Am J Cancer Res 2016;6(2):238-248
www.ajcr.us /ISSN:2156-6976/ajcr0018970

Original Article

Androgen receptor expands the population of cancer stem cells in upper urinary tract urothelial cell carcinoma cells

Chi-Cheng Chen[1,2], Teng-Fu Hsieh[1,2], Chi-Ping Huang[1], Ai-Lin Yu[1], Wen-Lin Chang[1], Chih-Rong Shyr[1]

[1]*Sex Hormone Research Center, Departments of Urology/Surgery and Medical Laboratory Science and Biotechnology, Graduate Institute of Clinical Medical Science, China Medical University/Hospital, Taichung 404, Taiwan;* [2]*Department of Urology, Taichung Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taichung 404, Taiwan*

Received November 2, 2015; Accepted November 27, 2015; Epub January 15, 2016; Published February 1, 2016

Abstract: Androgen receptor (AR) affects the development and progression of upper urinary tract urothelial cell carcinoma (UUTUC). However, the regulatory mechanism exerted by AR to affect UUTUC cells remains unclear. Here we investigated whether AR promotes UUTUC development and progression, possibly by expanding the population of cancer stem cells (CSCs), which are a particular population of cells within cancer cells responsible for tumor initiation, drug resistance and metastasis. We compared UUTUC cells with or without the addition of AR on their CSC population with flow cytometry, colony formation and sphere formation assay to determine the effect of AR on CSC activity, and real-time PCR was used to detect the expression stemness genes and miRNAs. In vivo tumor formation was evaluated with the implantation of cancer cells in nude mice. We found that the addition of AR in UUTUC cells, significantly increased the population of CSC, clonogenicity, sphere formation and the expression of stemness genes (Oct4, Bmi1 and Nanog), altered CSC-related miRNA profile, as well as promoted epithelial mesenchymal transition (EMT). And AR inhibitor, enzalutamide was shown to suppress AR's effect on tumorsphere formation. Furthermore, in an immune-deficient mouse model, the addition of AR in UUTUC cells also increased the tumor formation capacity. This study will help us better understand the extent to which AR contributes to UUTUC progression by expanding their CSC population and capacity. Our findings could explain high incidence of UUTUC observed in males. And targeting AR may lead to novel therapeutic approaches for genetically diversified urothelial carcinomas in precision medicine era.

Keywords: Androgen receptor, upper urinary tract urothelial cell carcinoma, cancer stem cells, tumorsphere

Introduction

UUTUC originating in the urothelium, is an aggressive and lethal urologic malignancy since patients with primary stage T4 tumors have 5-year disease-specific survival rate less than 10% and a median survival of 6 months only [1-3]. UUTUC patients initially respond to surgery and chemotherapy, but the long-term prognosis is generally unfavorable with recurrence and metastasis [4, 5]. Therefore, it is critical to identify new therapeutic targets that prevent recurrence and prolong survival for UUTUC patients.

CSCs are a small population of cells in heterogeneous tumor tissues, which could self-renew and differentiate [6]. CSCs could initiate tumors and are more resistant to chemotherapy and radiotherapy and linked to metastasis and poor prognosis [7]. CSCs have been reported to be present in multiple types of solid tumor and cultured cancer cell lines, including brain, breast, colon, prostate and urothelial tumors [6, 8-11]. The existence of CSCs in urothelial tumors is further substantiated by the presence of urothelial stem cells in the basal cell layers of urothelium, which are responsible for regenerating the damaged urothelium caused by physical, chemical or biological stresses [12]. Urothelial CSCs are similar to normal urothelial stem cells with the ability to self-renew and generate all of the heterogeneous cells that comprise a tumor [11]. The expression of CSC makers have been linked to the recurrence, invasive progression and worse prognosis of urothelial carcinomas [13-17], suggesting CSCs are involved in the progression and recurrence of UUTUC. The

understanding of how CSCs arise is critical to develop a novel therapeutic approach to block their presence and provide long-term disease-free survival to afflicted patients.

UUTUC afflicts more men than woman with a male-to-female ratio of 2~3 to 1 [1, 18, 19], and females were associated with a better survival [4], indicating that male hormones, androgens may play an important role in the progression of UUTUC. Our previous studies have demonstrated the androgen receptor (AR), which mediates androgen actions, affects the UUTUC cell migration and invasion as well as the response to anti-cancer drugs [20, 21]. Other study also indicates that the actions of AR could be linked to the regulation of CSCs since AR was shown to occupy the CD44 loci [22], which is a urothelial CSC maker [11]. Therefore, the objectives of our study were to examine whether CSC population and capacities in UUTUC cells could be altered by AR.

In this study, we firstly aimed to determine whether AR could affect the CSC population and functions by evaluating CD44 population, sphere and colony formation as well as stemness gene expression under the influence of AR. We also examined the expression of miRNAs and EMT makers affected by AR. In this study we identify that AR is an important factor to affect the development and progression of UUTUC.

Materials and methods

Cell culture

The human UUTUC cell line, BFTC 909, (from a UUTUC of renal pelvis patient) was a generous gift from Dr. Tzeng CC [23] and 7630 cells (also established from a UUTUC of renal pelvis patient) [24] were cultured in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin. The cells were maintained in a humidified 5% CO_2 incubator at 37°C. The generation of BFTC pWPI and BFTC hAR cells from parental BFTC 909 cells or the 7630 pWPI and 7630 hAR cells from parental 7630 cells was described previously [20, 21].

Flow cytometry of CD44+ cells

Cell suspensions obtained from BFTC pWPI and BFTC hAR cells were tested for fluorescent labeled monoclonal antibodies for CD44 antigens and respective IgG isotype controls. The antibodies were incubated for 30 min at 4°C. After washing, the labeled cells were analyzed by flow cytometry using a BD LSR II flow cytometer (Becton & Dickinson, Mountain View, CA, USA).

Quantitative reverse transcriptase-polymerase chain reaction (Q-RT-PCR)

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and used for first-strand cDNA synthesis. The mRNA levels were measured using CFX96™ real-time system (Bio-Rad Laboratories) using KAPA SYBR® fast qPCR kits (Kapa Biosystems, Inc., Woburn, MA, USA). The mRNA and miRNA expression level were determined using the $2^{(\Delta\Delta Ct)}$ method.

Colony formation assay

The cells were plated at a density of 1×10^3 cells on 6 cm plate. After a 10-day incubation at 37°C in a humidified atmosphere containing 5% CO_2, cell monolayers was washed with saline, fixed with 100% methanol, and stained with 0.5% crystal violet solution. Colonies, defined as being more than 50 cells, were counted with the use of a light microscope.

Sphere formation assay

The cells were plated as single cells in ultra-low attachment six-well plates at a density of 5,000 viable cells/well. Cells were grown in a serum-free medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA), 20 ng/ml human recombinant epidermal growth factor (hrEGF), 10 ng/ml human recombinant basic fibroblast growth factor (hrbFGF), 5 μg/mL insulin and 0.4% BSA (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator (5% CO_2) at 37°C. For secondary tumorsphere culture, the primary tumorspheres were dissociated into single cells filtered through a 40 μm cell strainer to ensure the single cells suspension. 2,500 cells were seeded into each well of six-well plate. Tumorsphere was observed under microscope 10-15 days later.

The subcutaneous xenografts in nude mice

BALB/c nude mice (BALB/cAnN.Cg-Foxn1nu/CrlNarl) purchased National Laboratory Animal Center (Taipei, Taiwan) were injected with 1 ×

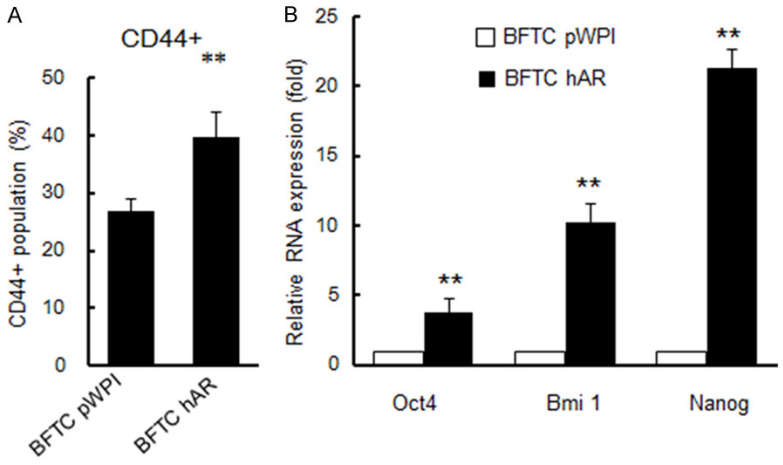

Figure 1. Expansion of CD44+ population and increase of stemness genes in BFTC 909 hAR cells. A. BFTC pWPI cells and BFTC hAR cells were analyzed for bladder CSC marker CD44, cell surface antigen by flow cytometry using a CD44 APC -labelled antibody and IgG1 isotype control antibody. B. Cancer stem cell stemness gene Oct4, Bmi-a and Nanog was examined by Q-RT-PCR between BFTC pWPI and BFTC hAR cells. Data are expressed as mean ± SD from three independent experiments (n = 3) (**P<0.01. vs control).

10^6 and 5 × 10^6 BFTC pWPI or BFTC hAR cells on both the right and left flanks were injected with BFTC pWPI or BFTC hAR cells respectively, for a total of 12 injection sites per condition. Mice were monitored for tumor formation up to 3 months post-injection, and each injection site was scored as either positive (palpable tumor) or negative (no palpable tumor). All animal procedures followed the Guide for the Care and Use of Laboratory Animal of the Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, U.S.A and were approved by the animal care and use committee of the China Medical University, Taiwan.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on paraffin embedded sections (5 μm thick). Paraffin sections were dewaxed and rehydrated through a graded series of ethanol washes, and maintained in phosphate buffered saline (pH 7.4; PBS). Sections were treated with primary anti OCT4 or anti-MMP9 antibodies, followed by biotin conjugated secondary antibody. Vectorstain ABC kit from Vector Laboratories (Burlingame, CA, USA) was used for immunohistochemistry, followed by DAB to visualize immunocomplexes. For quantification of immunostaining, the number of positively stained cells was counted in 4 random fields at a magnification of 40 ×.

Statistical analysis

For in vitro cell based experiments, data are expressed as mean ± SD from at least three independent experiments. Results were analyzed using two-tailed paired t-tests. For animal studies, tumor-free survival in BFTC pWPI and BFTC hAR cells implanted mice was determined via Kaplan-Meier analysis with comparison of curves using the survival log-rank test. A *P*-value < 0.05 was considered to be statistically significant.

Results

AR enriches the CD44 population and increases stemness gene expression of UUTUC cells

Since AR has been demonstrated to affect the migration, invasion and chemoresistance of UUTUC cells [20, 21, 25], we suspected that these actions could be linked to CSCs because CSCs are involved in cancer progression and chemoresistance. First we examined the proportion of CD44 population in UUTUC cells, BFTC 909 cells with or without the addition of AR. CD44+ cells are found in the basal layer of normal urothelium as well as urothelial carcinoma and it has been used as a CSC marker [8, 11]. Flow cytometry was performed to determine the CD44+ population of BFTC pWPI and BFTC hAR cells. The BFTC hAR cells contained higher cell fraction of the CD44+ cells than BFTC pWPI cells (**Figure 1A**), suggesting that AR in BFTC 909 cells is able to expand CSC population. To further confirm that AR is able to increase the CSC population in UUTUC cells, we examined the expression of several CSC stemness maker genes (Oct4, Bmi1 and Nanog), which were shown to be expressed in urothelial carcinomas [11]. The quantitative RT-PCR (Q-RT-PCR) analysis showed that the BFTC hAR cells expressed higher mRNA levels of Oct4, Bmi1 and Nanog (**Figure 1B**). This result showed that the stemness genes in UUTUC cells could also be enhanced by the addition of AR, which is consistent with the flow cytometry data, indicating that higher expression of CSC makers in BFTC hAR cells.

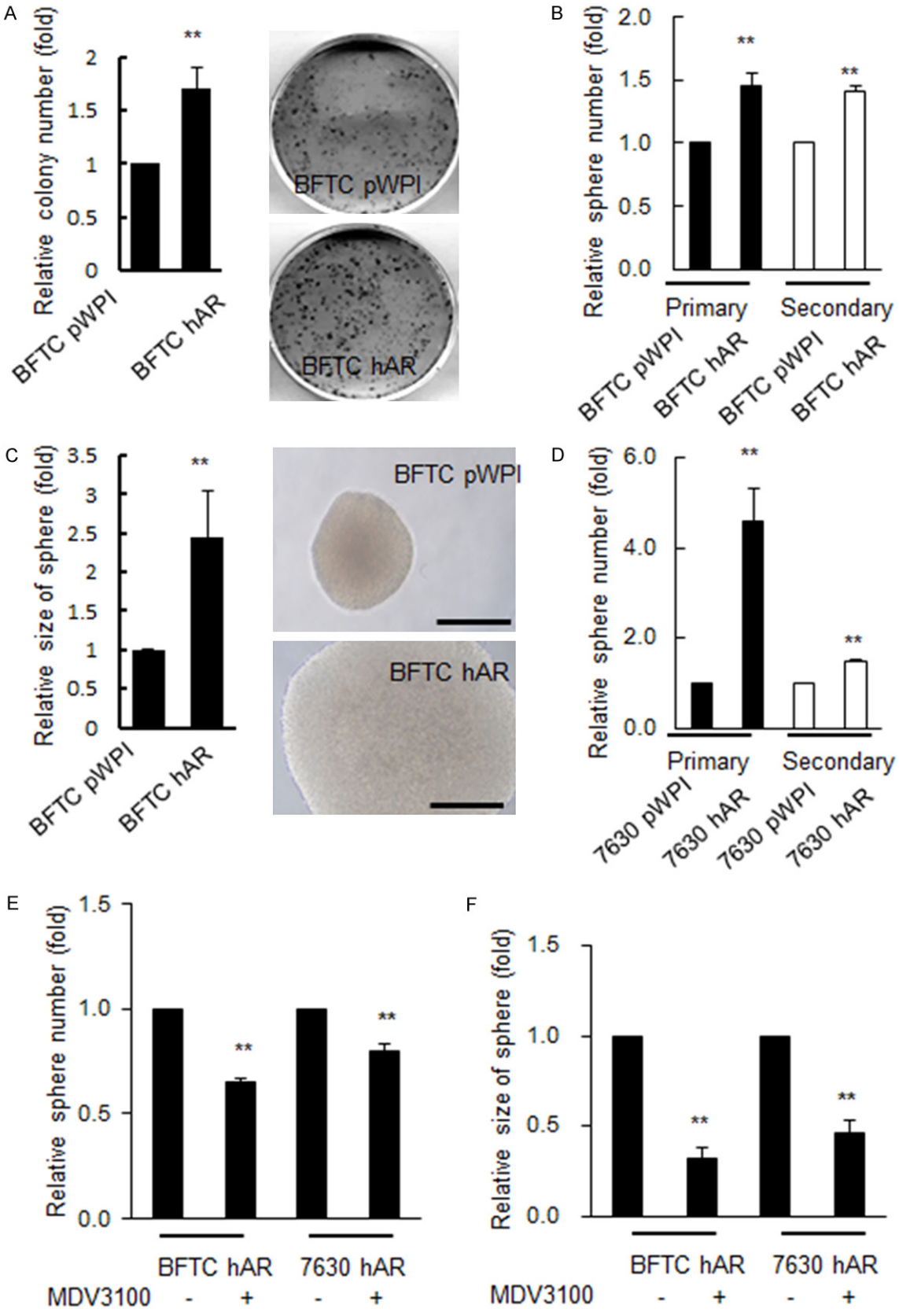

Figure 2. The clonogenicity and tumorsphere formation were increased in BFTC hAR cells. A. The colony formation of BFTC pWPI cells or BFTC hAR cells was photographed after being cultured for 10-14 days. BFTC pWPI cells or BFTC

hAR cells were seeded on 6 cm tissue culture dishes and incubated for 10-14 days. At the end of incubation period, numbers of colonies were counted. The relative colony number was calculated by setting the control (BFTC pWPI cells) as one-fold. B. The tumor sphere formation analysis of BFTC pWPI or BFTC hAR cells. The BFTC pWPI or BFTC hAR cells were grown in six-well ultralow attachment plates at a density of 5,000 cells/well in defined medium at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 10-15 days. At the end of incubation period, spheres were counted. The relative sphere number of primary spheres and secondary spheres was calculated by setting the control (BFTC pWPI cells) as one-fold. C. The size of spheres was also measured. The relative sphere size was calculated by setting the control (BFTC pWPI cells) as one-fold. Scale bar, 20 μm. D. The tumor sphere formation analysis of 7630 pWPI or 7630 hAR cells. E. Effect of enzalutamide (MDV3100) on tumorshpere formation of BFTC hAR and 7630 hAR cells. BFTC hAR or 7630 hAR cells were treated with 10 μM enzalutamide in media and assayed for tumorsphere formation and the relative sphere number was calculated after 10-15 day incubation. F. The relative size of tumorspheres from BFTC hAR or 7630 hAR cells with or without enzalutamide treatment were compared. Data are expressed as mean ± SD from three independent experiments (n = 3) (**P<0.01 vs control).

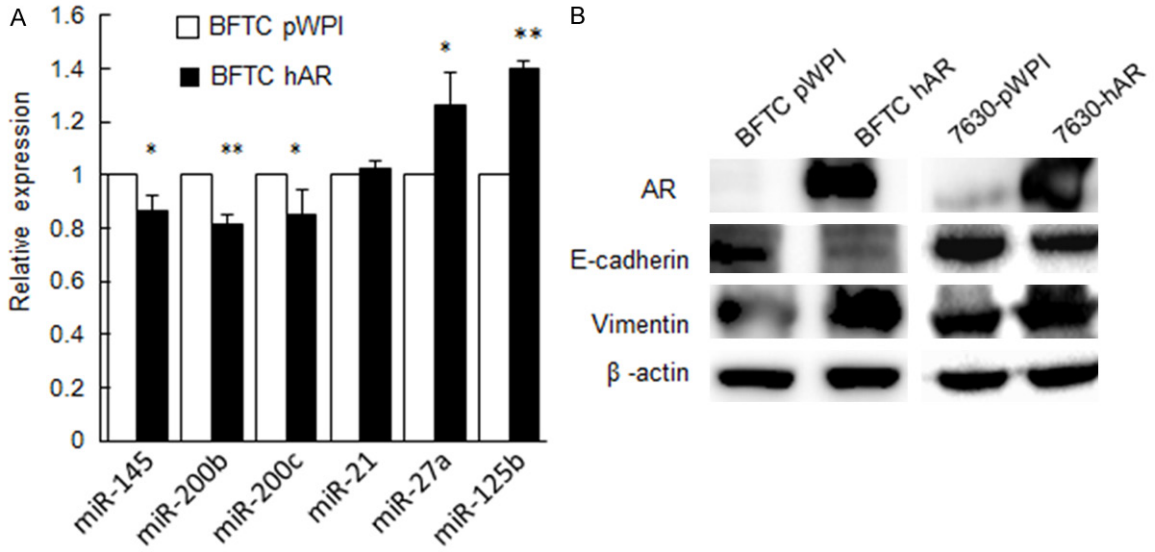

Figure 3. The CSC-related miRNA expression profile and EMT marker expression in BFTC pWPI and BFTC hAR. A. CSC-related miRNAs in BFTC pWPI and BFTC hAR were determined using Q-RT-PCR. Data are expressed as mean ± SD from three independent experiments (n = 3) (*P<0.05, **P<0.01. vs control). B. The expression of E-cadherin and Vimentin in BFTC pWPI and BFTC 909 hAR cells as well as 7630 pWPI or 7630 hAR cells. The cells were lysed. Equal amounts of protein were analyzed by SDS-PAGE and immunoblotted with anti-E-cadherin, anti-Vimentin, and anti-β-actin antibodies. The representative blot from three independent experiments is shown.

AR affects the colony and tumorsphere formation of UUTUC cells

To determine whether the clonogenicity of UUTUC cells are affected by AR, colony formation assay was performed to examine cell ability to repopulate themselves. The result showed that by the BFTC hAR cells increased their capacity to form colony with higher number of colonies when compared to BFTC pWPI cells (**Figure 2A**). Another characteristic of stem cells is the ability to form spheres. We therefore, further examined the function of AR in promoting the capacity of CSCs to survive and proliferate in anchorage-independent conditions and ability to form tumorspheres. Our results demonstrated that BFTC hAR cells had higher tumorsphere forming capacity both for primary and secondary tumor spheres (**Figure 2B**) and produced larger size of tumorsphere (**Figure 2C**) than BFTC pWPI cells. Similarly, when we used another UUTUC cells generated from a UUTUC of pelvis patient, 7630 cells, the addition of AR also increased the ability of 7630 cells to form primary and secondary tumorspheres (**Figure 2D**). Furthermore, since the presence of AR affects the colony and tumorsphere formation in UUTUC cells, the inhibition of AR could suppress he colony and tumorsphere formation. To test this notion, we treated BFTC hAR and 7630 hAR cells with a potent AR inhibitor, enzalutamide (MDV3100) which binds to the androgen receptor with greater relative affinity than the clinically used antiandrogen bicalutamide and reduce the efficiency of its nuclear translocation as well as impair both DNA binding to

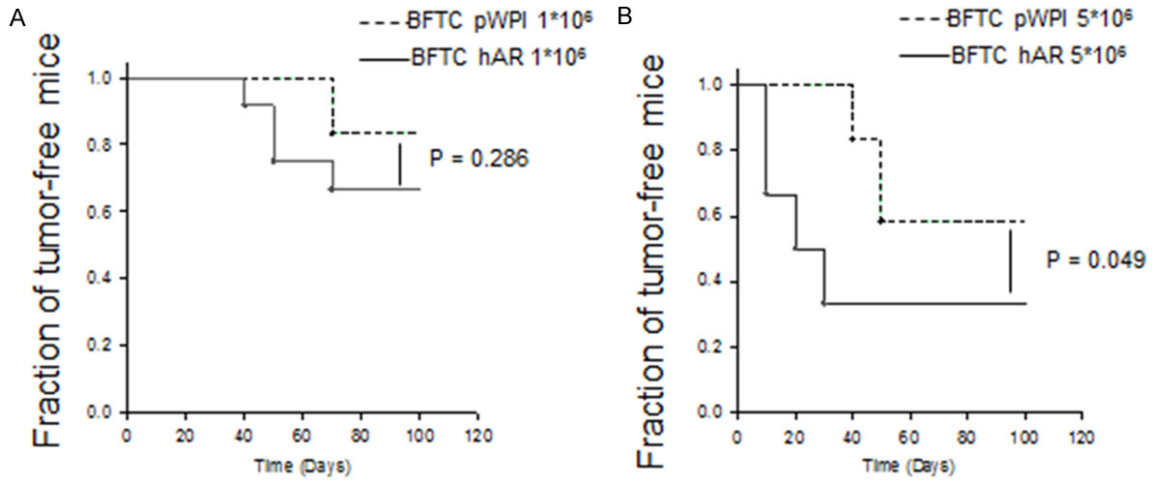

Figure 4. Comparison of tumor occurrence after injection of variable number of BFTC pWPI and BFTC hAR cells in nude mice. A. Injection of $1*10^6$ cells. B. Injection of $5*10^6$ cells.

androgen response elements and recruitment of coactivators [26]. And we found that both the number and size of tumorspheres were decreased by enzalutamide treatment (**Figure 2E**, **2F**), indicating that enzalutamide antagonized the effects of AR in increasing tumorsphere formation of UUTUC cells. Therefore, we demonstrated that AR in UUTUC cells could increase cellular capacity of CSC in UUTUCs.

Differential miRNA expression and EMT transition in BFTC hAR and BFTC pWPI cells

To examine the potential mechanism by which AR expand CSC population and promote their capacities of clonogenicity and tumorsphere formation, we looked at whether AR could affect the expression of micro RNAs (miRNA), which are 21-25 nucleotides long, non-coding RNAs that post-transcriptionally regulate gene expression [27]. The functions of miRNAs are not only involved in the initiation and progression of cancer, but also essential in regulating self-renewal and differentiation of CSCs [28, 29]. Therefore, we used Q-RT-PCR to compare the expression several CSC-related miRNAs in BFTC pWPI and BFTC hAR cells. As the result shown, the BFTC hAR cells had higher expression of miR-27a and miR-125b, but lower expression of miR-145, miR-200b and miR200c (**Figure 3A**). There was no significant difference on miR-21 expression between BFTC pWPI and BFTC hAR cells. Up-regulation of microRNA-125b has been also shown to expand the population of CSCs and increases chemoresistance [30]. miR-27a function as an antiapoptotic and proliferation-promoting factor in liver cancer cells [31]. miR-145 was shown to suppress tumor sphere formation and expression of CSC surface markers and stemness genes in prostate cancer cells [32]. In the other hand, miR-200 family were significantly downregulated in both breast CSCs and normal mammary stem and/or progenitor and furthermore, the overexpression of miR-200c reduced the clonogenic and tumor-initiation activities breast CSCs [28]. Therefore, from the miRNA expression patterns between BFTC pWPI and BFTC hAR cells, the result demonstrated that AR upregulates miRNAs that promote CSC population and downregulates miRNAs that suppress CSC population in UUTUC cells. Besides investigating the expression of miRNA, we also examined whether the addition of AR in BFTC cells would promote epithelial-mesenchymal transition (EMT), another characteristic of cancer stem cells [33]. We determined the expression of the epithelial cell marker, E-cadherin and mesenchymal cell marker, vimentin [34] in BFTC pWPI and BFTC hAR cells as well as in 7630 pWPI and 7630 hAR cells. And the result showed that lower E-cadherin, but higher vimentin expression were observed in BFTC hAR cells comparing to those expressions in BFTC pWPI cells and this finding was also demonstrated in 7630 cells when 7630 hAR cells had higher vimentin expression, but lower E-cadherin expression that 7630 pWPI cells (**Figure 3B**), further supporting the role of AR in promoting the expansion of CSC population in UUTUC cells.

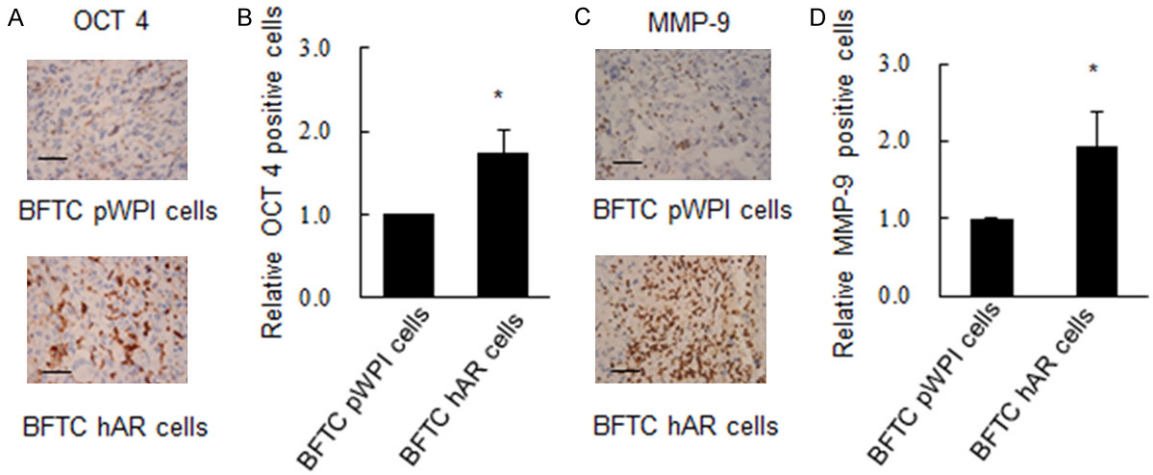

Figure 5. The OCT4 and MMP9 expression in BFTC pWPI and BFTC hAR cell xenograft tumors in nude mice. A. IHC staining of OCT4 expressing (brown-staining) cells in BFTC pWPI or BFTC hAR cell xenograft tumor in nude mice. Scale bar = 50 μm, 40 × magnification. B. Quantification of IHC-stained OCT 4+ cell counts in tissues from BFTC pWPI or BFTC hAR cell xenograft tumors in nude mice. (n = 4). C. IHC staining of MMP9 expressing (brown-staining) cells in BFTC pWPI or BFTC hAR cell xenograft tumor in nude mice. Scale bar = 50 μm, 40 × magnification. D. Quantification of IHC-stained MMP9+ cell counts in tissues from BFTC pWPI or BFTC hAR cell xenograft tumors in nude mice. (n = 4). The results were shown as the mean ± SD (*P<0.05 vs control).

BFTC hAR cells display highly tumorigenic behavior and high MMP-9 expression on xenografted tumors in vivo

To examine the effect of AR on CSC population in vivo, we compared the tumorigenic potential of BFTC pWPI and BFTC hAR cells since inside cancer cells, there are a heterogeneous collection of cell types including differentiated cancer cells and CSCs that are tumorigenic, resulting in higher tumor take rates [9]. Nude mice were injected subcutaneously with either $1*10^6$ or $5*10^6$ of BFTC pWPI and BFTC hAR cells in left and right flanks, respectively and tumor formation were observed. Tumors were detected when both $1*10^6$ and $5*10^6$ cells had been injected. Both number of cells injected, BFTC hAR cells were significantly more tumorigenic than BFTC pWPI cells at $5*10^6$ injected cells (**Figure 4B**). However, at $1*10^6$ injected cells, the significant difference was not reached between the two groups, but BFTC hAR cells still showed higher tumorigenic potential (**Figure 4A**). The result demonstrated that BFTC hAR cells were more tumorigenic in nude mice than BFTC pWPI cells, indicating that there are more tumorigenic CSCs in BFTC hAR cells. This finding that the addition of AR in BFTC 909 cells, compared to vector controls, resulted in higher tumor incidence in low cell number or high cell number implantation, correlates well with in vitro results. To further confirm the above in vitro cell line data in the in vivo mouse model, we examined the expression of OCT4 and MMP9 in BFTC pWPI and BFTC hAR cell xenograft tumors in nude mice with IHC staining. MMP-9 (matrix metalloproteinase-9) with the activity to proteolyze the extracellular matrix components for cell mobilization is a key molecular for cancer stem cells in the process of cancer cell metastasis key molecules involved in the process of cancer cell metastasis [35, 36]. The IHC staining data indicated that BFTC hAR tumors had more OCT4 (**Figure 5A**, **5B**) and MMP-9 (**Figure 5C**, **5D**) expressing cells than BFTC pWPI tumors did. These in vivo results further imply that AR may play an important role in expanding the CSC population.

Discussion

The current CSC model states that CSCs in cancer cells are responsible for tumor initiation, recurrence, metastasis and chemoresistance [6]. However, the regulation on how CSCs arise remains unclear. In this study, we found that the CD44+ population, stemness gene expression, and the capacity to repopulate of BFTC hAR cells are all higher than to BFTC pWPI cells, indicating that the CSC properties in UUTUC cells are enhanced by AR. These findings demonstrate the role of AR in UUTUC cells as a key regulator to increase CSC population, suggesting a potential therapeutic target to contain the population of CSCs by targeting AR actions. In addition to our finding that AR regulates CSC

population in UUTUC cell, the study on genome wide distribution of the AR binding sites using chromatin immunoprecipitation (ChIP) studies coupled with genomic microarray analysis (ChIP-chip) identify that AR occupancy of the CD44, CD49f/integrin-α6, CD133 and PTEN loci, which have been shown to be involved in regulating the rise of CSC population [22].

Furthermore, we also identified several stemness genes (Oct4, Bim 1 and Nanog) were expressed higher in BFTC hAR cells (**Figure 1B**). The transcription factors Oct4 and Nanog form a core regulatory network that coordinately control the capacity to self-renew and the multipotential differentiation of embryonic stem cells and cancer cells [37]. Bmi1, a polycomb group repressor, plays a key regulatory role in the self-renewal of maintenance of both normal and cancer stem cells [38]. We have also demonstrated that the addition of AR increase the self-renewal capacity of UUTUC cells as measured by colony and sphere formation (**Figure 2**) as well as tumor formation in nude mice (**Figure 4**). These data showed that AR in UUTUC cells can enhance CSC characteristics in gene expression and function, suggesting AR could be a regulator on the expansion of CSC-like cells through a set of transcription factors involved in maintaining the pluripotency of stem cells.

Beside changes in the expression of the stemness genes by AR, we also found that the profile of microRNAs was also altered by AR (**Figure 3A**). The ability of miRNAs to regulate expression of hundreds of target mRNAs gives miRNAs the control over a variety of cell functions including cell proliferation, stem cell maintenance, and differentiation in normal tissues and cancers [39]. In this study, we have found that there are two cluster of miRNA affected by AR in our miRNA analysis: one (miR-145, miR-200b and miR-200c) was downregulatd, but the other (miR-27a and miR-125b) is upreguatled by AR. In the downregulated group, miR-145 has been demonstrated to suppress tumor sphere formation and expression of CSC markers and 'stemness' factors including CD133, CD44, Oct4, c-Myc and Klf4 in PC-3 cells [32]. miR-200b expression in breast cancer cells was shown to block the formation and maintenance of mammospheres [40]. miR-200c decreased the expression of BMI1, and inhibited the clonal expansion of breast cancer cells [28]. In the upregulated group, miR-125b was shown to be required for Snail-induced breast cancer stem cell enrichment [30]. miR-27a was reported to promote cell proliferation and suppress apoptosis in liver cancer cells [31]. Since the population of CSCs could be regulated by miRNA networks, and AR was found in this study to control the profile of miRNA, how AR modulates CSCs through the regulation of miRNA networks warrants further investigation.

The acquisition of EMT characteristics in BFTC hAR and 7630 hARcells (**Figure 3B**) and increased expression of OCT4 and MMP-9 (**Figure 5**) in BFTC hAR cell xenograft tumors imply that the roles of AR in UUTUC cells grant the cells with higher ability to migrate and invade since EMT is a key step for cancer cell migration, invasion, and metastasis [33] and MMP-9 also play an important role in facilitating cancer cell migration and invasion [41]. Indeed, our previous findings have demonstrated that AR promotes the migration and invasion of UUTUC cell through the up-regulation of MMP9 [20], which could be explained that the addition of AR in UUTUC cells could promote EMT in UUTUC, which is also a characteristic of cancer stem cells [33, 42]. Furthermore, CSCs in bladder cancer have been linked to chemoresistance due to the CSC repopulation [43]. In our previous study, we demonstrated that the addition of AR in UUTUC cells increased chemoresistance of UUTUC cells to anti-cancer drugs [21]. The similar finding was reported that in AR expressing bladder cancer cells, androgen/AR signaling promoted cell growth and resistance to doxorubicin [44]. These results corroborate the findings in this study to claim the role of AR in modulating CSC population in UUTUCs.

Current anticancer therapies, such as chemotherapy, radiotherapy, or target therapy, fail to suppress CSCs and develop resistant clones [45]. The therapy to prevent the expansion of the CSC population could improve the outcome of cancer patients. Our findings that AR in UUTUC cells promotes the expansion of CSC population and increases clonogenicity and sphere formation through modulating the stemness genes and miRNAs provide a novel and potential approach to treat urothelial carcinomas through the blockade of AR signaling with antiandrogens such as enzalutamide used in our study. This could be a breakthrough in cancer treatment by adding the hormonal therapy in current treatment regime to provide a syner-

gistic combination therapy for genetically diversified urothelial carcinomas [46] in precision medicine era.

Acknowledgements

This work was supported by the grant from Ministry of Science and Technology, Taiwan (MOST 103-2314-B-303-009&103-2633-B-039-003-), China Medical University grant (CMU102-BC-8), Buddhist Tzu Chi General Hospital grant (TTCRD 103-09), and in part by Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence (MOHW104-TDU-B-212-113002).

Disclosure of conflict of interest

None.

Addresse correspondence to: Chih-Rong Shyr, Sex Hormone Research Center, China Medical University Hospital, Cancer Center Building, 6 Hsiuh-Shih Rd, Taichung, 40454, Taiwan. Tel: 886-4-22052121 Ext. 7514; Fax: 886-4-22057414; E-mail: t20608@mail.cmuh.org.tw

References

[1] Roupret M, Zigeuner R, Palou J, Boehle A, Kaasinen E, Sylvester R, Babjuk M and Oosterlinck W. European guidelines for the diagnosis and management of upper urinary tract urothelial cell carcinomas: 2011 update. Eur Urol 2011; 59: 584-594.

[2] Hall MC, Womack JS, Roehrborn CG, Carmody T and Sagalowsky AI. Advanced transitional cell carcinoma of the upper urinary tract: patterns of failure, survival and impact of postoperative adjuvant radiotherapy. J Urol 1998; 160: 703-706.

[3] Abouassaly R, Alibhai SM, Shah N, Timilshina N, Fleshner N and Finelli A. Troubling outcomes from population-level analysis of surgery for upper tract urothelial carcinoma. Urology 2010; 76: 895-901.

[4] Papatsoris AG, Chrisofos M, Skolarikos A, Varkarakis I, Lekas A, Dellis A, Koritsiadis S and Deliveliotis C. Upper urinary tract transitional cell carcinoma. A 10-year experience. Tumori 2008; 94: 75-78.

[5] Audenet F, Yates DR, Cussenot O and Roupret M. The role of chemotherapy in the treatment of urothelial cell carcinoma of the upper urinary tract (UUT-UCC). Urol Oncol 2013; 31: 407-413.

[6] Clevers H. The cancer stem cell: premises, promises and challenges. Nat Med 2011; 17: 313-319.

[7] Cho RW and Clarke MF. Recent advances in cancer stem cells. Curr Opin Genet Dev 2008; 18: 48-53.

[8] Yang YM and Chang JW. Bladder cancer initiating cells (BCICs) are among EMA-CD44v6+ subset: novel methods for isolating undetermined cancer stem (initiating) cells. Cancer Invest 2008; 26: 725-733.

[9] Nguyen LV, Vanner R, Dirks P and Eaves CJ. Cancer stem cells: an evolving concept. Nat Rev Cancer 2012; 12: 133-143.

[10] Chen YH, Hung MC and Shyu WC. Role of cancer stem cells in brain tumors. Biomedicine 2012; 2: 84-91.

[11] Chan KS, Espinosa I, Chao M, Wong D, Ailles L, Diehn M, Gill H, Presti J Jr, Chang HY, van de Rijn M, Shortliffe L and Weissman IL. Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells. Proc Natl Acad Sci U S A 2009; 106: 14016-14021.

[12] Hatina J and Schulz WA. Stem cells in the biology of normal urothelium and urothelial carcinoma. Neoplasma 2012; 59: 728-736.

[13] Huang P, Chen J, Wang L, Na Y, Kaku H, Ueki H, Sasaki K, Yamaguchi K, Zhang K, Saika T, Nasu Y, Watanabe M and Kumon H. Implications of transcriptional factor, OCT-4, in human bladder malignancy and tumor recurrence. Med Oncol 2012; 29: 829-834.

[14] Chang CC, Shieh GS, Wu P, Lin CC, Shiau AL and Wu CL. Oct-3/4 expression reflects tumor progression and regulates motility of bladder cancer cells. Cancer Res 2008; 68: 6281-6291.

[15] Su Y, Qiu Q, Zhang X, Jiang Z, Leng Q, Liu Z, Stass SA and Jiang F. Aldehyde dehydrogenase 1 A1-positive cell population is enriched in tumor-initiating cells and associated with progression of bladder cancer. Cancer Epidemiol Biomarkers Prev 2010; 19: 327-337.

[16] Volkmer JP, Sahoo D, Chin RK, Ho PL, Tang C, Kurtova AV, Willingham SB, Pazhanisamy SK, Contreras-Trujillo H, Storm TA, Lotan Y, Beck AH, Chung BI, Alizadeh AA, Godoy G, Lerner SP, van de Rijn M, Shortliffe LD, Weissman IL and Chan KS. Three differentiation states risk-stratify bladder cancer into distinct subtypes. Proc Natl Acad Sci U S A 2012; 109: 2078-2083.

[17] Kitamura H, Torigoe T, Hirohashi Y, Asanuma H, Inoue R, Nishida S, Tanaka T, Fukuta F, Masumori N, Sato N and Tsukamoto T. Prognostic impact of the expression of ALDH1 and SOX2 in urothelial cancer of the upper urinary tract. Mod Pathol 2013; 26: 117-124.

[18] Munoz JJ and Ellison LM. Upper tract urothelial neoplasms: incidence and survival during the last 2 decades. J Urol 2000; 164: 1523-1525.

[19] Hall MC, Womack S, Sagalowsky AI, Carmody T, Erickstad MD and Roehrborn CG. Prognostic factors, recurrence, and survival in transitional cell carcinoma of the upper urinary tract: a 30-year experience in 252 patients. Urology 1998; 52: 594-601.

[20] Chen CC, Hsieh TF, Chang CH, Ma WL, Hung XF, Tsai YR, Lin MH, Zhang C, Chang C and Shyr CR. Androgen receptor promotes the migration and invasion of upper urinary tract urothelial carcinoma cells through the upregulation of MMP-9 and COX-2. Oncol Rep 2013; 30: 979-985.

[21] Hsieh TF, Chen CC, Yu AL, Ma WL, Zhang C, Shyr CR and Chang C. Androgen receptor decreases the cytotoxic effects of chemotherapeutic drugs in upper urinary tract urothelial carcinoma cells. Oncol Lett 2013; 5: 1325-1330.

[22] Marcinkiewicz K, Scotland KB, Boorjian SA, Nilsson EM, Persson JL, Abrahamsson PA, Allegrucci C, Hughes IA, Gudas LJ and Mongan NP. The androgen receptor and stem cell pathways in prostate and bladder cancers (review). Int J Oncol 2012; 40: 5-12.

[23] Tzeng CC, Liu HS, Li C, Jin YT, Chen RM, Yang WH and Lin JS. Characterization of two urothelium cancer cell lines derived from a blackfoot disease endemic area in Taiwan. Anticancer Res 1996; 16: 1797-1804.

[24] Hsieh TF, Chen CC, Chang CH, Yu AL, Ma WL and Shyr CR. The potential use of primary human upper urinary tract urothelial cell carcinoma (UUT-UCC) cultured cells for prognostic indicators and chemosensitivity test. Exp Toxicol Pathol 2013; 65: 703-708.

[25] Shyr CR, Chen CC, Hsieh TF, Chang CH, Ma WL, Yeh S, Messing E, Li TH, Li FY and Chang C. The expression and actions of androgen receptor in upper urinary tract urothelial carcinoma (UUTUC) tissues and the primary cultured cells. Endocrine 2013; 43: 191-199.

[26] Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT, Scher HI, Jung ME and Sawyers CL. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science 2009; 324: 787-790.

[27] Garg M. MicroRNAs, stem cells and cancer stem cells. World J Stem Cells 2012; 4: 62-70.

[28] Shimono Y, Zabala M, Cho RW, Lobo N, Dalerba P, Qian D, Diehn M, Liu H, Panula SP, Chiao E, Dirbas FM, Somlo G, Pera RA, Lao K and Clarke MF. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell 2009; 138: 592-603.

[29] Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, Patrawala L, Yan H, Jeter C, Honorio S, Wiggins JF, Bader AG, Fagin R, Brown D and Tang DG. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med 2011; 17: 211-215.

[30] Liu Z, Liu H, Desai S, Schmitt DC, Zhou M, Khong HT, Klos KS, McClellan S, Fodstad O and Tan M. miR-125b functions as a key mediator for snail-induced stem cell propagation and chemoresistance. J Biol Chem 2013; 288: 4334-4345.

[31] Huang S, He X, Ding J, Liang L, Zhao Y, Zhang Z, Yao X, Pan Z, Zhang P, Li J, Wan D and Gu J. Upregulation of miR-23a approximately 27a approximately 24 decreases transforming growth factor-beta-induced tumor-suppressive activities in human hepatocellular carcinoma cells. Int J Cancer 2008; 123: 972-978.

[32] Huang S, Guo W, Tang Y, Ren D, Zou X and Peng X. miR-143 and miR-145 inhibit stem cell characteristics of PC-3 prostate cancer cells. Oncol Rep 2012; 28: 1831-1837.

[33] Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J and Weinberg RA. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008; 133: 704-715.

[34] Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F and Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2000; 2: 76-83.

[35] Li F, Tiede B, Massague J and Kang Y. Beyond tumorigenesis: cancer stem cells in metastasis. Cell Res 2007; 17: 3-14.

[36] Li L and Neaves WB. Normal stem cells and cancer stem cells: the niche matters. Cancer Res 2006; 66: 4553-4557.

[37] Jeter CR, Badeaux M, Choy G, Chandra D, Patrawala L, Liu C, Calhoun-Davis T, Zaehres H, Daley GQ and Tang DG. Functional evidence that the self-renewal gene NANOG regulates human tumor development. Stem Cells 2009; 27: 993-1005.

[38] Park IK, Morrison SJ and Clarke MF. Bmi1, stem cells, and senescence regulation. J Clin Invest 2004; 113: 175-179.

[39] Liu C and Tang DG. MicroRNA regulation of cancer stem cells. Cancer Res 2011; 71: 5950-5954.

[40] Iliopoulos D, Lindahl-Allen M, Polytarchou C, Hirsch HA, Tsichlis PN and Struhl K. Loss of miR-200 inhibition of Suz12 leads to polycomb-mediated repression required for the formation and maintenance of cancer stem cells. Mol Cell 2010; 39: 761-772.

[41] Nelson AR, Fingleton B, Rothenberg ML and Matrisian LM. Matrix metalloproteinases: bio-

logic activity and clinical implications. J Clin Oncol 2000; 18: 1135-1149.
[42] Scheel C, Eaton EN, Li SH, Chaffer CL, Reinhardt F, Kah KJ, Bell G, Guo W, Rubin J, Richardson AL and Weinberg RA. Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. Cell 2011; 145: 926-940.
[43] Kurtova AV, Xiao J, Mo Q, Pazhanisamy S, Krasnow R, Lerner SP, Chen F, Roh TT, Lay E, Ho PL and Chan KS. Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. Nature 2015; 517: 209-213.
[44] Shiota M, Takeuchi A, Yokomizo A, Kashiwagi E, Tatsugami K, Kuroiwa K and Naito S. Androgen Receptor Signaling Regulates Cell Growth and Vulnerability to Doxorubicin in Bladder Cancer. J Urol 2012; 188: 276-286.
[45] Maugeri-Sacca M, Vigneri P and De Maria R. Cancer stem cells and chemosensitivity. Clin Cancer Res 2011; 17: 4942-4947.
[46] Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014; 507: 315-22.