Constitutive β-catenin Activation Induces Male-Specific Tumorigenesis in the Bladder Urothelium

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ABSTRACT

The incidence for bladder urothelial carcinoma (UC), a common malignancy of the urinary tract, is about three times higher in men than in women. Although this gender difference has been primarily attributed to differential exposures, it is likely that underlying biological causes contribute to the gender inequality. In this study, we report a transgenic mouse bladder tumor model upon induction of constitutively activated β-catenin signaling in the adult urothelium. We showed that the histopathology of the tumors observed in our model closely resembled that of the human low grade urothelial carcinoma. Additionally, we provided evidence supporting the KRT5-positive;KRT7-negative basal cells as the putative cells-of-origin for β-catenin-induced luminal tumor. Intriguingly, the tumorigenesis in this model demonstrated a marked difference between opposite sexes; forty percent of males developed macroscopically detectable luminal tumors in twelve weeks, whereas only three percent of females developed tumors. We investigated the mechanisms underlying this sexual dimorphism in pathogenesis and demonstrated that nuclear translocation of the androgen receptor (AR) in the urothelial cells is a critical mechanism contributing to tumor development in male mice. Finally, we performed global gene profiling experiments and defined the molecular signature for the β-catenin-induced tumorigenesis in males. Altogether, we have established a model for investigating sexual dimorphism in UC development, and implicated synergy between β-catenin signaling and androgen/AR signaling in carcinogenesis of the basal urothelial cells.
INTRODUCTION

Bladder cancer is the fourth most common cancer type among men, and ninth among women (1). Life time cost for bladder cancer patients is the highest among all cancer types on a per-patient basis (2). Despite its high prevalence and burden, the etiology of bladder cancer is poorly understood. One important feature of bladder cancer is that its incidence is three times higher in men than in women (1), despite the fact that the development and function of the bladder are not thought to be linked to sex hormones. Historically, this male predilection, similar to lung cancer, was explained by higher rates of tobacco smoking in men. However, this male predilection existed in the non-smoking population, and contrary to lung cancers, the gender difference in bladder cancers remained stable even when the smoking prevalence markedly increased in women (3). These observations suggested intrinsic mechanisms underpinning gender-based differential susceptibility to bladder cancer.

The most common type of bladder cancer is urothelial carcinoma (UC) arising from the bladder urothelium. Normally, mature urothelium renews itself slowly but continuously by producing new cells in the basal layer, which differentiate into intermediate cells and terminally differentiated apical umbrella cells. However, under pathogenic conditions, the basal urothelial cells can rapidly proliferate to restore homeostasis (4, 5). Therefore, the basal cell layer has been proposed to be the urothelial stem cell niche (4). Interestingly, the tumor-initiating cells isolated from UC demonstrate basal cell characteristics (6, 7). However, the cells-of-origin for UC remains to be determined.

Abnormal activation of the canonical WNT/β-catenin pathway is implicated in many cancer types, especially those affecting epithelia of endodermal origin, such as prostate (8) and
colorectal cancers (9). Elevated WNT activity, possibly through promoter hypermethylation-mediated repression of WNT inhibitors, has been reported in a subset of bladder cancers (10, 11). Moreover, augmented expression of WNT pathway genes (6) and nuclear localization of β-catenin (12) have been observed in the bladder tumor-initiating cells. In mice, WNT/β-catenin signaling is required for basal urothelial proliferation during injury-induced regeneration (13), and forced expression of a constitutively activated form of β-catenin using UPII-Cre caused urothelial overgrowth, although no tumor developed in the absence of additional mutations (14, 15). Together, these observations indicate a dynamic role for WNT/β-catenin signaling in regulating homeostasis and carcinogenesis of the urothelium.

Androgen and androgen-receptor (AR) signaling has also been linked to bladder cancers, as AR knockout mice showed resistance to N-butyl-N-(4-hydroxybutyl)nitrosamine-induced carcinogenesis (16). Crosstalk between WNT/β-catenin and androgen-androgen receptor (AR) pathways has been reported both during development (17) and in carcinogenesis, particularly in prostate cancer (8, 18, 19). Notably, a recent study showed a strong association between nuclear AR and β-catenin expression in human bladder cancers (20), and the authors further revealed an interaction between these two proteins in bladder cancer cell lines. These findings prompted us to hypothesize that β-catenin is involved in mediating sexual dimorphism in bladder tumorigenesis. Herein, by using an inducible transgenic system to force persistent β-catenin activation in adult urothelium, we established a mouse bladder tumor model that exhibits strong male predilection as a consequence of synergy between β-catenin and androgen/AR signaling.
MATERIALS AND METHODS

Animal Maintenance

All animals were housed in the animal facility at Washington University according to NIH and Animal Care and Use Committee guidelines. Dox food pellets were purchased from Bio-Serv (Frenchtown, NJ). Castration was performed following standard surgical procedures.

Histological Analyses

Bisected bladder were embedded in paraffin or OCT and cut into 5μm sections. Immunohistochemical and immunofluorescence staining were performed according to standard protocols.

RNA Isolation, Microarray Analysis, and Real-time RT-PCR

The urothelium was physically separated from the bladder for RNA extraction, which used the RNeasy kit (Qiagen, Valencia, CA). Illumina (San Diego, CA) mouse-6 chips were used for microarray analysis.

Cell Culture, Treatments and MTT Assay

Bladder cancer cell lines, UMUC3 and 5637, and WNT3a producing L-cells were obtained from American Type Culture Collection (Rockville, MD). Cancer cells were maintained in phenol red-free DMEM supplemented with 10% charcoal stripped FBS. A 10μM testosterone stock solution in ethanol was diluted 1000-fold in culture medium to a final concentration of 10nM. WNT3a conditioned media was prepared according to suppliers instructions. For MTT assays, bladder cancer cells were seeded on five 96-well plates (2000 cells/well) in identical layouts and let attach overnight. The next day (time 0), normal medium was replaced according to treatment
plan and MTT assay performed on one plate every 24 hours as previously described. Each treatment was performed in triplicates for at least three times, and representative results were presented.

Luciferase Assay

Bladder cancer cells were seeded on white 96-well plates (10000 cells/well) and let attach overnight. The next day, cells were transfected with canonical WNT activity reporter LEF-LUC and pRL-TK vector (Promega, Madison, WI) using X-tremeGENE HP® (Roche Applied Science, Indianapolis, IN) following manufacturer's instructions. Culture media containing transfection reagent was replaced 24 hours later with conditioned media and cells were incubated for additional 48 hours. Luciferase activity was measured using the Dual-Glo® luciferase assay system (Promega) and relative LEF-LUC activity was calculated as the ratio of luminescence from LEF-LUC (firefly luciferase) to pRL-TK (Renilla luciferase).

Statistical Analyses

Student t-tests were performed to compare proliferative indices between experimental groups.

*Additional information is available in supplemental methods.
RESULTS Forced Expression of Stabilized β-catenin in Mature Urothelium Induced Luminal Bladder Tumors in Male Mice

Previous studies have investigated the role for sustained β-catenin activation in the urothelium by using a urothelial-specific UPII-Cre (14, 15). However, the urothelial expression of this Cre is patchy and restricted to the upper layer of differentiated cells; the basal layer, which contains progenitor cells that have high proliferative reserve and therefore are more likely to be the cells-of-origin for tumors, is largely excluded (Fig. 3C b-d in (21)). Importantly, the basal layer is also the endogenous site of WNT activation during injury-induced regeneration. Therefore, we sought to use an alternative genetic tool that can target basal urothelial cells with high efficiency. We adopted an Msx2rtTA;tetO-Cre system, which confers Cre expression in Msx2-expressing cells upon doxycycline (Dox) treatment (22). We first tested the efficacy of the system using an R26<sup>mT/mG</sup> reporter, which expresses membrane-targeted EGFP in the presence of Cre and red fluorescent tdTomato (mT) protein in the absence of Cre (23) (Fig. 1A). Trigenic Msx2rtTA;tetO-Cre;R26<sup>mT/mG</sup> mice were treated with Dox at 4-weeks of age, and their bladders were collected five days later. Immunofluorescence analyses demonstrated that all cells above the basement membrane, marked by laminin staining (Fig. 1B), including all cells positive for the basal-cell marker KRT5 (Fig. 1C), expressed EGFP. Conversely, all mesenchymal cells retained tdTomato (Fig. 1B, C). To test whether these EGFP-positive cells include urothelial stem cells, we performed lineage tracing in which the animals were treated with Dox for 5 days, and bladders were collected 20 weeks later for expression analysis. Because the turnover time for mouse urothelium is around 40 weeks (24), the percentage of EGFP-positive cells would drop if stem cells were not labeled during the initial treatment. In contrast, we observed that all
urothelial cells remained EGFP-positive (Fig. 1D), indicating that urothelial stem cells were also targeted by this system.

To assess the effects of persistent β-catenin activation, we generated trigenic

\(\text{Msx2rta}^{-/} \text{tetO-Cre}^{-/} \text{;β-Cat}^{\text{Ex3/+}}\) animals (hereafter referred to as GOF mutants). The \(\text{β-Cat}^{\text{Ex3/+}}\) allele (25), which was also used in previous studies to study β-catenin activation (14, 15), allows expression of stabilized β-catenin upon Cre-mediated exon3 excision. These GOF mice, along with their littermate controls, were put on Dox-containing diet for at least three weeks to allow complete Cre-mediated recombination, starting from postnatal day 25 when the urothelium has fully matured, and their bladders were analyzed after twelve weeks. Thirty-five percent of the GOF animals died during this period because they suffered from a variety of conditions including alopecia and craniofacial malformations (Supplemental Fig. 1), reflecting the consequences of abnormal β-catenin activation in the corresponding Msx2-expressing organs. Twenty-four percent of the remaining GOF mutants (29 out of 122) had macroscopically discernable tumors within the bladder lumen; 11 had a single tumor and 18 had multi-foci tumors (Fig. 1E). These luminal tumors closely resembled low-grade papillary urothelial carcinoma (Fig. 1F, G). They often exhibited polypoid structure, and the urothelium lost its typical polarity and demonstrated nuclear atypia, high mitogenic activity, and considerable vascular infiltration. The vast majority of tumor cells stained positive for E-Cadherin, indicating their epithelial origin (Fig. 1H). However, no nuclear pleomorphism or muscle invasion was observed. Notably, our results are in contrast with previous studies that used \(\text{UPII-Cre}^{-/}\) to overexpress the same \(\text{β-Cat}^{\text{Ex3}}\) allele but detected no tumors in the absence of additional mutations (14, 15). We suspect that this discrepancy reflects key difference in expression pattern of the Cre-drivers.
Intriguingly, we noticed a striking sex difference in the rate of tumorigenesis, as we found that 45% (27/60) male GOF mice developed tumors, compared to a mere 3% (2/62) in females (Fig. 1I). Histopathological analysis also demonstrated that urothelia in GOF males were more severely affected than those in GOF females (Supplemental Fig. 2). Notably, this difference was not due to differential expression of rtTA or β-catenin, as western blots revealed equal β-catenin expression in GOF males and females (Supplemental Fig. 3). The fatality noted above was not due to sex-related causes, however, as females and males died at similar rates (data not shown).

**The KRT5+;KRT7- Basal Urothelial Cells are Putative Cells-of-Origin for β-catenin-Induced Bladder Tumors**

We next sought to identify the cells-of-origin for the tumors. First, to better understand urothelial differentiation, immunofluorescence (IF) staining against three differentiation markers, keratin-5 (KRT5), keratin-7 (KRT7), and uroplakinIII (UPK3), was performed to define different cell types. We observed four distinct cell populations: the KRT5+;KRT7- basal cells (Fig. 2C, red), KRT5+;KRT7+ basal-intermediate cells (Fig. 2C, yellow), KRT5-;KRT7+ intermediate cells (Fig. 2C, green), and KRT5-;UPK3+ umbrella cells (Supplemental Fig. 4C, green). Similar results were also obtained from staining using alternative basal marker CD44 and intermediate cell marker Loricrin (Supplemental Fig. 4D-I). This graded marker expression pattern, similar to that observed in the prostate epithelium (26), reflects a continuous differentiation process and a hierarchical relationship among urothelial cells, and suggests that the KRT5+;KRT7- basal cells are likely early urothelial progenitor/stem cells. Next, we determined which cell population is responsible for over proliferation driven by β-catenin mutation. Five days after Dox treatment,
elevated β-catenin expression and nuclear localization of the protein was observed throughout the GOF urothelium (Supplemental Fig. 5B). The GOF urothelia were highly proliferative as 33% of urothelial cells were Ki67-positive, compared to near-zero in controls (Supplemental Fig. 5C, D and G). In addition, we also detected considerable mitotic activity in the GOF urothelium, evidenced by the presence of Phospho-Histone H3-positive cells (Supplemental Fig. 5E, F). Notably, these Ki67-positive cells were exclusively KRT5+ basal cells, and this proliferation is accompanied by an expansion of the KRT5+;KRT7- basal cell layer (Supplemental Fig. 6). These results indicate that activation of β-catenin signaling promotes basal cell proliferation.

To understand the dynamics of β-catenin-induced tumorigenesis, and define contribution from different urothelial cell types, we examined GOF mouse bladders every two to three weeks over a period of twelve weeks. Five weeks after starting Dox, GOF urothelia exhibited remarkable histopathological changes. They were thicker than controls (Fig. 2D and Supplemental Fig. 7B) and contained histologically distinct cell nodules within the urothelium (arrows in Fig. 2D-D’’ and Supplemental Fig. 7B). These nodular cells were small, and histologically resembled the basal cells (Supplemental Fig. 7C). The nodular cells displayed a unique signature of low KRT5 (Fig. 2D’) and absence of KRT7 expression (Fig. 2D’’). Co-staining of KRT7 and Ki67 revealed high proliferative activities in these KRT5-low;KRT7-negative cells (Fig. 2G). Moreover, these cells showed distinct signaling activity, evidenced by p-S6 expression, an established target of the mTOR pathway (Supplemental Fig. 7D). These histological/molecular signatures of the GOF urothelia largely persisted throughout tumorigenesis (Fig. 2D-F, D’-F’, D’’-F’’), and the majority of the cells from fully developed tumor shared the same KRT5-low;KRT7-negative signature as the dysplastic nodules/lesions observed early on (Fig. 2F-F’’). These results suggested that the KRT5+;KRT7- basal cells were
the cells-of-origin for β-catenin induced luminal tumors (model in Fig. 2J). Notably, we found that although most tumor cells retained their original keratin signature, only cells adjacent to the basement membrane were highly proliferative (Fig. 2H), and most of these proliferating cells were stained positive for KRT14 (Fig. 2I), an early progenitor cell marker in bladder cancers (27, 28). Our finding that the basal cells are responsible for tumor formation suggests that the failure of earlier studies to detect tumors upon expression of activated β-catenin using UPII-Cre may be explained by the differential oncogenic potential of progenitor and differentiated urothelial cell types.

**Aberrant Androgen Receptor Expression in the β-catenin-GOF Urothelium**

Next, we sought to determine the mechanisms underlying the male-predilection in this bladder tumor model. We observed no difference in bladders between GOF males and females three weeks after Dox treatment (data not shown), but bladders of GOF males five weeks after treatment showed a higher proliferation index and more KRT7-negative cells than those of GOF females at the same stage (Fig. 3A). This corresponds to the time when circulating androgen level peaks in male mice (29). Considering the potential interaction between β-catenin and androgen/AR signaling, we speculated that androgen signaling might function to enhance the male phenotype. We first analyzed AR expression by immunohistochemistry and found that in control males, AR was preferentially expressed in the cytoplasm of the intermediate cells (Fig. 3B). In contrast, prominent nuclear AR expression was evident in the dysplastic/tumor cells of GOF males (Fig. 3C). Further analysis demonstrated a mutually exclusive expression pattern of KRT7 and nuclear AR (Fig. 3E). Indeed, nuclear AR expression clearly defined the boundary between tumor/dysplastic epithelium and the neighboring epithelium (Supplemental Fig. 8).
females, AR expression was much weaker in both controls and the GOF mutants compared to males (data not shown and Fig. 3D). Notably, this nuclear AR translocation was not a result of elevated testosterone in the GOF males (Fig. 3F).

Next, we tested whether β-catenin overexpression is sufficient to induce AR nuclear translocation in bladder cancer cells in a previously described AR-positive bladder cancer cell line UMUC3 (16, 20). We grew these cells in charcoal-stripped DMEM medium, and assayed for AR expression upon different treatment by immunofluorescence staining. Without treatment, AR was mainly expressed in the cytoplasm. This cytoplasmic expression was not changed after 24 hours of WNT3a treatment (Fig. 3G), which can markedly increase β-catenin protein level (Fig 4E). On the other hand, treatment of 10nM testosterone induced nuclear AR expression in around 15% of AR-positive cells. Intriguingly, co-treatment of 10nM testosterone and WNT3a markedly increased the number of cells with nuclear AR expression by more than twofold to above 40%. We further tested whether this β-catenin-induced AR nuclear translocation rely on de novo protein synthesis, by treating the cells with 25μg/ml cycloheximide along with testosterone and WNT3a. We found that cycloheximide did not reduce the percentage of cells with nuclear AR expression (Fig. 3G and Supplemental Fig. 9). Altogether, these data indicated that β-catenin can mediate AR nuclear translocation independent of its transcriptional activity. Therefore, we speculate that the dysplastic cells in the GOF urothelium with nuclear AR expression might express higher level of β-catenin protein compared to their neighboring cells. To test the hypothesis, we stained bladder sections from 2-month and 4-month-old male β-catenin-GOF mice along with a wild type control with an anti-β-catenin antibody. Intriguingly, the basal cells in the 2-month-old GOF mice clearly showed much higher expression (Fig. 3I, arrows) than their neighboring cells, which also demonstrated stronger staining than the control
(Fig. 3H). The uneven β-catenin protein expression was also observed in the 4-month-old GOF male mouse with multi-foci bladder tumors. The tumor cells and some dysplastic basal cells (Fig. 3J), the same cell population with nuclear AR expression (Supplemental Fig. 8), clearly showed a stronger β-catenin staining than the rest of the urothelium. These data suggest that the nuclear AR translocation may be mediated by high level of β-catenin protein expression.

**Androgen-Androgen Receptor Signaling Enhances β-catenin-Induced Bladder Tumorigenesis**

To determine whether abnormally expressed AR plays a role in promoting β-catenin-induced tumorigenesis in male mice, we castrated GOF male mice 15 days after starting Dox treatment and determined its effect on tumor development (Fig. 4A). We first analyzed AR expression by real-time RT-PCR analysis, and found that its urothelial expression in castrated male GOF mice was more than threefold lower than non-castrated counterparts (Fig. 4B), indicating a markedly reduced level of androgen-AR signaling activity. Next, we examined the bladders from these castrated GOF animals after three months of Dox treatment, and found that only 12.5% (2/16) of castrated males developed luminal tumors (Fig. 4C). In addition, cell proliferation of castrated GOF males was 20% less than non-castrated counterparts three weeks after castration (data not shown). These results indicated that misregulation of androgen-AR signaling contributed to β-catenin-induced male-specific bladder tumorigenesis.

To test whether this finding is relevant in human bladder carcinogenesis, we analyzed the roles for AR and β-catenin signaling in two human bladder cancer cell lines, the AR-positive UMUC3 and the AR-negative 5637 cells. Both cell lines were treated with either WNT3a conditioned medium, 10nM testosterone, or the combination of both for 96 hours, and cell growth and
viability were assessed by MTT assay in 24-hour intervals. In neither cell line did 10nM testosterone treatment show any overt effect on cell growth. On the other hand, WNT3a conditioned medium which led to increased β-catenin protein expression (Fig. 4D and E), significantly enhanced growth of both cell lines at 96 hours (Fig. 4F, green triangles). Combined treatment of testosterone and WNT3a, however, augmented the growth-promoting effect of WNT3a conditioned media only in AR-positive UMUC3 cells at 96 hours (1215.6±20.0% vs. 1087.7±27.7%, \( P<0.02 \) in UMUC3; 1182.1±19.7% vs. 1174.4±29.8 %, \( P=0.86 \) in 5637 cells).

Collectively, these data demonstrated that androgen-AR signaling synergize with β-catenin to promote growth of the bladder cancer cells.

**Androgen-Androgen Receptor Signaling Enhances the Transcriptional Activity of β-catenin Mediated Canonical WNT Signaling Pathway**

To gain insights into the molecular mechanisms through which AR synergizes with β-catenin in promoting tumorigenesis, we performed expression profiling of urothelial cells from control and GOF animals. Urothelial samples were collected from animals three weeks after starting Dox treatment, a stage when androgen starts to elevate but sexual dimorphism in bladder phenotype has yet to develop. We analyzed four groups of samples representing different sex and genotypes, each consisting of three pooled samples (Fig. 5A and B), and identified three clusters of differentially regulated genes (DRGs) (Fig. 5A): A) genes differentially regulated between genotypes, i.e., β-catenin targets, B) genes differentially regulated between opposite sexes, i.e., sex-specific genes, and C) genes differentially expressed between GOF males and GOF females, i.e., genes causing male-predilection in β-catenin-induced bladder tumorigenesis. We sought to determine the relationship among these three groups of DRGs, in order to understand the
potential mechanisms through which AR impacts the downstream signaling of β-catenin.

Comparing data acquired from GOF to control, we uncovered 576 genes (β-catenin-targets, supplemental Fig. 1) that differed in expression level by more than 2-fold, including 24 previously identified direct canonical TCF/LEF-dependent WNT signaling targets (highlighted in Supplemental Table 1). This result validated our model and indicated that abnormal activation of canonical WNT signaling is one of the events caused by β-catenin-GOF mutation. The rest majority of the genes were likely either targets of non-WNT related β-catenin signaling, or genes further downstream of the β-catenin pathway. Next, we compared data sets from GOF males to GOF females to identify genes underpinning β-catenin-induced sexual dimorphic tumorigenesis. We found 130 DRGs (male tumor genes) in this group, among which were 68 β-catenin targets, an additional 17 possible (changed in the GOF males relative to the control males by 1.5-fold-2-fold) β-catenin targets, and 11 genes differentially expressed in opposite sexes in controls (Fig. 5B, C, and Supplemental table 2). Close examination of the 85 β-catenin targets revealed that 83 showed the same trend of, but greater changes in the GOF males than in females (Fig. 5D), suggesting that β-catenin responsiveness is higher in males than in females. These genes were further categorized by their biological functions (Fig. 5E). Notably, among these genes there were several well-characterized direct downstream targets of canonical TCF/LEF-dependent WNT signaling pathway including Axin2, Stra6, Sp5 and Gpr49, suggesting that AR might synergize with β-catenin by enhancing β-catenin-mediated transcriptional activity. To test this hypothesis, we treated the bladder cancer cells with 10nM testosterone to induce nuclear AR activity, and then determined its impact on the transcriptional activity of TCF/LEF-dependent canonical WNT/β-catenin signaling by assaying for Luciferase activity of the well-characterized LEF-LUC reporter (TOPFLASH) (30). LEF-LUC contains a Luciferase gene driven by a
minimal promoter and enhancer with seven TCF/LEF binding sites, and therefore is used to
analyze activity of the TCF/β-catenin-mediated transcription. As a positive readout, WNT3a
treatment enhanced luciferase activity in both cancer cell lines (fold-change is 4.25±1.81 and
3.36±0.72 in UMUC3 and 5637 cells, respectively, Fig. 6A). On the other hand, 10nM
testosterone alone did not have any impact on either cell line (Fig. 6A). Intriguingly, co-
treatment of WNT3A and testosterone augmented growth of AR-positive UMUC3 cells for an
additional 74.1% than WNT3A alone (fold-change 7.40±0.34, P<0.05), but had no additional
effect on WNT3A-treated AR-negative 5637 cells. Similar results were also obtained when we
analyzed the expression of direct TCF/β-catenin downstream target Axin2 in these cells (Fig. 6B).
These data are largely consistent with a recent independent publication by Li et. al.(20), in which
the authors also indicate this synergy is at least in part mediated by elevated nuclear β-catenin
expression upon AR activation. We next tested whether this is also the case in our study by
assaying nuclear expression of β-catenin by both western blot analysis (Fig. 6C) and
immunofluorescence staining (Fig. 6E). However, we found no evidence of enhanced β-catenin
protein expression in either assay (Fig. 6C-E). Therefore, we speculate that the activation of
transcription by AR may be mediated through regulation of β-catenin co-factor TCF/LEF family
proteins. We examined the expression of LEF1 in testosterone-treated UMUC3 cells by
immunofluorescence (Fig. 6F and G). Indeed, the nuclear LEF1 expression was highly elevated
after testosterone treatment (Fig. 6G). We next analyzed LEF1 expression in control and β-
catenin-GOF male bladders, and found that the nuclear LEF1 expression can also be observed in
the dysplastic cells in the GOF urothelium (Fig. 6I) but not the control urothelium (Fig. 6H).
These data suggested that AR may enhance β-catenin-mediated transcription by activating its co-
factor LEF1.
In addition to facilitating transcription mediated by β-catenin, AR might also contribute to tumor growth by regulating its own downstream target genes. Therefore, we compared the 130 genes differentially regulated between GOF males and females, with existing microarray databases and found 8 known downstream targets of AR in the male tumor genes (Supplemental Table 3). Some of these genes play important roles in mediating normal biological processes and carcinogenesis (Supplemental table 3), and their expression was further verified by real-time PCR (Fig. 6J). It is possible that aberrant expression of these genes might have contributed to enhanced bladder tumorigenesis in males. Together, these data suggested two potential mechanisms mediated by AR and contribute to β-catenin-induced male specific bladder tumorigenesis: 1) AR augments β-catenin responsiveness in males, and 2) AR activates a distinct set of downstream target genes in the presence of ectopic β-catenin. Notably, we did not observe co-activation of SHH and FGF signaling, indicating that β-catenin-induced bladder tumor development is independent of these oncogenic pathways (Supplemental Fig. 10).

DISCUSSION

A Novel Mouse Model for Bladder Cancer Research

In this study, we established a bladder tumor model in which a constitutively activated form of β-catenin is induced to express in all urothelial cells in adult mice. This mouse model has the following distinctive features: 1) the genetics is defined, and the histopathology of the luminal tumors faithfully recapitulates that of the low grade papillary UC, 2) the tumor development is relatively fast, and 3) the incidence is much higher in males, which makes it an excellent tool to investigate sexual dimorphism in bladder pathogenesis.
Notably, the bladder phenotype of this model are clearly different from previous report showing that forced expression of $\beta$–Catenin-ex3 allele alone using UPII-Cre does not cause malignancy (14). This phenotypic variation is likely caused by key differences in spatiotemporal expression pattern of the Cre drivers. The endogenous UPKII is preferentially expressed in the upper urothelial layers (21). Therefore, we suspect that the UPII-Cre, although driven by a partially inverted promoter (31), targets a more differentiated cell population with limited proliferative reserve, which is more resilient to oncogenic mutation than the stem/progenitor cells targeted by Msx2-rtTA. Indeed, from the low-magnification images presented in the original paper, it appeared that UPII-Cre expression is patchy and the basal cells were largely spared (Fig. 3C b-d in (21)). On the other hand, our findings has strongly suggested that the cells-of-origin for the urothelial tumors in the Msx2-rtTA model are indeed the KRT5+;KRT7- basal cells. Moreover, the UPII-Cre is expressed during embryonic/neonatal development when urothelium acquires differentiation. In contrast, Cre activity is not induced in the Msx2-rtTA model until doxycycline is given after urothelia have matured. Whether $\beta$–catenin responsiveness of the developing urothelium channels that of the adult urothelium remains unknown. Notably, an AhCreER$^T$ (14) was also used to conditional activate $\beta$–catenin in the urothelium. However, the intestinal hyperplasia caused by intestinal Cre expression in this model precluded the use of AhCreER$^T$ for long term tumor studies (14). Collectively, the phenotypic differences among these models likely reflect the dynamics of the urothelium in response to oncogenic $\beta$-catenin mutation.

Unfortunately, the detailed stage and cell-type specific characterization of the UPII-Cre expression has not been published. Further analyses determining spatiotemporal expression pattern of the UPII-Cre within the urothelium will provide valuable insights into cellular mechanisms underlying the differential tumorigenesis in these models.
**Basal Cells as Cells-of-Origin for Urothelial Carcinoma**

Bladder urothelium contains heterogeneous cell populations with distinct functions. Previous studies have demonstrated that the urothelial stem cells or progenitor cells reside in the KRT5-positive;SHH-positive basal layers, and these cells are capable of differentiating into umbrella cells and reconstitute urothelium under both homeostatic and wound conditions (4, 13). In support of this notion, our marker analyses have revealed a graded shifting of cell surface markers from basal cells to the fully differentiated luminal umbrella cells (Fig. 2A-C, Supplemental Fig. 4), which is consistent with a gradual basal to apical differentiation process. However, the KRT5-positive cells constitute more than half of all urothelial cells and likely contain not only progenitor cells including urothelial stem cells/transit amplifying cells, but also intermediate cell types. Our analysis revealed a KRT5+;KRT7- subpopulation that represents a small fraction of KRT5 cells which localizes right adjacent to the basement membrane. Whether they are the true urothelial stem cells and/or early transit amplifying cells remains to be tested by lineage tracing experiments. Nonetheless, this cell population demonstrates the most primitive differentiation state evidenced by the lack of KRT7, Loricrin, and UPK3 expression (Fig. 2A-C, Supplemental Fig. 4). In addition, through histological analyses at sequential time points, we found that the exophytic bladder tumors developed in the GOF mutant were indeed evolved from small but highly proliferative clusters of dysplastic KRT5+;KRT7- cells (Fig. 2). Despite all these studies, the definitive evidence of these cells as cells-of-origins for bladder tumors still has to come from lineage tracing experiments which require genetic tools (Cre lines) that can specifically target these cells. Establishing these tools should be an important aim of future studies.

**Molecular Bases for Male-Predilection in Bladder Tumorigenesis**
Male-predilection is an important issue in bladder cancer research because more than three quarters of bladder cancer patients are men. Interestingly, this gender difference in disease susceptibility has also been observed in BBN-induced mouse model (16) and our model, suggesting it cannot be simply explained by higher rates of tobacco smoking in men. The involvement of androgen-AR signaling in bladder carcinogenesis has started to be revealed by several recent studies (16, 20, 32). However, the exact mechanism(s) through which AR promotes bladder tumor development remain to be elucidated. Through both in vivo and in vitro analyses, our study demonstrates a profound synergy between AR and β-catenin in bladder tumorigenesis, which is supported by the following three important discoveries: 1) overexpression of β-catenin protein in the urothelial cells induces nuclear translocation of the AR, 2) coordinated AR and β-catenin activation enhances bladder tumor growth, and 3) AR facilitates β-catenin-induced bladder tumorigenesis by enhancing its transcriptional activity. These findings clearly indicate a multifaceted interaction between AR and β-catenin during bladder carcinogenesis.

The function of AR has been linked to β-catenin by numerous studies on prostate cancer cells (18, 33-39). β-catenin can directly bind to liganded-AR (18, 33) and enhances AR-mediated transcriptional activity (18, 33) in addition to regulate AR expression (39). On the other hand, liganded-AR can facilitate nuclear translocation of β-catenin (34) and its binding to AR responsive promoter elements (34). It has been shown that AR can compete with TCF factors (35, 36), in particular TCF4 for β-catenin. Consequently, activation of AR can suppress the transcriptional activity of the canonical TCF/LEF-dependent WNT/β-catenin signaling. Our study, in contrast, has shown that nuclear AR can enhance the activity of β-catenin-mediated
canonical WNT signaling in urothelial cells, both in vivo (Fig. 5 and supplemental Table 2) and in vitro (Fig. 6A and B). These data indicate that the signaling output of this reciprocal interaction between AR and β-catenin is highly cell context-dependent. Notably, same result has been obtained by a recent independent report by Li et al. (20). However, unlike what was described in that study, we did not find an associated increase in nuclear β-catenin expression upon AR activation (Fig. 6C-E). Instead, we observed an increased expression of β-catenin co-factor LEF1 protein in the nuclei (Fig. 6F-I). Whether this LEF1 activation plays a role in augmented canonical WNT signaling activity, and how AR regulates LEF1 and/or other TCF factors, are currently being investigated. It is also noteworthy that β-catenin can activate not only TCF-dependent WNT signaling, but also other downstream signaling cascades. In our microarray analyses, we also found many non-WNT related genes that showed greater expression changes in GOF males than females (Supplemental Table 1). It is plausible that the binding of AR to β-catenin can recruit other co-factors, such as histone methyltransferases or histone acetyltransferases, to modulate downstream gene expression.

Another interesting finding is that β-catenin accumulation can mediate AR nuclear translocation, but only in basal cells-derived dysplastic/tumor urothelial cells (Fig. 3D-E). This differential AR expression could be a result of higher amount of β-catenin observed in these dysplastic cells (Fig. 3I and J). Importantly, the β-catenin<sup>ex3</sup> allele is transcribed not only in basal cells and their derivatives but in all urothelial cells. Therefore, the elevated protein expression may reflect higher transcription from the β-catenin locus and/or increased protein stability, in these particular cells. Alternatively, the nuclear translocation of AR may require not only β-catenin, but also
other co-factor(s) that only present in this particular cell type, which are yet to be identified. These speculations require further investigations.

Altogether, our study indicates that the synergistic activation of β-catenin and AR may be the underlying mechanisms for male-predilection in bladder carcinogenesis. The clinical relevance of this finding is supported by a recent paper demonstrating a strong association between nuclear expression of these two proteins in human bladder cancers (20). However, abnormal β-catenin activation is only observed in a small fraction of the urothelial cancers (11, 12), which is obviously not sufficient to explain the 3:1 male predilection. Whether and how androgen-AR signaling also contributes to the rest of the male bladder cancers without β-catenin activation remain to be determined by future studies.

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REFERENCES

FIGURE LEGENDS

Fig. 1 Forced β-catenin activation induces luminal tumors in mouse bladder urothelia. (A) A schematic of Msx2rtTA;tetO-Cre;R26emT/mG system. (B-D) IF staining on reporter line using antibodies indicated showing ubiquitous EGFP expression throughout the urothelium 5 days (B-C), and 5 months (D) after Dox treatment. (E) Macroscopic view of single tumor (left) and multifoci tumors (right) in GOF males 12 weeks after Dox treatment. (F-H) Histological analyses of the tumor showing vascularization, polypoid structure (F-G) and E-Cadherin expression (H) in tumors. (I) Tumor incidence in male and female GOF mutants. Scale bars in B-D represent 60μm, in F-H represent 500μm.

Fig. 2 KRT5-positive;KRT7-negative basal cells are the cells-of-origin for β-catenin-induced urothelial tumors. (A-C) Double-IF using antibodies indicated. Note that the red KRT5+;KRT7-basal cells, yellow KRT5+;KRT7+ basal-intermediate cells, green KRT5−;KRT7+ intermediate cells in C. (D-D′′) Histological and double-IF analyses on adjacent sections of GOF urothelia five weeks after starting treatment showing dysplastic nodules (arrows in D-D′′) with low-KRT5 expression (D′), and absence of KRT7 (D′′) expression. (E-F) Histological and KRT5/7 double-IF analyses 8- (E-E′′) and 12- (F-F′′) weeks after treatment showing tumor progression along with expansion of the KRT5-low;KRT7-negative dysplastic cells. (G-I) Double-IF analyses using antibodies indicated. (G) Co-staining of KRT7 and Ki67 revealing that the KRT7-negative nodular cells were highly proliferative. (H-I) Double IF staining showing Ki67-positive cells were localized close to basement membrane (H), and expressing KRT14 (I). (J) A model for WNT-induced tumorigenesis of basal urothelial cells. Scale bars in A-C represent 60 μm, in D-F represent 250μm, in G-I represent 125μm.
Fig. 3 Misregulation of AR in the urothelial cells upon elevated β-catenin expression. (A) Proliferation and KRT7-indice showing that GOF male urothelia are more severely affected, *p<0.025. (B-D) IHC against AR showing cytoplasmic expression in the intermediate cells of control males (B), nuclear expression in the dysplastic urothelial cells of GOF males (C), and weak cytoplasmic expression in GOF females (D). (E) Double-IF showing complementary expression of AR and KRT7. (F) Serum testosterone level of control and GOF mutant animals 8 weeks after Dox treatment measured by ELISA assay. Note that there is no significant difference between control males and GOF males. (G) Immunofluorescence AR staining in UMUC3 cells upon different treatment as indicated. All cells were collected 24 hours after treatment. (H-J) β-catenin IHC staining on urothelia of control (H), 2-month-old GOF (I), and 4-month-old GOF (J) male animals. Note the elevated β-catenin expression in the dysplastic basal cells (arrows in I) and tumor cells (arrows in J) in the GOF animals. All scale bars represent 60μm.

Fig. 4 AR enhances β-catenin-induced bladder tumorigenesis. (A) A schematic diagram of the time line of doxycycline regimen and castration schedule. (B) Quantitative real-time RT-PCR showing reduced AR expression in the castrated GOF males compared to non-castrated GOF males. (C) Tumor incidence in castrated and non-castrated GOF males. (D, E) β-catenin Immunofluorescence showing markedly increased protein level in UMUC3 cells after incubation in WNT3a conditioned media. (F) Representative MTT assays demonstrated increased cell growth in both cell lines by WNT3a-treatment at 96 hours (1087.7±27.7% WNT3a vs. 943.1±35.1 control, P<0.01 in UMUC3, 1182.1±19.7% WNT3a vs. 1026.2±72.4 %, P<0.03 in 5637 cells). Testosterone alone did not affect cell growth in either cell line (P=0.71
and 0.77 respectively). Combined treatment of WNT3a and testosterone only augmented the WNT3a effect in UMUC3 cells (1215.6±20.0% vs. 1087.7±27.7%, P<0.02 in UMUC3; 1182.1±19.7% vs. 1174.4±29.8 %, P=0.86 in 5637 cells). TST, testosterone.

Fig. 5 Sexual dimorphic gene regulation by hyperactive WNT/β-catenin signaling in the bladder urothelium. (A) A schematic for microarray analyses. (B) Hierarchical clustering heat map of the 130 male tumor genes. Note that most genes are not distinguishable between WT males and females, but are changed prominently in GOF males. (C) Composition of male tumor genes. (D) Star glyph distribution of the fold-change of the 85 male tumor WNT targets revealed sexual dimorphic response to WNT activation. Log2 values of fold-change for each gene were shown. Note that the peaks were eminent in male for all genes except for two (asterisks). This change reflects a higher WNT-responsiveness in males. (E) Classification of the 85 genes based on GO biological processes. Note that some genes have multiple functions and may be represented in more than one category.

Fig. 6 AR enhances β-catenin mediated transcriptional activity. (A) LEF-LUC luciferase activity was induced 4.25-fold by WNT3a conditioned medium in UMUC3 cells, and was increased 7.40-fold by co-treatment with TST (WNT3a 4.25±1.81 , P<0.03 compared to control; WNT3a+TST 7.40±0.34, P<0.05 compared to WNT3a alone). This synergistic effect was not observed in AR-negative 5637 cells (WNT3a 3.36±0.72; WNT3a+TST 2.92±0.58, P=0.46). (B) Expression of canonical WNT downstream target, Axin2, upon same treatments measured by real time RT-PCR (C) Western blot against β-catenin revealed no increase in protein level in nuclear extracts of UMUC3 cells treated with control or TST media. (D, E) Immunofluorescence showing similar levels of β-catenin in UMUC3 cells with or without TST treatment. (F, G) Immunofluorescence showing increased LEF1 protein in the TST-treated UMUC3 cell nuclei (G
compared to F). (H, I) IHC showing nuclear LEF1 expression in the β-catenin GOF urothelial cells (I), but not control urothelial cells (H). (J) Realtime RT-PCR analyses on putative AR downstream targets using urothelial RNA isolated from animals with sex and genotype indicated.
Figure 1

A

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Dox

5-day Dox

B. mT/mG laminin

C. mT/mG KRT5

5-month post Dox

D. mT/mG

E.

F. H&E

G. H&E

12-wk Dox

H.

I. Tumor Incidence

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Figure 3

A

Proliferation Index (percent K67+/100)

B

KRT7 Index (percent KRT7+/100)

C

D

E

F

G

H

I

J

10nM TST - - + + +
WNT3a - + - + +
cycloheximide - - - - +

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Figure 4

A

5 wk castration

P0

3 wk Dox diet

4 mon bladder collection

B

AR relative mRNA level

GOF

GOF
castrated

C

Tumor Incidence (%)

GOF

GOF
castrated

D

β-catenin DAPI

UMUC3

E

UMUC3+WNT3a

F

UMUC3

CTRL

10nM TST

WNT3a

WNT3a+TST

5637

CTRL

10nM TST

WNT3a

WNT3a+TST
Constitutive \( \beta \)-catenin Activation Induces Male-Specific Tumorigenesis in the Bladder Urothelium

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