Biased Expression of the FOXP3Δ3 Isoform in Aggressive Bladder Cancer Mediates Differentiation and Cisplatin Chemotherapy Resistance

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Abstract

Purpose: The transcriptional regulation mediating cancer cell differentiation into distinct molecular subtypes and modulating sensitivity to existing treatments is an enticing therapeutic target. Our objective was to characterize the ability of the forkhead/winged transcription factor FOXP3 to modulate the differentiation of bladder cancer.

Experimental Design: Expression of FOXP3 was analyzed by immunohistochemistry in a tumor microarray of 587 samples and overall survival in a subset of 187 patients following radical cystectomy. Functional assays were performed in SW780 and HT1376 cell lines in vitro and in vivo and gene expression profiling performed by RNA-Seq. Validation was undertaken using gene expression profiles of 131 patients from The Cancer Genome Atlas (TCGA) consortium in bladder cancer.

Results: FOXP3 expression correlates with bladder cancer stage and inversely with overall survival, with biased expression of the FOXP3Δ3 isoform. Functional assays of FOXP3Δ3 demonstrated resistance to chemotherapy in vitro, whereas subcutaneous xenografts overexpressing FOXP3Δ3 developed larger and more poorly differentiated bladder cancers. RNA expression profiling revealed a unique FOXP3Δ3 gene signature supporting a role in chemotherapy resistance. Accordingly, knockdown of Foxp3 by siRNA in HT1376 cells conferred sensitivity to cisplatin- and gemcitabine-induced cytotoxicity. Validation in TCGA dataset demonstrated increased expression of FOXP3 in subtypes II to IV and skewing of molecular subtypes based on FOXP3Δ3-specific gene expression.

Conclusions: (i) Biased expression of the FOXP3Δ3 isoform in bladder cancer inversely correlates with overall survival, (ii) FOXP3Δ3 induces a unique gene program that mediates cancer differentiation, and (iii) FOXP3Δ3 may augment chemotherapy resistance. Clin Cancer Res; 22(21); 5349–61. ©2016 AACR.

Introduction

Chemotherapy resistance remains pervasive in the systemic treatment of advanced malignancies. Challenges arise in understanding the molecular mechanisms that mediate treatment resistance as well as in identifying individuals that will best respond to therapy. These observations resonate in the field of bladder cancer, as cisplatin-based systemic chemotherapy remains the mainstay both to treat advanced disease and as neoadjuvant therapy prior to radical cystectomy yet only provides a 40% to 60% objective response rate, a modest improvement in overall survival rate of 5%, and lacks companion biomarkers to predict and monitor treatments (1). Urothelial carcinoma of the bladder comprises the majority of bladder cancer cases and represents the ninth leading cause of cancer mortality in the world at more than 165,000 per year and the eighth most common cause of cancer mortality in the United States at more than 15,000 per year (2, 3). Although the recent influx of the genetic characterization of bladder cancers has advanced bladder cancer staging into the molecular era and provided insight into disease biology, the limited treatment options in bladder cancer emphasize the need for new therapeutic modalities (4).

Forkhead box p3 (FOXP3), a member of the forkhead/winged helix transcription factor family initially described in development of CD4⁺CD25⁺ T regulatory cells integral in immune homeostasis, functions as a transcriptional activator and repressor of more than 700 genes in mice and 5,000 genes in humans (5–7). Located on the short arm of the X chromosome, FOXP3 consists of 11 coding exons and three noncoding exons generating a 431–amino acid protein with a structure consisting of an N-terminal repressor domain and a C-terminal forkhead domain initially described in development of CD4⁺CD25⁺ T regulatory cells integral in immune homeostasis.
Clin Cancer Res; 22(21) November 1, 2016

Clinical Cancer Research

Translational Relevance

Cisplatin-based systemic chemotherapy is the standard therapy for advanced urothelial carcinoma of the bladder, despite clinical response rates in only half of patients and complete responses in 5% of patients. To improve the selection of patients who undergo chemotherapy and to increase treatment efficacy, we need to understand the molecular basis for chemotherapy resistance in bladder cancer. This study shows for the first time that the FOXP3A3 isoform is preferentially expressed in bladder cancer, induces cancer differentiation, and confers chemotherapy resistance. These findings support the transcriptional regulation of cancer differentiation and the plasticity among cancer subtypes which can be leveraged to optimize therapeutic regimens. FOXP3A3 may represent a novel companion diagnostic to guide therapy and target to undermine chemotherapy resistance.

domain (FKH), flanking a zinc finger and leucine zipper (8). Two naturally occurring isoforms FOXP3A3 and FOXP3A3A8 are found in human Tregs, with exon 3 located in the repressor domain and exon 8 located within the leucine zipper domain important for homo- and heterodimerization. FOXP3A3 is expressed at relatively similar ratios to FOXP3 and exhibit overlapping but distinct abilities to differentiate into functional Tregs (9, 10). While tumor-infiltrating Tregs have been associated with cancer progression, recent studies have described FOXP3 expression in multiple epithelial lineages including those of the breast, prostate, lung, ovary, pancreas, melanoma, and bladder (11–16). Evidence in breast and prostate cancer suggests a role as a tumor suppressor gene, supported by the low expression of FOXP3 relative to the normal epithelial counter-part and the predominance of missense mutations in patients with breast and prostate cancer, whereas wild-type Foxp3 repressed ErbB2 and Skp2 in breast cancer and c-Myc in prostate cancer models (13, 16, 17). In contrast, clinical data have shown increased expression of FOXP3 in breast cancer epithelial cells correlating with worse overall survival, as well as increased expression in pancreatic cancer and melanoma (11, 12, 18). These observations promote the hypothesis that FOXP3 may trigger the expression of an epithelial differentiation program that induces aggressive disease. To test this hypothesis in bladder cancer, we examined expression of Foxp3 in a tumor microarray and clinical specimens and characterized its function in in vitro and in vivo model systems.

Materials and Methods

Tumor microarray

Representative regions of formalin-fixed, paraffin-embedded tumors and adjacent normal urothelium obtained from 1985 to 1995 at UCLA were constructed into a tissue microarray by the Tissue Array Core Facility in the UCLA Department of Pathology (Los Angeles, CA; ref. 19). Transurethral resected and radical cystectomy samples from 341 patients were represented as 587 urothelial carcinomas including carcinoma in situ, 258 adjacent urothelium, and 38 lymph node metastases, each in triplicate tissue spots. Pathologic staging was performed in accordance with the 2002 TNM classification and grading according to the WHO criteria. Clinical and pathologic data on 187 cystectomy patients were reported including age, gender, T and N stage, grade, presence of lymphovascular invasion, and overall survival and updated in 2010. Expression of FOXP3 percentage in epithelial cells was scored into quartiles in accordance to our genitourinary pathologist.

Percentage expression of FOXP3 represented the pooled mean from all eligible spots of the same tumor. FOXP3 percentage was categorized into quartiles and correlated with tumor classification by the Mantel–Haenszel χ2 test. Overall survival in patients following radical cystectomy was defined as the duration from the date of cystectomy to either death or the date of last follow-up. A recursive partitioning survival tree was built to obtain a cutoff value to dichotomize FOXP3 percentage into positive and negative groups, which differentiated overall survival between the two groups. Kaplan–Meier curves and log-rank tests were used to compare the survival between the positive and the negative groups. Univariate and multivariate Cox proportional hazard regression models were constructed by including the dichotomized FOXP3 percentage score based on the cutoff value (positive vs. negative), T classification (Tis vs. Ta, T1 vs. T2, T3, and T4), lymph node status (positive vs. zero), lymphovascular invasion status (yes vs. no), gender (female vs. male), and age. For all statistical investigations, tests for significance were two-tailed, with a statistically significant P value threshold of 0.05. Statistical analyses were carried out using SAS version 9.2 (SAS Institute Inc.).

Cell culture

Human bladder cancer specimens were dissociated in collagenase IV (Invitrogen) and filtered through 40-μm nylon mesh to generate a single-cell suspension as previously described (20). Bladder cancer (SW780, T24, R14, HT1376, HTB5) and prostate cancer cell lines (LNCaP, PC3) were a gift from R. Reiter, UCLA; benign urothelial cell line (SV-HUC) was a gift from J. Rao, UCLA; and lung cancer cell lines (H441, H596, H1703) were a gift from B. Gomperts, UCLA, all originally authenticated from ATCC (karyotyping and morphology) and used within 6 months of resuscitation. FOXP3 and FOXP3A3 were cloned into the pTARGEN expression vector (Promega) and transfected into 293T (ATCC), SW780, T24, and HT1376 cell lines using Xfect (Clontech). Stable cells were generated with selection by 500 μg/mL G418 and maintained with 100 μg/mL G418. For sphere cultures, cells were imbedded in Matrigel (BD Biosciences) with 40 μL of 2.5 x 105 cells/mL with 60 μL Matrigel in a 12-well plate and supplemented with sphere medium (RPMI-1640 with B27 [Invitrogen], 20 ng/mL EGF [Sigma], 20 ng/mL FGF [Sigma], and 2 μg/mL heparin [Sigma]). Cells were treated with an IC50 dose of cisplatin (Sigma, 15,663-27-1).

Immunoblot analysis

Tumors were homogenized and lysates prepared using 1% Triton X-100 and separated on a Bis-Tris Gel (Bio-Rad). Blots were incubated with primary anti-FOXP3 Ab (1:5,000, ABCAM 54501) or anti-FOXP3A3 Ab (1:1,000, Novus Biological NBP2-24953SS) and secondary goat anti-rabbit or anti-mouse IgG horseradish peroxidase–conjugated Ab (Southern Biotech), with membranes visualized on film enhanced by chemiluminescence (GE Healthcare). Blots were quantitated using ImageJ.
Quantitative RT-PCR

cDNA synthesized using high-capacity cDNA Reverse Transcription Kits (Applied Biosystems) from 1 μg total RNA extracted with TRIzol (Invitrogen). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) in triplicate using a Viia 7 Real-Time PCR (Life Technologies) with data normalized by GAPDH expression. Primer pairs: FOXP3 full-length spanning exons 2–3, 5'-CATTCAACGCACCTCTC-3', 5'-GGTCTGACCTCAGTATGG; FOXP3 Δ3 spanning exons 2–4, 5'- AGCTGAGCCTCAAGGCTGT-3', 5'-GTTGACCGGAGATCATTG-3'. FOXP3 full-length at exon 8, 5'-AGCAGCTGTCCTGAGAAG-3', 5'-CTTGTCCGCCATGAGCCAG; FOXP3Δ8 5'-CACAGGGCATCACCAGCA-3', 5'-GCAAACGGCTGTCAGG-3'; ALDH1, 5'-TACAATGTCCTGATTAA-3', 5'-TATCTTCAAATAGCACGATA-3'; NANOG, 5'-CTCATCCATCCTGCAAATCTCTT-3', 5'-CAGCTTGGCCTTCTGTCITGA-3'; OCT4, 5'-ATCAANCTCGTCGACAAA-3', 5'-CTTGAAGATTTTCAGTTGTCGACATCT-3'; SHH, 5'-GCGGAAGGTATGAGGGCAAG-3', 5'-GCGAAAGGCTCAGCAGTCTC-3'; SOX2, 5'-GGAGAATTTCCTAGTTGAG-3', 5'-TAACTAAATAAGTCTCCACCCAAAAGA-3'; GAPDH 5'-GCTTCCTGCTCCTCCTGTG-3', 5'-ACGACCAAATCCGCTGTGAC-3'.

FOXP3 genomic sequencing

Primer pairs to sequence FOXP3 exons including intron-exon junctions are depicted schematically (Supplementary Fig. S1).

Flow cytometry

Cells were incubated with anti-CD44-PE (1:100, BD Biosciences #550989) and anti-CD49f-FITC (1:200, BD Biosciences #550989) and anti-CD49f-FITC (1:200, BD Biosciences #550989) and anti-FOXP3 (1:1,000, Abcam 54501, anti-FOXP3 Δ3 (1:500, Novus Biological NBP2-24953SS), anti-Ki67 (1:50, DAKO M7240), anti-pan-C (1:1,000, DAKO M3515), anti-C5K (1:50, Invitrogen 180267), anti-C-K20 (1:50, DAKO M7019), anti-E-cadherin (1:100, DAKO M3612), and anti-N-cadherin (1:50, Invitrogen 33-3900). Imaging was performed using an Axio Imager 2 (Zeiss).

RNA-Seq data analysis

Paired-end 50 basepair reads were performed on a HiSeq 2000 (Illumina). Uniquely aligned reads mapped to the Homo sapiens reference genome (UCSC hg19) with the gapped aligner Tophat allowing up to two mismatches were collected and quantified by RPMK using customized scripts written in Perl after normalization on the basis of the geometric means. Differential expression analysis was performed using DESeq and edgeR in R. TCGA datasets were downloaded from RNA-Seq data The Cancer Genome Atlas (TCGA) Data Portal and merged and normalized by R package to reduce batch effects. Unsupervised hierarchical clustering was performed to examine the correlation and linkage of samples using R.

siRNA knockdown

Knockdown of FOXP3 in HT1376 cells was accomplished using a mixture of 4 FOXP3-specific siRNAs (ON-TARGET plus SMART pool, Thermo Scientific I-009307-09) or scrambled siRNA using DharmaFECT (Thermo Scientific) according to manufacturer’s instructions. After 48 hours, cells were plated at equal densities for cell viability. FOXP3 siRNA target sequences: GAAACACUGCCUGCCUGUC, UCAGAGAUAGAUCUAUCACG, CGCACUUGCGCGCGAA, and CAAAAGCGUGGUGCCAAAAG.

Cell viability assay and wound closure assay

Cells seeded at 1 × 10^5 cells in 12-well plates overnight were treated with titrated doses of cisplatin or ganciclovir for 48 hours and incubated with MTT (10 mg/mL, Sigma) for 4 hours and lysed with 0.1% NP40 in 4 mmol/L HCl and isopropanol and absorbance detected at 590 nm to determine cell viability (Tecan). Cells seeded at 1 × 10^5 cells in 12-well plates overnight to 80% confluency were scratched to generate a 200-μm-wide linear defect. Then changed to low serum media containing 2% FBS to inhibit cell proliferation, and assessed for wound closure at various time points.

Statistical analysis

When comparing two groups, the two-tailed Student t test was performed, whereas when comparing multiple groups, one-way ANOVA was used (significance P < 0.05). For in vitro and in vivo experiments, at least two independent experiments were performed as indicated with errors bars representing SD and P values indicated.

Study approval

The bladder tissue microarray was utilized under the approval of the UCLA Institutional Review Board IRB#11-000423; human bladder cancer specimens were obtained following informed consent under approval of the UCLA Institutional Review Board IRB#11-001363. All animals were housed in specific pathogen-free conditions in the UCLA Division of Laboratory Animal Medicine according to Animal Research Committee protocol #2010-023-13E.
Figure 1. FOXP3D3 isoform expression predominates in bladder cancer. A, FOXP3 expression was examined by immunohistochemistry in a tumor microarray (n = 587 samples) correlating with tumor classification by the Mantel-Haenszel $\chi^2$ test (P < 0.05; between Tis and Ta/T1, T2-4 or LN; between Ta/T1 and T2-4 or LN). B, Kaplan-Meier survival curve in a subset of patients following radical cystectomy (n = 187). A recursive partitioning survival tree was built to obtain a cutoff value to dichotomize FOXP3 percentage into positive and negative groups at 10%, differentiating overall survival between the two groups (log-rank P < 0.04), risk table shown below. C, expression of FOXP3 in selected primary bladder cancers by stage compared with FOXP3 and FOXP3D3 overexpressed in 293T cells was detected by immunoblot using a pan-FOX3 and FOXP3D3 antibody as indicated. (Continued on the following page.)
Results

Expression of FOXP3 negatively correlates with overall survival in human urothelial carcinoma following radical cystectomy

To investigate the clinical significance of FOXP3 expression in bladder cancer, we interrogated a bladder cancer tissue microarray of 587 tumors including carcinoma in situ, 258 adjacent normal urothelium, and 38 lymph node metastasis (n = 341). Compared with the low expression in adjacent normal urothelium, the percentage of FOXP3 expression in tumor epithelium progressively increased with advancing stage from carcinoma in situ, non–muscle-invasive Ta–T1 disease, and muscle-invasive T2–T4 disease, which expressed FOXP3 at similar levels as in metastatic lymph nodes (Fig. 1A). Overall, approximately 25% of patients expressed minimal levels of FOXP3 with a lowest quartile expression at 1.2%, highest quartile expression at 33.6%, and median expression of 13.3% (range, 0%–93%). Kaplan–Meier survival curve in a subset of patients who underwent radical cystectomy (n = 187) demonstrated an inverse correlation of FOXP3 expression to overall survival with a median survival of 9.3 years [FOXP3 < 10%, n = 82; 95% confidence interval (CI), 6.3–11] compared with 2.8 years (FOXP3 > 10%, n = 105; 95% CI, 2.0–5.9; Fig. 1B). Patient demographics were similar to other cystectomy series (Supplementary Table S1; ref. 21). Univariate, but not covariate analysis, indicated that percentage of FOXP3 expression is a predictor of overall survival (P < 0.04 and P = 0.11, respectively; Supplementary Table S2). Representative tumor staining of FOXP3 revealed either predominant nuclear expression or both nuclear and cytoplasmic expression patterns which is similar to pancreatic cancers, but distinct to the predominant cytoplasmic expression observed in breast cancers (refs. 11, 16; Supplementary Fig. S2A). FOXP3 expression in stromal cells did not correlate with T stage (data now shown).

Predominant expression of FOXP3A3 isoform in bladder cancer

To examine the presence of mutations and isoforms of FOXP3 in bladder cancers, primary bladder tumors were examined by immunoblot (n = 5), and although higher-stage tumors revealed increased protein expression as expected, the size at 43 kDa corresponded to the FOXP3A isoform rather than the expected full-length protein (47 kDa). This was confirmed by immunoblot using a FOXP3A3-specific antibody (Fig. 1C; Supplementary Fig. 2B). To distinguish among the two FOXP3 isoforms found in Tregs, a PCR strategy detected predominant expression of FOXP3A3 compared with both full-length and deletion of exon 8 in a distinct set of primary bladder tumors, with ratios of 15–26:1 in high-grade pathologic T1–T3 tumors tested, but not in peripheral blood, adjacent normal urothelium, or fetal bladder mesenchymal cells (Fig. 1D; Supplementary Fig. S2C). This was consistent in bladder cancer cell lines SW780, T24, HT1376, HTB5, and RT4 with ratios of FOXP3A3 to FOXP3 at 8–42:1 but not in the benign urothelial cell line SV-HUC (1.6:1) or prostate cancer cell lines LNCaP and PC3 (1.3–2.3:1; Fig. 1E). Immunohistochemical staining of primary bladder tumors using a pan-FOXP3 antibody and a FOXP3A3-specific antibody showed similar expression patterns using either antibody, supporting the predominant expression of FOXP3A3 (Fig. 1F). Sequencing of all 11 FOXP3 coding exons including intron–exon junctions of bladder cancer cell lines SW780, RT4, T24, HTB5, and HT1376 and a distinct set of primary bladder cancers from patients (pT1 = 1, pT2 = 3, and pT3–4N1 = 3) revealed no somatic mutations (Supplementary Fig. S1).

Overexpression of FOXP3A3 in SW780 cells induces spheroid formation and unique gene expression

We screened bladder cancer cell lines for expression of CD44, CD49f, and FOXP3 by flow cytometry and revealed lower expression of FOXP3 from bladder cancer cell lines derived from low-grade tumors (SW780, T24) at 1% to 4% and higher expression from cell lines derived from high-grade bladder cancers (HTB5, HT1376) at 7% to 13% (Supplementary Fig. S3A). To test the hypothesis that overexpression of FOXP3 or FOXP3A3 may induce a more aggressive phenotype, we generated stable cell lines expressing FOXP3 and FOXP3A3 from a CMV promoter in SW780 cells, selected in part because of its low basal expression of FOXP3, denoted as SW780FOXP3 and SW780FOXP3A3, respectively (Supplementary Fig. S3B, S3C). SW780FOXP3A3 lines consistently expressed lower levels of FOXP3, suggesting a potential selection bias. Overexpressed FOXP3A3 localized to the nucleus, consistent with our previous observations (Supplementary Fig. S3D). Functional testing in Matrigel sphere assays revealed that while cultured SW780FOXP3 cells generated fewer spheres, SW780FOXP3A3 cells generated more numerous spheres compared with parental cells (Fig. 2A). Likewise, qPCR analysis of a spectrum of stem cell genes showed increased expression of SOX2 and SIIH in SW780FOXP3A3, but not SW780FOXP3, cells, whereas both SW780FOXP3A3 and SW780FOXP3 cells expressed decreased OCT4 and similar levels of ALDH1 and NANOG compared with parental SW780 cells (Fig. 2B). Culturing of native SW780 cells in spheres enriched for FOXP3 expression in the CD44+/CD49f− and CD44+/CD49f+ population (25% to 94% in CD44+/CD49f+ and 3% to 17% in CD44−CD49f− populations; Fig. 2C).

To assess global transcriptional changes in the FOXP3A3 gene program that could explain the biased and preferential expression in human bladder cancers, we surveyed gene expression signatures between FOXP3 and FOXP3A3 in bladder cancer via RNA-Seq (Supplementary Fig. S5). Comparing SW780FOXP3A3 and SW780 cells, 1,064 distinct genes including 634 upregulated and 430 downregulated were identified, whereas comparing SW780FOXP3A3 and SW780FOXP3 cells expressed decreased OCT4 and similar levels of ALDH1 and NANOG compared with parental SW780 cells (Fig. 2B). Culturing of native SW780 cells in spheres enriched for FOXP3 expression in the CD44−CD49f− and CD44+/CD49f+ population (25% to 94% in CD44+/CD49f+ and 3% to 17% in CD44−CD49f− populations; Fig. 2C).

(Continued) D and E, expression of FOXP3, FOXP3A3 and Δ8 isoforms by quantitative PCR of primary cells (B) and cell lines (C) as indicated. Ratios of FOXP3A3 to FOXP3 are indicated above brackets. Results represent means of triplicates with peripheral blood mononuclear cells (PBMC), normal urothelium, and fetal bladder mesenchymal cells (FBM), and cell lines representative of three independent experiments. Minimal deviations in error bars for triplicate samples were excluded for clarity, F, representative high-grade urothelial carcinoma (T3N1) stained with a pan-FOXP3 antibody and FOXP3A3-specific antibody shown at 400×. Inset shows magnification with arrows indicating Foxp3-positive tumor-infiltrating lymphocytes.
Results are representative of two independent experiments. Embedded with Matrigel to assess for spheroid formation and quantitated after 7 days. Columns depict means of triplicates and error bars denote SD.

Gene ontology (GO) term clusters are depicted on the left column with representative gene groups. Significant functional groups induced by SW780 FOXP3 compared with SW780 cells include cell movement, cell-to-cell signaling and interaction, molecular transport, and amino acid metabolism.

Signaling network analysis using Gene Ontology (GO) terms revealed unique gene expression patterns with increased expression in positive regulators of transcription and chromatin-related genes by qPCR performed in SW780, SW780FOXP3, and SW780FOXP3Δ3 cells. Columns depict means of triplicates and error bars denote SD. Results are representative of 2 independent experiments.

Left, percentage of positive stained cells is indicated in quadrants; middle, total FOXP3 expression by percentage and mean fluorescence intensity (MFI) as indicated; right, expression of FOXP3 in each CD44 and CD49 quadrant graphically depicted with percentage of FOXP3 in CD44+CD49F-, CD44+CD49F+, CD44-CD49F+, and CD44-CD49F- populations as indicated.

One-way ANOVA was used for group comparisons with $P$ values as indicated.

Signaling network analysis using SW780FOXP3 and SW780FOXP3Δ3 compared with SW780-specific genes identified associations with p53 and AKT, two key pathways in bladder cancer. Only the p53, but not AKT network, was associated with SW780FOXP3 compared with SW780-specific genes, supporting a FOXP3 unique mechanism to induce high-grade bladder cancer (Supplementary Fig. S7) (4).
Expression of FOXP3Δ3 leads to larger bladder tumors and influences differentiation in vivo

As cancer stem cells may represent a hierarchical organization of cells with an ability to differentiate into all cell types of a particular cancer, we asked whether FOXP3Δ3 could influence bladder cancer differentiation using an established xenograft model combining stable cell lines with fetal bladder mesenchymal cells (22). Both SW780<sup>FOXP3</sup> and SW780<sup>FOXP3Δ3</sup> cells subcutaneously implanted in NOD-scid IL2Rgamma<sup>null</sup> (NSG) mice grew larger tumors than SW780 cells (Fig. 3A). Under hematoxylin and eosin (H&E) staining by light microscopy, tumors derived from SW780 cells showed typical histology of low-grade urothelial carcinoma with prominent squamous differentiation, homogeneous and small nuclei.
A  

![SW780 FOXP3∆3](image1)  

![HT1376 FOXP3∆3](image2)  

![T24 FOXP3∆3](image3)  

Control  

Cisplatin

B  

![FOXP3∆3 SW780](image4)  

![FOXP3∆3 HT1376](image5)  

![FOXP3∆3 T24](image6)  

CT  

Cis

C  

SW780  

HT1376  

T24

% Survival

Cisplatin 10 μmol/L

Day 3  

Day 5  

Day 7

P < 0.01

D  

![SW780 FOXP3∆3](image7)  

![SW780 parental](image8)  

Cisplatin (μmol/L)

P < 0.01

![HT1376 FOXP3∆3](image9)  

HT1376 parental

T24 FOXP3∆3

E  

![HT1376 scrambled siRNA](image10)  

![HT1376 FOXP3 siRNA](image11)

P < 0.01

F  

![Chemotherapy naïve](image12)  

Cisplatin sensitive

Cisplatin resistant

CD44⁺CD49F⁻  

CD44⁻CD49F⁺  

FOXP3

CD44⁺CD49F⁺  

CD44⁻CD49F⁻
with little nuclear pleomorphism and abundant cytoplasm, resulting in a low nuclear/cytoplasmic (N/C) ratio. Tumors derived from SW780(Foxp3/−) cells showed features of high-grade urothelial carcinoma, with larger and more darkly stained nuclei exhibiting a high degree of nuclear pleomorphism and scant cytoplasm, resulting in a high N/C ratio. Immunohistochemistry further distinguished SW780(Foxp3/+) from parental tumors, with increased expression of the proliferation marker of Ki-67 associated with more aggressive disease in clinical bladder cancer histopathology and decreased expression of pan-Ck and Ck5, suggesting more poorly differentiated and aggressive tumors (23–26). The process of epithelial-to-mesenchymal (EMT) differentiation marks a fundamental change in cellular structure linked with tumor invasion and worse bladder cancer prognosis (27, 28). With a decrease in CK expression, we sought to examine the influence of FOXP3 on EMT and found decreased expression of E-Cadherin and increased expression of N-Cadherin in SW780(Foxp3/+) compared with SW780 cells (Fig. 3B). No quantitative differences were observed between SW780(Foxp3−) and SW780(Foxp3+) tumors, suggesting that the gene expression differences induced by overexpressed Foxp3 and Foxp3− do not grossly alter cellular growth and differentiation.

Chemotherapy-induced expression of FOXP3Δ3 mediates treatment resistance

Analysis of differentially induced genes confirmed FOXP3Δ3-specific regulation of SOX2 and SHH compared with FOXP3 and further revealed pathways and genes involved in drug transport including p-glycoprotein (ABC11) and ABCB11, ABCA10, ABCA13, and ABCC4, suggesting a role of FOXP3Δ3 in mediating chemotherapy resistance (29). Induction of FOXP3 has been shown in breast and colon cancer cell lines by chemotherapeutic agents such as doxorubicin, etoposide, and cisplatin in a p53-dependent manner and by anisomycin through ATF-2 and JNK pathways, suggesting an autocrine resistance mechanism (30, 31). To determine the effects of cisplatin on FOXP3 expression in bladder cancer cell lines, an LD50 dose administered to SW780, HT1376, and T24 bladder cancer cell lines revealed an induction of FOXP3 protein by flow cytometry (Fig. 4A), whereas expression of FOXP3 mRNA by qPCR confirmed a time-dependent induction of FOXP3 by cisplatin with predominant expression of FOXP3Δ3 compared with the full-length isoform (31–67:1 in SW780; 11–30:1 in HT1376; 14–21:1 in T24 cells; Fig. 4B). To assess whether modulation of FOXP3 expression mediated resistance or sensitivity to cisplatin, we treated SW780 and SW780(Foxp3/+) spheres with a time course of cisplatin and showed that SW780(Foxp3/+) spheres were more resistant to cisplatin (Fig. 4C). To examine the generality of our findings in multiple cell lines and other agents, we also generated FOXP3Δ3 stable lines in T24 and HT1376 cells and demonstrated that FOXP3Δ3 overexpression induced resistance to both cisplatin and gemcitabine (Fig. 4D). Similarly, knockdown of FOXP3 by approximately 40% in HT1376 cells using siRNA and flow cytometry sensitized bladder cancer cell lines to cisplatin and gemcitabine (Fig. 4E, Supplementary Fig. S8). To examine this association in a preliminary sample of primary tumors, we observed that in cisplatin-sensitive patients (n = 2), defined as downstaging of their tumors from muscle-invasive bladder cancer to residual non–muscle-invasive disease following neoadjuvant chemotherapy at the time of radical cystectomy, the percentage of FOXP3 in the remaining cells was relatively lower (3.7%–19%) than in untreated high-risk patients (n = 2). This is in contrast to cisplatin-resistant patients (n = 3) with residual muscle-invasive disease and a high expression of FOXP3+ cells (47%–65%), supporting a role in chemotherapy resistance (Fig 4F).

FOXP3Δ3-induced genes mediate differentiation of urothelial carcinoma subtypes

As an external validation of our results, we queried the gene expression from the first 131 bladder cancer tumors of the TCGA dataset and found FOXP3 to be expressed at significantly higher levels in bladder tumors compared with normal urothelium (P = 0.029). The TCGA clustered bladder cancer into four molecular subtypes, with cluster I representing papillary tumors enriched with FGFR3 mutations often associated with lower grade tumors, cluster II enriched with luminal markers, cluster III enriched with stem and progenitor cytokertains and squamous histology, and cluster IV enriched with features of basal cells (4). Increased FOXP3 expression was observed in cluster II–IV subtypes but not subtype I (Fig. 5A). To examine the gene expression profiles induced by FOXP3Δ3 in both low- and high-grade cancers, we generated stable cell lines expressing FOXP3 and FOXP3Δ3 in HT1376 cells, denoted as HT1376(Foxp3+) and HT1376(Foxp3Δ3−) respectively, and examined gene expression profiling by RNA-Seq (Fig. 5B, Supplementary Fig. S9A). Comparing HT1376(Foxp3+) and HT1376 cells, 4077 distinct genes
including 2,100 upregulated and 1,977 downregulated were identified, while comparing HT1376FOXP3 and HT1376 cells, 779 distinct genes including 405 upregulated and 374 downregulated were identified (adjusted P < 0.05, fold change > 2, and RPKM > 1). Correlation plots show highly significant correlation between FOXP3- and FOXP3A3-induced genes in SW780 and HT1376 cell lines (Supplementary Fig. S9B). Comparisons of expression profiles of unique SW780FOXP3 genes compared with SW780 genes against the TCGA dataset depicted using a dendrogram segregated SW780FOXP3 unique gene expression profiles predominantly with the cluster II subtype, followed by close association with the cluster IV subtype, whereas expression of these genes in SW780 and SW780FOXP3 cells associated with the cluster III subtype. Interestingly, a similar comparison in HT1376 cells show segregation of HT1376FOXP3 unique genes with the cluster III subtype, whereas expression of these genes in HT1376 and HT1376FOXP3 cells associated with the cluster I subtype (Fig. 5C). Examining SW780FOXP3 to SW780 unique genes, gene expression profiles in SW780 cells again associated with the cluster III subtype, but both SW780FOXP3A3 and SW780FOXP3 cells aligned with the cluster II subtype. In comparing HT1376FOXP3 to HT1376 unique genes, gene expression profiles in HT1376 and HT1376FOXP3 cells associated with the cluster II subtype, whereas HT1376FOXP3A3 cells aligned with the cluster IV subtype (Supplementary Fig. S10). Taken together, this suggests the ability of FOXP3 to program a gene expression signature that regulates bladder cancer differentiation as represented by skewing the molecular subtype contingent to the intrinsic genetic profile of a cell line. Furthermore, FOXP3A3-induced genes have a unique differentiation program from FOXP3, perhaps allowing an as-of-yet unidentified competitive advantage to external selection.

Lung cancers may have similarities to bladder cancer secondary to commonalities in environmental hazards such as smoking as well as high mutational densities (32). To generalize our observations, we examined FOXP3 expression between normal and tumor tissue from the lung adenocarcinoma TCGA dataset, which we predicted to be similar to bladder cancer, and the prostate cancer TCGA dataset, which we anticipated to have no association (33). As expected, FOXP3 expression is elevated between normal and lung adenocarcinoma across all molecular subtypes. To our surprise, while we suspected no or decreased expression of FOXP3 in prostate cancer compared with normal prostate epithelium, we found a significant difference between normal and cancer only in the rare but highly aggressive Gleason 5+5 patients (Fig. 5D). Representative lung adenocarcinoma cell lines H596, H11703, and H441 derived from patients with adenocarcinoma, squamous cell carcinoma, and papillary adenocarcinoma, respectively, were examined for FOXP3 expression. H596 and H11703 cell lines were found to have high ratios of FOXP3A3 to FOXP3 in the 6–9:1 range with no somatic mutations in these cell lines following exon sequencing (Fig. 5E).

Discussion

Our findings shed new light to the functional role of FOXP3 in epithelial cancers. We have shown a predominant expression of the FOXP3A3 isoform over full-length FOXP3 at the mRNA and protein level from 8- to 40-fold in bladder cancer in the absence of somatic mutations and with a unique FOXP3A3-dependent gene expression signature. Our observations are in contrast to the array of mutations and deletions described in breast and prostate cancer patients where FOXP3 is thought to function as a tumor suppressor gene (34). Sequencing of various breast cancer cell lines and primary tumors identified multiple mutations including absence of exons 3, 4, and 8, and missense mutations in the forkhead or zinc finger functional domains, with minimal expression of FOXP3A3 (16). In our RNA-Seq analysis in SW780 and HT1376 cells, FOXP3 or FOXP3A3 did not regulate ERBB2 expression, whereas FOXP3, but not FOXP3A3, downregulated c-MYC in SW780 cells. Correlation of FOXP3 expression in the bladder and lung TCGA show similar patterns in lung adenocarcinomas and affirms the similarities in bladder and lung cancers ranging from smoking exposure as well as high mutational heterogeneity, suggesting a deeper biologic commonality that will need to be leveraged in the future (32).

Molecular characterization of bladder cancers have identified expression signatures unique to basal and luminal subtypes that raises the fundamental question whether these subtypes originate from a single cancer stem cell population or rise from distinct lineage-specific cells (4, 25, 26). A hierarchical organization in bladder cancer is supported by the identity of a population of bladder cancer cells, ranging in percentage of 3% to 36% of primary tumors and expressing the surface markers CD44+ and CD49f+, that have the ability to self-renew and differentiate into basal and luminal urothelial cell types (35). In our studies, up to 50% of dissociated primary tumor cells express CD44 and CD49f, with FOXP3 enriched, but not exclusive to the CD44+CD49f+ population. Despite the high expression of FOXP3 in the CD44+ population in vitro, the decrease in expression of basal cytokeratin CK5 in vivo that we observed is not unprecedented, as prior studies have shown that CD44+ could not be detected in 60% of 300 tumors examined, suggesting a differentiation program occurring in vivo (35). Cluster II bladder cancers have been linked with luminal cell differentiation, as represented by p53-like cells that harbor chemotherapy resistance, challenging the notion that more aggressive tumors express basal cell markers (36). We demonstrate that expression of a single transcription factor can modify a gene signature from one subtype to a distinct subtype that is inherent to the baseline genetic alterations and gene programs of specific cell lines, suggesting a plasticity of bladder cancer subtypes. For instance, overexpression of FOXP3A3 biased the expression profile of SW780 cells from subtype III to subtype II, whereas HT1376 cells biased the expression profile from subtype I to subtype III. This raises the question whether bladder cancer subtypes are predetermined at the initiation of bladder cancer at the level of the bladder cancer stem cell or that subtypes can be transcriptionally regulated. What mediates the selection pressures influencing alternatively spliced variants of FOXP3 will need to be evaluated.

The induction of FOXP3 in the peripheral immune system requires TGFβ in context of an enhanceosome including Smad3 and NFAT (37, 38). This regulation of FOXP3 may also be influenced by epigenetic changes such as histone acetylation of Smad3- and NFAT-binding areas or methylation of FOXP3 intronic regions (39). In epithelial cells, the regulation of FOXP3 is less described but shown to be induced by various chemotherapeutics and anisomycin, whereas knockdown of...
FOXP3 by shRNA inhibited anisomycin-induced apoptosis in breast cancer cells (31). We showed that chemotherapy has the ability to induce FOXP3 expression in bladder cancer cell lines, which itself specifies a gene program that induces expression of genes such as p-glycoprotein and other ABC transporters that may mediate chemotherapy resistance. Our findings did not show an obvious dependency on p53, as both p53 wild-type (SW780) and mutant (T24, HT1376) cell lines induced FOXP3. This suggests an autocrine mechanism that mediates chemotherapy resistance through FOXP3-specific gene expression. Although the p53-like bladder cancer subtype corresponds to TCGA cluster II and enriches for chemotherapy-resistant tumors, chemotherapy resistance does not draw a line across molecular subtypes and is observed in up to 40% to 70% of the remaining bladder cancer subtypes (4, 26, 40). We queried the p53-like subtype and identified 19 genes that were in common. Interestingly, when comparing SW780FOXP3 to p53-like genes, only four genes
were shared, three of which were common with subtype II genes. Between p53-like and HT1376/FOXP3A-specific genes, 18 genes were shared (Supplementary Table S3).

The treatment of bladder cancer faces many challenges, in part, secondary to its molecular heterogeneity and lack of companion diagnostics and therapeutic targets. Our findings reveal the novel expression of FOXP3A3 in bladder cancer, which modulates bladder cancer differentiation and mediates resistance to cisplatin and gemcitabine. The FOXP3A3 isoform or members of its downstream transcriptome may prove to be novel therapeutic targets and markers for treatment efficacy. Perhaps FOXP3 may function as a rheostat to direct treatment sensitivity. Understanding the intrinsic and extrinsic mechanisms that influence cancer cells to express FOXP3A3 will provide insight in tumor heterogeneity, differentiation, and resistance to therapy. Many open questions exist, including the regulation of FOXP3 isoform expression, heterogeneity of FOXP3 expression and mutations among different cancers, and direct evidence confirming the driver role FOXP3A3 in bladder cancer and remain the subject of our ongoing work.

Disclosures of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank F. Codrea and J. Scholes and the UCLA Broad Stem Cell Research Center Flow Cytometry Core, the UCLA Jonsson Comprehensive Cancer Center Tissue Array Core, and N. Doan and the Translational Pathology Core Laboratory (NIH/NCI P30CA016668) for providing core facility support. G. Cheng and O. Witte for critical review and helpful discussions; J. Wang and S. Wang for transportation of clinical specimens, and L. Du for additional statistical support.

Grant Support
This study was supported by the National Cancer Institute (A.I. Chin), Broad Stem Cell Research Center and UCLA Jonsson Comprehensive Cancer Center (A.I. Chin, E.M. Peek), and STOP Cancer (A.I. Chin). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 23, 2015; revised May 9, 2016; accepted May 11, 2016; published OnlineFirst May 17, 2016.

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doi:10.1158/1078-0432.CCR-15-2581

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