyzed using one-way analysis of variance (ANOVA). A *P* value less than 0.05 was considered to indicate a significant difference.

## Results

### *AR expression in T24 and UM-UC-3 cell lines*

The mRNA level and protein level of AR in T24 and UM-UC-3 cells were detected, and results showed that AR was slightly expressed in T24 cells but was overexpressed in UM-UC-3 cells (**Figure 1A**). In addition, the expression of AR in metastasis bladder tissues was higher than that in non-metastasis bladder tissues (**Figure 1B**).

### *AR expression was correlated with bladder cancer cell proliferation*

When T24 cells were transfected with pcDNA-AR vector, cell proliferation was significantly

increased compared with the control group from 48 h till 96 h ( $P < 0.05$ , **Figure 2A**), number of T24 colony was also significantly increased compared with the control group ( $P < 0.05$ , **Figure 2B**). Besides, effects of AR expression on UM-UC-3 cell proliferation was assessed. The results showed that when UM-UC-3 cells were transfected with siRNA-AR vector, cell proliferation ability was significantly declined compared to that in control, as well as the number of colony ( $P < 0.05$ , **Figure 2C** and **2D**). These results indicated that AR overexpression could promote bladder cancer cell proliferation.

### *AR promotes cell proliferation by regulating CD24 in T24 cells*

Cell proliferation of T24 cells in each group was analyzed using siRNA transfection and pcDNA-AR transfection method (**Figure 3**). Compared with the control group, T24 cell proliferation was significantly suppressed by silencing CD24 but was significantly increased by overexpression of AR at the time of 48 h ( $P < 0.05$ ). When T24 cells were transfected with both si-CD24 and pcDNA-AR vectors, cell proliferation ability was significantly decreased compared with that in control group ( $P < 0.05$ ), suggesting that AR may play certain role in CD24-mediated cell proliferation in T24 cells. Besides, the tendency of number of colony formation in each experimental group was similar to the tendency change of cell proliferation ability (**Figure 3B**).

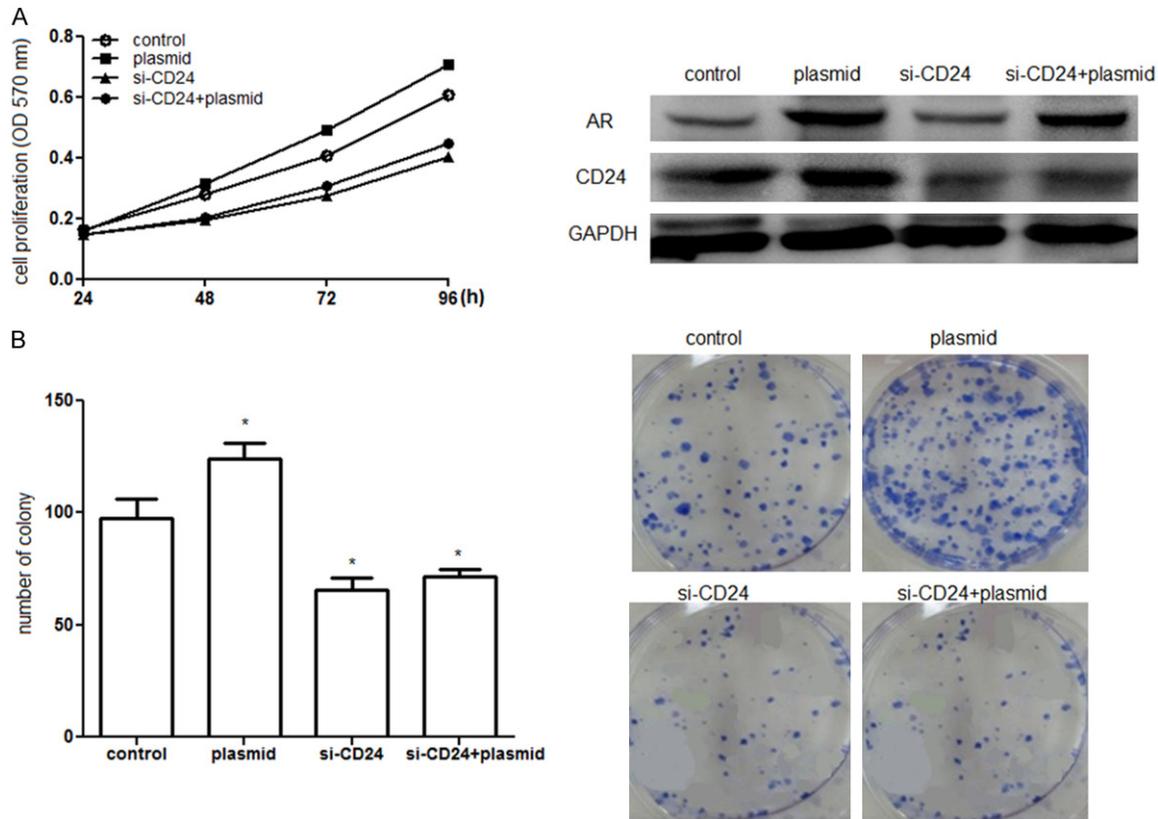
### *AR expression was correlated with bladder cancer cell migration*

We then investigated the effects of AR expression on bladder cancer cell migration in T24 or UM-UC-3 cells via overexpressing or silencing AR (**Figure 4**). The number of migrated T24 cells was significantly increased compared with the control by overexpression of AR in T24 cells ( $P < 0.05$ , **Figure 4A**). However, the number of migrated cells was declined compared to the control by silencing AR in UM-UC-3 cells ( $P < 0.05$ , **Figure 4B**), suggesting that high AR level may contribute bladder cell migration.

### *Expression of VEGF and MMP9 in bladder cancer cells*

Previous evidence has shown that vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP9) are two major cell

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**Figure 3.** AR regulates T24 cell proliferation via affecting CD24 expression. A: Cell proliferation ability was increased by AR overexpression but was suppressed by silencing CD24 compared to the control. When cells transfected with siRNA-CD24 and pcDNA-AR vectors, cell proliferation ability was suppressed by co-transfection of CD24 and AR, indicating the negatively correlation between CD24 and AR in regulating cell proliferation. B: The number of colony was significantly increased by the overexpressed CD24, but was significantly decreased by the silencing of CD24.

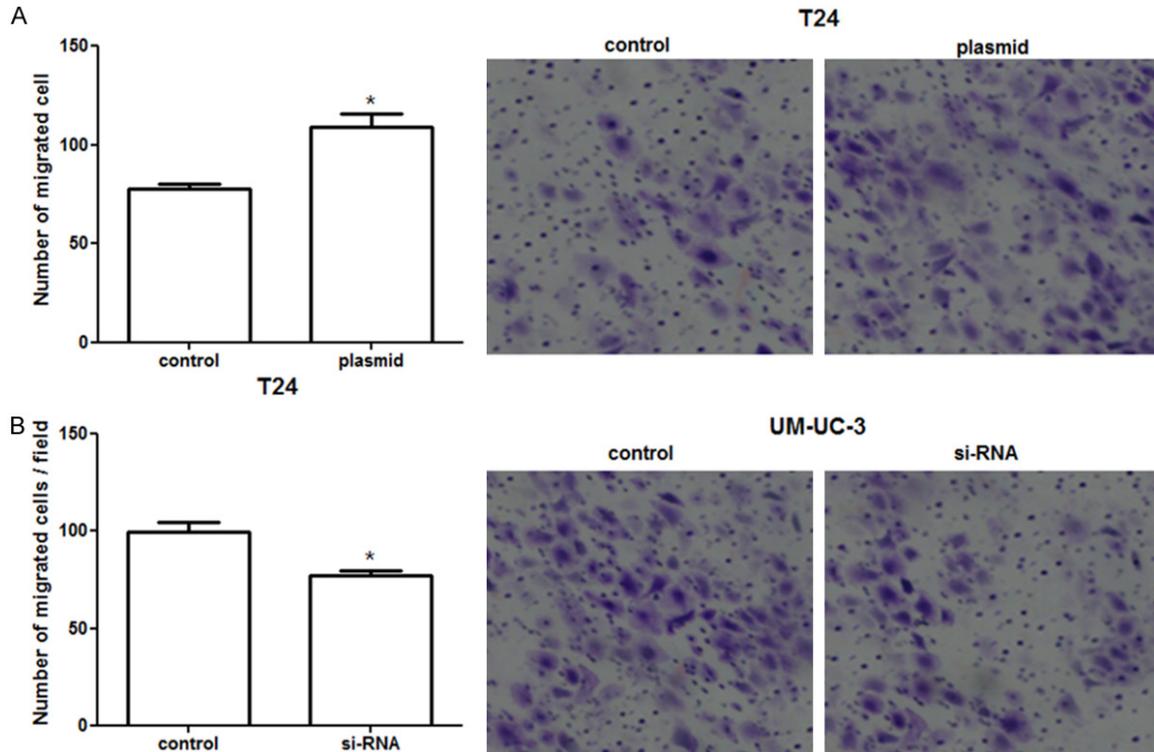
migration-related factors, and they are regulated by AR in several kinds of diseases, namely they are target genes for AR [23, 24]. The results showed that the number of migrated cells was decreased by silencing CD24 compared to the control ( $P < 0.05$ ), also, migrated cells was decreased by silencing VEGF ( $P < 0.05$ ), indicating that VEGF or CD24 expression was positively correlated with cell migration (Figure 5A and 5C). Subsequently, the VEGF level was highly increased while CD24 was down-regulated by AR overexpression in T24 cells, however, VEGF level was drastically declined by silencing AR in UM-UC-3 cells (Figure 5B). Interestingly, the results showed that MMP9 level was slightly but not significantly declined by silencing AR, implying that AR expression may be positively correlated with VEGF expression in bladder cancer cells.

### Discussion

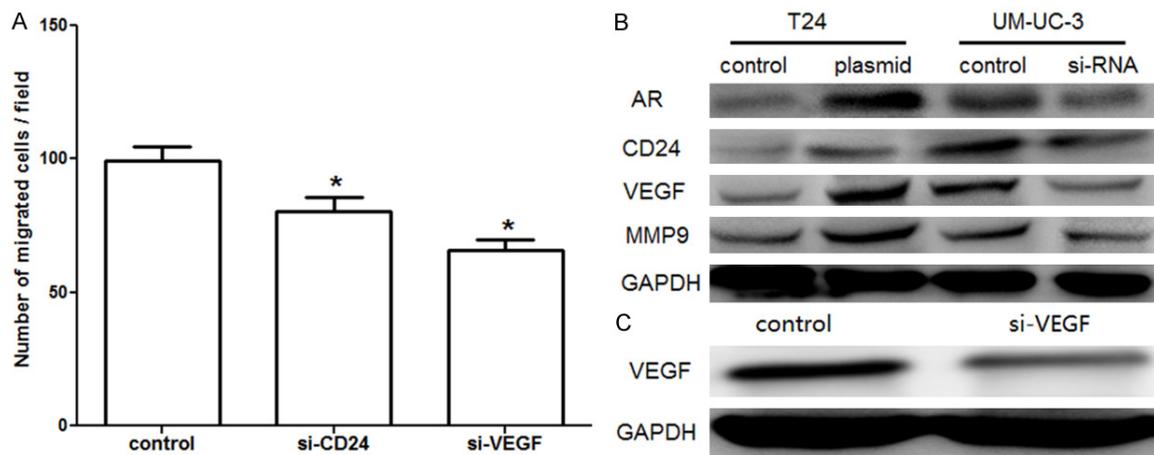
Previous evidence has revealed that there was gender difference of incidence for bladder cancer

[4, 25]. AR was involved in the biology of various diseases, including bladder cancer [4, 6, 26]. To date, few have reported the correlation between AR expression and the gender difference of incidence for bladder cancer. In this study, we investigated the roles of AR expression in male bladder cancer cell proliferation and metastasis using the T24 and UM-UC-3 cells and the siRNA-mediated gene and CRISPR activation plasmid transfection [20]. In agreement with previous study [5], the data presented in our study showed that AR was significantly increased in UM-UC-3 cell but significantly decreased in T24 cells, and AR was highly expressed in metastatic bladder cancer tissues, indicating the correlation between AR expression and bladder cancer pathogenesis. Moreover, T24 cell proliferation or migration was significantly increased by the overexpressed AR while the UM-UC-3 cell proliferation or migration was significantly suppressed by the silencing AR, however, the promote role of AR overexpression on T24 cell proliferation was partly blocked by silencing CD24 while the cell

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**Figure 4.** Effects of AR expression on bladder cancer cell migration. A: Overexpression of AR promotes the number of migrated T24 cells; B: Silencing AR declined the number of migrated UM-UC-3 cells. \*:  $P < 0.05$  compared with the control.



**Figure 5.** Influence of AR expression on cell migration-related factors expression in bladder cancer cells. A: The number of migrated cells was significantly decreased by silencing CD24 or silencing VEGF compared with that in control; B: Western blotting analysis showed that pcDNA-AR transfection resulted in the high expression of VEGF and MMP9 but the low expression of CD24 in T24 cells, but silencing AR lead to the down-regulation of VEGF and MMP9 in UM-UC-3 cells; C: Expression of VEGF in T24 cells by western blotting. \*:  $P < 0.05$  compared with the control.

migration was inhibited by silencing VEGF. In addition, the CD24 or VEGF expression was significantly increased by AR overexpression in T24 cells but was down-regulated by silencing AR in UM-UC-3 cells.

The promote roles of AR on tumor cell proliferation have been mentioned a lot in a variety of male-related diseases, such as prostate cancer and bladder cancer [5, 27]. Miyamoto et al. proved that the cell proliferation in mice with

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bladder cancer was promoted by the up-regulated AR [5]. Our results showed that AR overexpression promotes T24 cell proliferation while silencing AR suppresses UM-UC-3 cell proliferation (**Figure 2**), suggesting that the AR overexpression may contribute bladder cell proliferation. Meanwhile, it has been demonstrated that the up-regulated CD24, which is a cell proliferation-related factor, contributes bladder tumor growth and metastasis via affecting cell proliferation in male mice [16]. Our data showed that T24 cell proliferation was suppressed by silencing CD24, and the promote effect of AR on T24 cell viability was partly blocked by silencing CD24 (**Figure 3**); we speculated that AR overexpression may contribute T24 cell proliferation via modulating CD24.

On the other side, we further analyzed the correlation between AR and bladder metastasis. Hornberg et al. proved that the activated AR promotes castration-resistant prostate cancer bone metastasis [28]. However, few have been demonstrated the correlation between AR and bladder tumor metastasis. Our data showed that the migrated T24 or UM-UC-3 cells were promoted or suppressed by the up-regulated or silencing AR respectively (**Figure 4**), indicating that AR expression was associated with bladder cancer cell migration. Consequently, we detected the influence of AR expression on VEGF and MMP9 expression to identify the potential mechanism for AR on bladder metastasis. VEGF and MMP9 are two cell metastasis-related factors, thereinto, VEGF contributes vessel formation and then results in the tumor progression, while MMP9 promotes the release of VEGF via degrading matrix in cells and then contribute tumor progression [29, 30]. The endothelial cell proliferation was stimulated by androgen via AR-VEGF signals [31]. Wang et al. had proved that endothelial cells promotes prostate cancer cell metastasis via AR-MMP9 signals [24], and the recent report proves that bladder tumor metastasis and tumorigenesis are mediated by CD24 and AR [16]. Our results revealed that the migrated cells were decreased by silencing VEGF, and the VEGF expression was significantly increased by the up-regulated AR in T24 cells, but was opposite the that in UM-UC-3 cells (**Figure 5**), implying that AR overexpression may promote bladder cell migration through up-regulating VEGF and CD24. However, the MMP9 expression was slightly but not significantly increased by the up-regulated

AR, indicating that MMP9 may not be involved in bladder cell metastasis.

Taken together, this study used the worldwide newest CRISPR activation plasmid transfection and siRNA-mediated gene to analyze the effects of AR overexpression or suppression on bladder tumor proliferation and metastasis. The data presented in this study reveals that AR overexpression promotes the CD24-mediated bladder cell proliferation and the VEGF-mediated metastasis. This study may provide theoretical basis for the future exploration of gender difference of incidence for bladder tumor and provide the possible of target therapeutics for AR in bladder cancer. Further comprehensive experimental studies are still needed to search the deep mechanism.

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