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Original Article

The transcription factor sex-determining region Y-box 2 (SOX2) in bladder cancer

Rachel L Griffin[1], Lauren Shuman[2], Hironobu Yamashita[2], Qingqing Wu[3], Guoli Chen[3], Ryan M Brown[4], Don Vander Griend[4], David J DeGraff[2,3], Joshua I Warrick[2,3]

[1]Department of Comparative Medicine, Penn State College of Medicine, Hershey, PA, USA; [2]Department of Urology, Penn State College of Medicine, Hershey, PA, USA; [3]Department of Pathology, Penn State College of Medicine, Hershey, PA, USA; [4]Department of Pathology, University of Illinois at Chicago, Chicago, IL, USA

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Abstract: Sex-determining region Y-box 2 (SOX2) is a transcription factor with a central role in embryologic development. SOX2 is also an oncogene in several cancer types. Prior work by our group has shown SOX2 activity associates with cell cycle dysregulation in early-stage bladder cancer. The present study was thus undertaken to broadly investigate SOX2 in bladder cancer, with emphasis on associations with tumor stage, clinical outcomes, and tumorigenicity. Gene expression was quantified by immunohistochemistry in an established tissue microarray (n=303 cystectomy specimens, all stages) and whole tissue sections of noninvasive papillary urothelial carcinoma (n=25). Gene expression by RNA sequencing was evaluated in non-muscle invasive and muscle-invasive cohorts from publicly available repositories. By immunohistochemistry, SOX2 was expressed in 40% of whole tissue sections of noninvasive papillary carcinoma, which correlated with *SOX2* expression by RNA sequencing (r=0.6, P=0.001, Spearman correlation). Expression tended to be focal (median H-score =6). SOX2 was expressed in only 9% of TMA cases, consistent with focal expression. *SOX2* expression was substantially higher in muscle-invasive compared with noninvasive papillary urothelial carcinoma by RNA sequencing (P<0.001, Wilcoxon rank sum test). *SOX2* expression associated with stage progression in lamina-propria invasive cancers (hazard ratio =2, P=0.05, Cox model, binary, RNA sequencing) but not noninvasive papillary cancers (P=0.5, Cox model, binary, RNA sequencing). *SOX2* expression did not associate with overall survival in muscle-invasive carcinoma. Activity of SOX2 in bladder cancer was tested *in vivo* using murine allografts created with MB49 cells that express human SOX2 (MB49-SOX). MB49-SOX allografts expressed this protein focally by immunohistochemistry, much like human tumors. Compared with controls, MB49 allografts demonstrated larger tumor size (P=0.03, Wilcoxon rank sum test) and higher tumor burden in mesenteric metastases (P=0.009, Wilcoxon rank sum test). Though SOX2 expression is focal within tumors, it may drive tumorigenesis, increase growth rate, and promote aggressive features of bladder cancer, particularly stage progression of early-stage disease.

Keywords: Bladder cancer, urothelial carcinoma, sex-determining region Y-box 2, SOX2, non-muscle invasive

Introduction

Bladder cancer is the fourth most common cancer in men and the sixth most common cancer in the United States [1]. It is broadly classified into muscle-invasive and non-muscle invasive disease, based on invasion of the muscularis propria. The former has substantially higher mortality. Non-muscle invasive cancer is further classified into noninvasive papillary (stage Ta), flat carcinoma in situ (stage Tis), and lamina propria-invasive (T1) [2]. A subset of non-muscle invasive cancers progresses to muscle-invasive disease, though it is difficult to predict which cases will progress [3].

Sex-determining region Y-box 2 (SOX2) is a transcription factor that promotes pluripotency during embryogenesis [4, 5]. It also appears to be part of a transcriptional network that drives cell cycle dysregulation in noninvasive bladder cancer [6]. SOX2 is also expressed in many cancer type, including glioblastoma, ovarian, esophageal, oral, lung, prostate, bladder, and sinonasal carcinomas, and generally associates with worse outcomes (Reviewed in [7]).

We have previously shown that activity of SOX2 is higher in noninvasive papillary urothelial carcinoma with cell cycle dysregulation [6]. The finding suggested SOX2 may drive stage progression in early-stage disease. In this present study, we sought to further characterize the role SOX2 plays in bladder cancer. We focused on the relationships between SOX2 expression, tumor stage, and clinical outcomes.

Materials and methods

Protocols based on human tissue were performed under approval by the Penn State College of Medicine Human Subjects Protection Office (Institutional Review Board). All animal experiments were performed under the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University College of Medicine. Detailed protocols are presented in Supplementary Materials.

Immunohistochemistry

We began by performing immunohistochemistry (IHC) for SOX2 and Ki-67 on bladder cancers from a tissue microarray, which was created from a consecutive series of human cystectomy cases (n=303 patients), as previously described [8, 9]. These cases included noninvasive and invasive bladder cancers, the latter including several histologic variants. Inclusion criteria stated subjects included in the tissue microarray must be adults (18 years or older) who underwent cystectomy for bladder cancer at Penn State Hershey Medical Center from 2001 to 2014. This included patients who had received neoadjuvant chemotherapy. Exclusion criteria stated these subjects should not include pediatric patients (younger than 18 years old) or patients who had undergone cystectomy for reasons other than bladder cancer. Patient demographics and tumor staging data are presented in Table S1. Immunohistochemistry was also performed on 25 whole tissue sections of noninvasive papillary urothelial carcinoma. Inclusion criteria stated subjects included in this part of the study must be adults (18 years or older) diagnosed with noninvasive papillary urothelial carcinoma on transurethral resection of bladder tumor at Penn State Hershey Medical Center. Exclusion criteria stated these subjects should not include pediatric patients (younger than 18 years old) or those with a prior diagnosis of muscle-invasion bladder cancer.

Slides were prepared as previously described [10]. The primary antibodies used for IHC were anti-SOX2 (1:1000; D6D9; Cell Signaling Technology) and anti-Ki-67 (1:1000; 8D5; Cell Signaling Technology). Following overnight incubation, slides were washed 3 times for 10 minutes in PBS and sections were incubated in biotinylated secondary antibody diluted in PBS containing horse serum (1:200; Vector Labs) for 1 hour. Specific antibody binding was visualized using Vectastain Elite ABC Peroxidase kit (Vector Labs) according to the manufacturer protocol with diaminobenzidine substrate buffer as the chromogen (Thermo Scientific). Expression of SOX2 was quantified by H-score, an approach that multiplies the percentage of positive cells by the intensity of expression (range 0-3), giving possible scores of 0-300. Ki-67 was quantified as the percentage of positive cells, termed Ki-67 index.

Gene expression analysis on clinical cases

RNA sequencing data were extracted from publicly available sources from the Uromol study of non-muscle invasive bladder cancer [11] and the study of muscle-invasive bladder cancer by The Cancer Genome Atlas (TCGA) Consortium [12]. Cases in the TCGA study were excluded if they appeared noninvasive on the digitally scanned slides, as previously described [6]. Expression by immunohistochemistry was compared with expression by RNA sequencing using cases previously published by our group, specifically on the 25 cases on which expression of SOX2 was evaluated by immunohistochemistry [6].

Stable cell line generation and culture

MB49 cell lines were stably transfected with human *SOX2* using a lentivirus system. Control cell lines were transfected with identical GFP-based vector without *SOX2* (MB49-SOX, MB49-GFP). Briefly, the lentivirus vector (4 ug) was added to the target cells (MB49) in a transfection media of 5 ml OptiMEM, 9 ul ViraPower (Thermo Fisher Scientific, Waltham, MA), and 36 ul Lipofectamine (Thermo Fisher Scientific). At 24 hours following transfection, the media was replaced with complete DMEM. At 24 hours post-media change, media was passed through

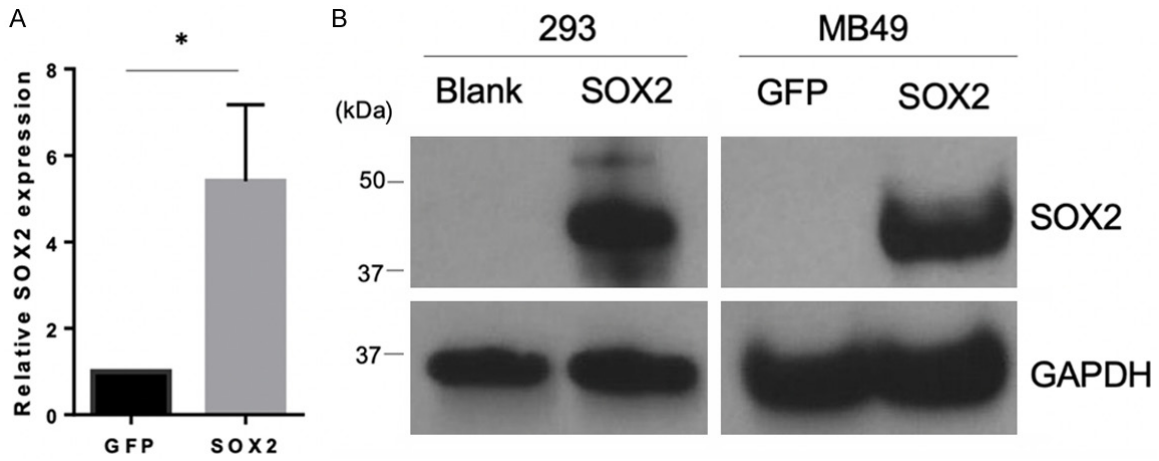

Figure 1. Ectopic overexpression of SOX2 in mouse MB49 bladder cancer cells. (A) Relative mRNA expression of *SOX2* in MB49 cell line in comparison to MB49-GFP as a negative control, (B) Protein expression of SOX2 in MB49 cell line as compared to 293T-SOX2 (positive control) and MB49-GFP (negative control).

a 0.45 um filter and diluted 1:1 in target cell media. Polybrene (Sigma-Aldrich, St. Louis, MO) was added to give a final concentration of 10 ug/mL, and target cells were incubated in this solution for 24 hours. Excess viral media was stored at 4°C to allow for 3 consecutive treatments, each 24 hours apart. Following viral infection, cells were treated with complete media containing 1 ug/mL puromycin for selection to stably express SOX2. Control cell lines were created in the same manner using pCMV6-AC-GFP vector (Origene, Rockville, MD). *SOX2* expression was validated with qPCR and Western blot (**Figure 1**).

Tissue recombination allografts

Allografts were generated by implanting MB49-GFP and MB49-SOX2 cells beneath the renal capsule of severe combined immunodeficiency C.B-17/IcrHsd-*Prkdc*^{scid} mice (SCID, Envigo) as previously described [10]. Scores were assigned for the following categories: ascites production, local tumor extension from the left kidney, tumor deposits in the mesentery, tumor deposits in the spleen, and tumor deposits in the liver. A combined score was also calculated. Tumor invasiveness and tumor size were evaluated by histology. **Tables 1** and **2** summarize the scoring system.

Statistical analysis

The statistical approach for gene expression in clinical cases utilized nonparametric measures to compare continuous variables, specifically the Wilcoxon rank sum test for two-group comparisons and the Kruskal-Wallis for comparisons of greater than 2 groups. Cox models were utilized for survival analysis. SOX2 expression in the survival analysis was divided into high and low groups, defined as those with SOX2 expression above and below the median SOX2 expression for the whole group.

For the mouse allograft models, data are presented as the mean ± standard deviation of the mean. An unpaired, two-sided student t-test was used to evaluate relative expression of SOX2 in each cell line. The total gross pathology and histology scores for each cell line overexpressing SOX2 were compared with the control group (GFP) using the Wilcoxon rank sum test. The individual categories were also compared between cell lines overexpressing SOX2 and the control cell lines using the Wilcoxon rank sum test. Differences between sex were evaluated by a multivariable model with the total score as the dependent variable and sex and SOX2 status as independent variables. All values of significance were set to P=0.05. Statistical analysis was performed using SPSS IMB© Copyright IBM Corporation 1994, 2023 or R version 4.1.0.

Results

SOX2 protein expression is focal in human bladder cancer

Immunohistochemistry was first performed on a human tissue microarray previously created by us, comprising noninvasive and invasive

Table 1. Gross pathology scoring system consisting of scores for morbidity and mortality, ascites production, local tumor extension, and tumor deposits in various organs

Category	Score	Description
Morbidity and Mortality	0	Bright, alert, active; normal behavior and ambulation, no clinical signs
	1	Mild lethargy or mild hunching, able to ambulate to reach food and water, active when observed cage side, no other clinical signs
	2	Moderate lethargy or moderate hunching, able to ambulate to reach food and water, responsive when cage is handled
	3	Moribund, minimal response when cage is handled, unable to ambulate to reach food and water
	4	Found dead or euthanized prior to experimental endpoint
Ascites	0	None
	1	Mild
	2	Moderate
	3	Severe
Local Tumor Extension	0	None
	1	Mild, <1 cm
	2	Moderate, 1-2 cm
	3	Severe, >2 cm
Tumor Deposits in Spleen, Mesentery, Liver	0	None
	1	Focal or <2 mm tumor nodules
	2	Multifocal or >2 mm tumor nodules
	3	Diffuse tumor nodules or infiltration of tumor into organ

Table 2. Histologic scoring system consisting of scores for tumor invasiveness into the kidney and spleen

Location	Score	Degree of Invasion
Tumor Invasiveness into Kidney	0	None
	1	<50% Tumor Infiltration
	2	>50% Tumor Infiltration
	3	No recognizable kidney tissue left
Tumor Invasiveness into Spleen	0	None
	1	Pressing on the organ
	2	Infiltration of the organ, <50%
	3	Infiltration of the organ, >50%

cancers sampled from 303 consecutive cystectomies/cystoprostatectomies performed for bladder cancer [9]. SOX2 was expressed in 9% of bladder cancers by immunohistochemistry in this analysis. Of the cores that were positive for SOX2, median H-score was 45 (IQR=115, range 5-300), indicating focal expression in positive cases. SOX2 expression did not differ significantly between invasive and noninvasive bladder cancer (P=0.7, Wilcoxon rank sum test) or between different histomorphologies of invasive bladder cancer (P=0.14, Kruskal-Wallis). SOX2 expression correlated with Ki-67 protein expression by immunohistochemistry when combining all cases (r=0.1, P<0.001, Spearman correlation).

The above findings suggested SOX2 is focally expressed in bladder, generating a falsely low rate of SOX2 positivity in the tissue microarray analysis. We thus performed SOX2 immunohistochemistry on whole tissue sections of noninvasive papillary urothelial carcinoma (n=25), on which RNA sequencing had previously been performed [6]. This showed SOX2 was expressed in 40% of cases. Expression tended to be focal and weak. Of the cases positive for SOX2, median H-score was 6 (**Figure 2**; IQR=8, range 1-90). Despite limited expression at the protein level, SOX2 expression by immunohistochemistry strongly correlated with *SOX2* expression by RNA sequencing (r=0.6, P<0.001, Spearman correlation). The findings suggest RNA sequencing is a reasonable proxy for expression at the protein level.

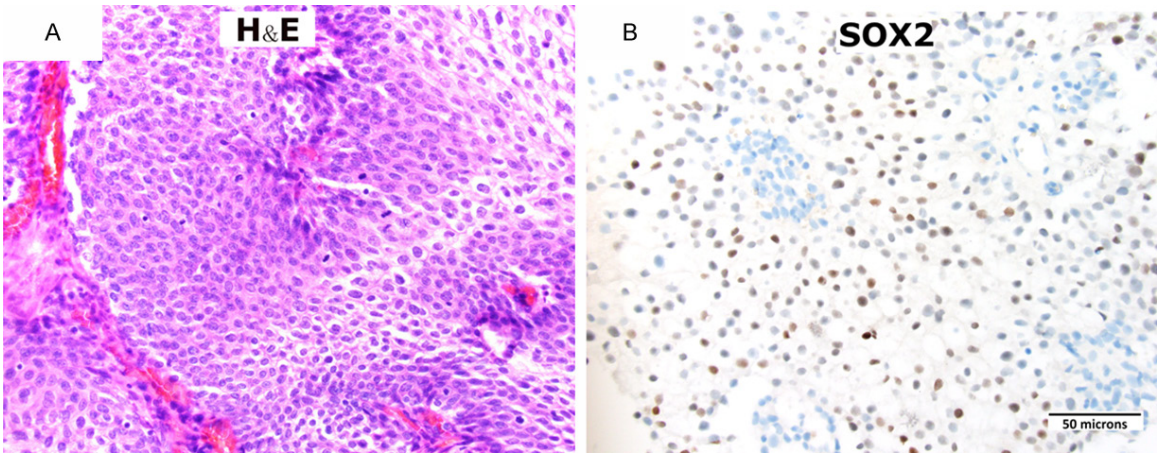

Figure 2. SOX2 expression in human bladder cancer. Sections of human noninvasive papillary urothelial carcinoma showed (A) typical histomorphologic features of the tumor on hematoxylin and eosin (H&E) stained sections, namely papillary structures composed of fibrovascular cores with overlying thickened urothelium, and fused papillae. (B) Tumors expressed SOX2 in a nuclear distribution by immunohistochemistry, which was focal in most cases. Both photomicrographs shown are 400X magnification.

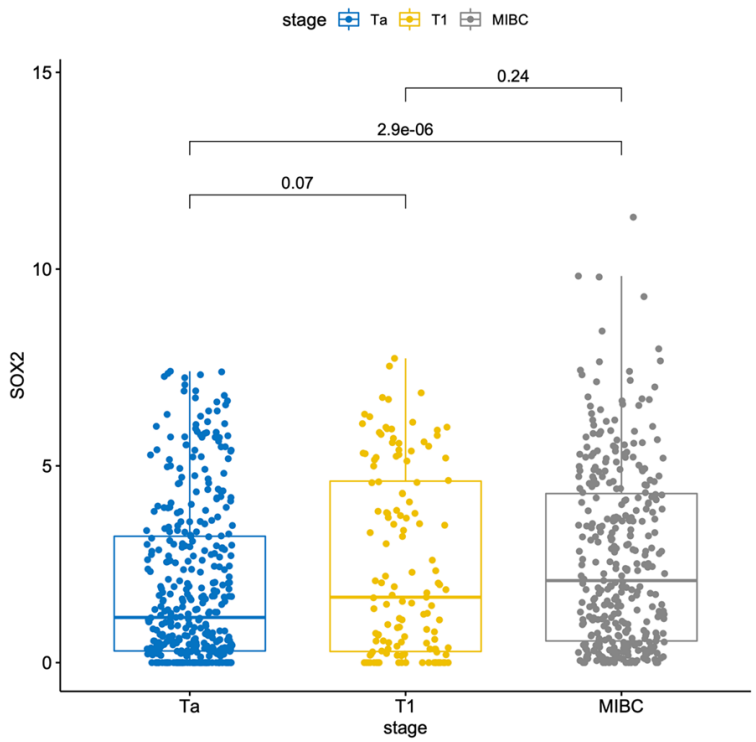

Figure 3. *SOX2* expression by tumor stage. *SOX2* expression was significantly higher in muscularis propria-invasive bladder cancer than noninvasive papillary bladder cancer. Expression in lamia propria-invasive bladder cancer is also higher than in noninvasive papillary bladder cancer, though this only approached statistical significance. There was no significant difference between lamina propria and muscularis propria invasive bladder cancers. Numbers above horizontal bars indicate *p*-values. Data taken from combined Uromol and TCGA cohorts, RNA sequencing.

SOX2 increases with higher tumor stage per RNA sequencing data

SOX2 expression at the transcriptional level was assessed in two large, publicly available data sets: the Uromol cohort, comprising noninvasive and lamina propria-invasive bladder cancers [11], and the TCGA cohort, comprising muscle-invasive bladders cancers [12]. Expression data from the Uromol and TCGA cohorts were combined and normalized, as previously described [6], to compare expression by tumor stage. Muscle-invasive cancers had higher expression of *SOX2* than noninvasive papillary urothelial carcinoma (**Figure 3**; P<0.001, Wilcoxon rank sum test). Similarly, lamina propria-invasive bladder cancer had higher *SOX2* expression than noninvasive papillary urothelial carcinoma, though the result only approached statistical significance (**Figure 3**; P=0.07, Wilcoxon

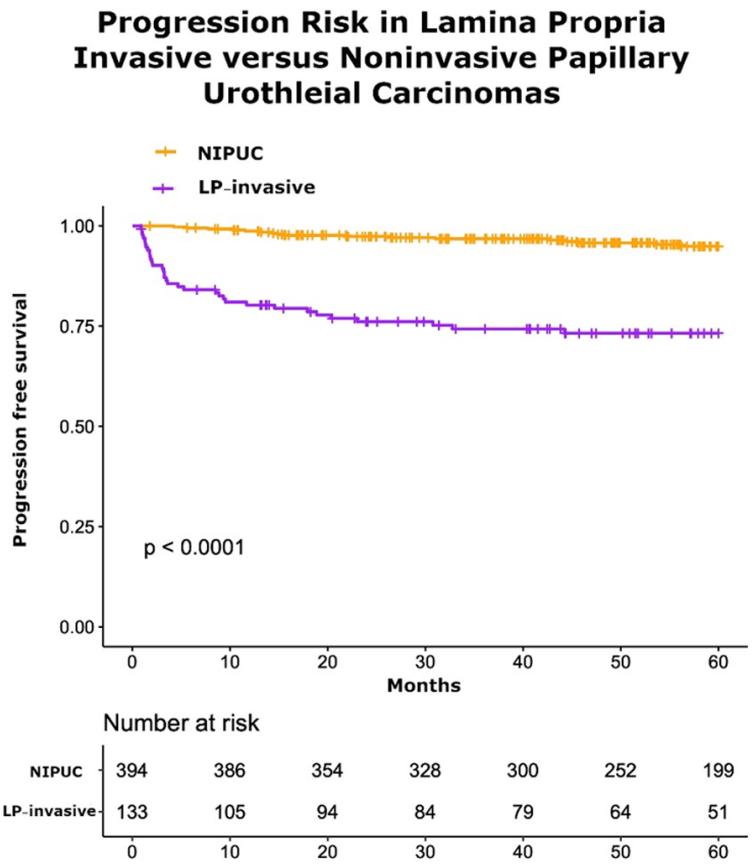

Figure 4. Progression risk and stage of non-muscle invasive bladder cancer. Risk of progression was risk was higher in lamina propria-invasive cancers (LP-invasive) compared with noninvasive papillary urothelial carcinomas (NIPUC) based the Uromol cohort, consistent with prior studies.

rank sum test). However, *SOX2* expression did not differ between muscle-invasive and lamina propria-invasive bladder cancer (**Figure 3**; P= 0.24, Wilcoxon rank sum test). *SOX2* expression in the combined cohort positively correlated with *MKI67* expression (r=0.2, P<0.001, Spearman correlation), consistent with our findings on tissue microarray. We found no difference in *SOX2* expression among molecular subtypes in the TCGA cohort (P=0.9, Kruskal Wallis, using the five subtype TCGA system) or the Uromol cohort (P=0.4, Kruskal Wallis, using the four-subtype Uromol system).

SOX2 expression identifies lamina-propria invasive bladder cancers with higher risk of progression to muscle invasive disease

Survival analysis demonstrated a higher risk of stage progression in lamina-propria invasive carcinoma versus noninvasive papillary urothelial carcinoma (**Figure 4**; HR= 7.1, P<0.0001, Cox model; Uromol Cohort [11]), consistent with the more aggressive nature of invasive tumors. Additionally, survival analysis showed that lamina-propria invasive bladder cancer with high *SOX2* expression had higher risk of progression than those with low *SOX2* expression (**Figure 5**; *SOX2* high vs low as defined in *Methods*, HR=2, P=0.05, Cox model; Uromol Cohort [11]). However, no difference with seen in noninvasive papillary urothelial carcinoma stratified by *SOX2* expression status (**Figure 5**; P=0.23, Cox model; Uromol Cohort [11]). In the TCGA cohort of muscle-invasive cancers [12], *SOX2* expression did not associate with overall survival (*SOX2* as continuous variable, P=0.5, Cox model).

SOX2 expression is focal in cell lines modified to express SOX2

The effects of SOX2 on bladder cancer were evaluated through genetic manipulation of the murine bladder cancer cell line MB49, modified to overexpress human SOX2 protein (MB49-SOX2), compared with controls (MB49-GFP). As expected, ectopic overexpression of SOX2 in MB49 resulted in increased detection of *SOX2* mRNA (**Figure 1A**; P=0.0126, Student's t-test, unpaired, two-sided) and SOX2 protein (**Figure 1B**, densitometry - SOX2:GAPDH 1.4:0.01 for 293 and 2.0:0.03 for MB49). SOX2 protein expression was focal in positive allografts by immunohistochemistry (**Figure 6**), consistently seen in approximately 2% of cells at 3+ intensity (H-score of 6 for all MB49-SOX allografts, H-score of 0 for all MB49-GFP allografts). This observation is similar to the pattern seen in human tumors.

SOX2 induces larger tumors and a more aggressive phenotype in allograft mouse models

Mice implanted with MB49-SOX2 allografts had significantly higher gross pathologic scores

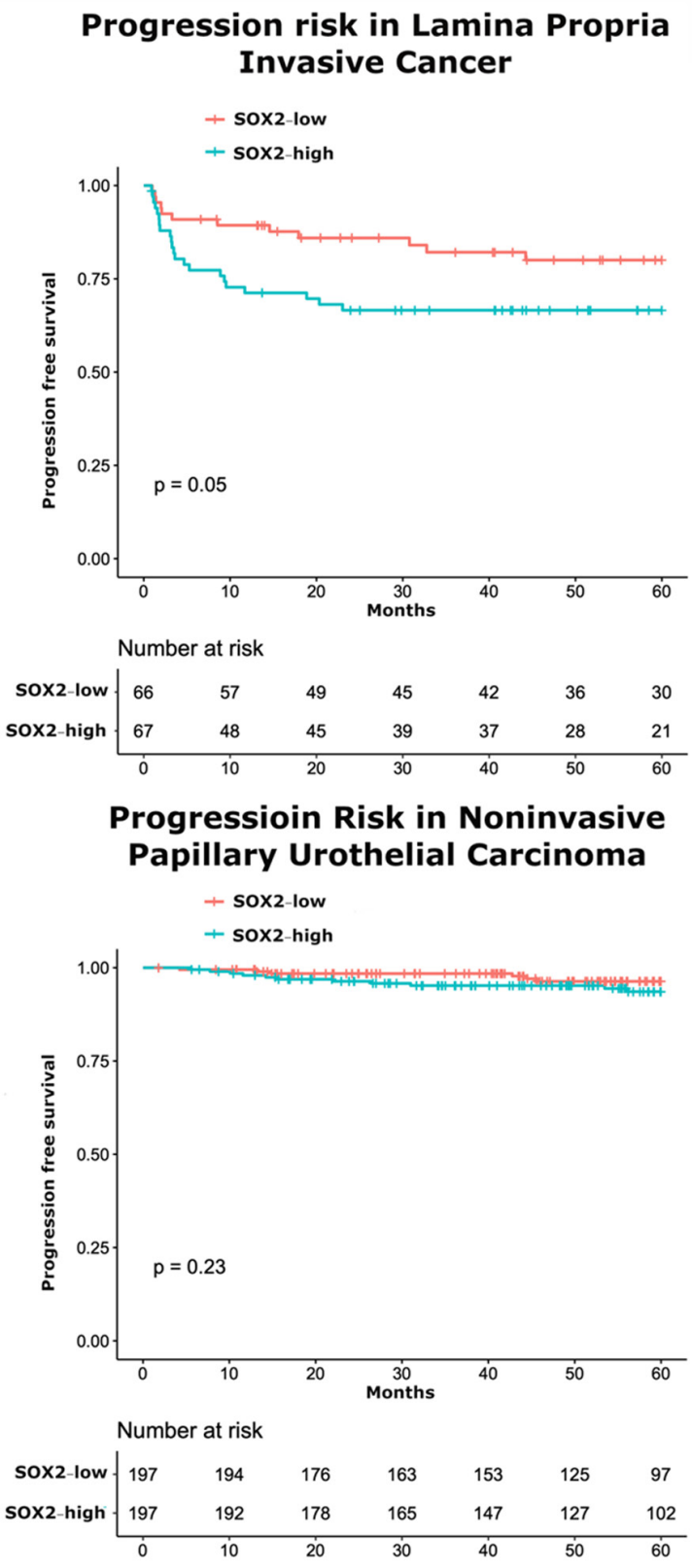

Figure 5. Stage progression and SOX2 expression. Progression risk was higher in lamina propria-invasive cancers with high *SOX2* expression versus those with low *SOX2* expression. SOX2 expression was classified as high versus low based on above or below median SOX2 expression, respectively. Data taken from the Uromol Cohort, RNA sequencing.

than the mice implanted with the control allograft, MB49-GFP (**Figure 7A**; P=0.05, Wilcoxon rank sum test, n=10). There were notably more tumor deposits in the mesentery for MB49-SOX2 as compared to the control group (**Figure 7E**; P=0.009, Wilcoxon rank sum test). Statistically significant differences were not seen for the other categories in isolation, including morbidity/mortality (**Figure 7B**; P=0.5, Wilcoxon rank sum test), ascites (**Figure 7C**; P=0.08, Wilcoxon rank sum test), local tumor extension (**Figure 7D**; P=0.9, Wilcoxon rank sum test), tumor deposits in the spleen (**Figure 7F**; P=0.2, Wilcoxon rank sum test), or tumor deposits in the liver (*p*-value not evaluated; the *p*-value for tumor deposits in the liver could not be calculated since no metastasis was detected for either group). There was not a statistically significant difference between male and female mice in either the MB49-SOX2 (P=0.4, Multivariable model) or controls (P=0.1, Multivariable model).

All tumors were evaluated under microscopic examination for invasiveness into the kidney and spleen (**Table 2**; **Figure 7G-I**). The size of the tumor was also measured as the longest diameter extending outwards from the left kidney. MB49-SOX2 grafts grew to a statistically significantly larger size than the MB49-GFP grafts (**Figure 7G**; P=0.025, Wilcoxon rank sum test, n=10). However, there was no statistical significance between the two groups for invasiveness into the kidney (**Figure 7H**; P=0.2, Wilcoxon rank sum test) or spleen (**Figure 7I**; P=0.8, Wilcoxon rank sum test).

Discussion

SOX2 is a well-characterized transcription factor that is vital for embryonic development, playing a major role in maintaining pluripo-

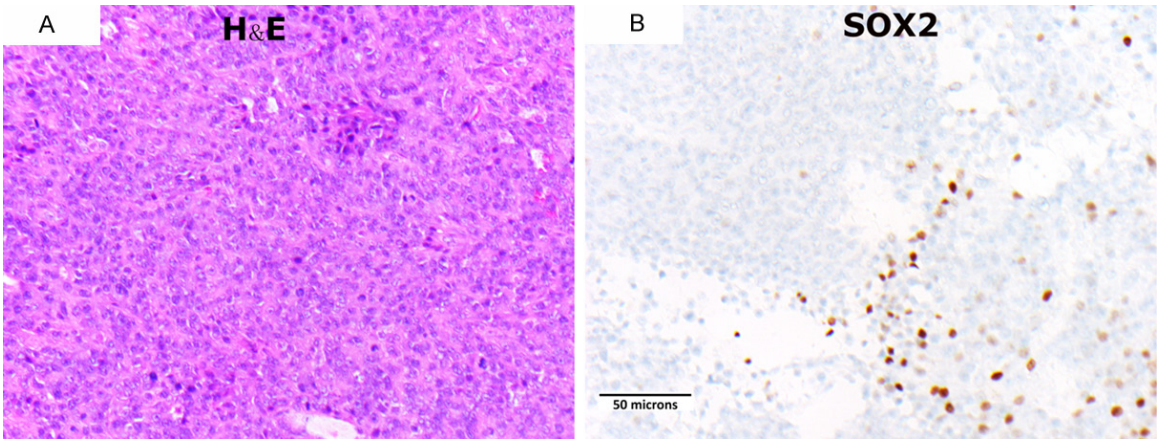

Figure 6. Expression of SOX2 in murine model tumors. Sections of MB49 allografts showed (A) malignant epithelial cells forming expansive sheets on hematoxylin and eosin (H&E) stained sections, consistent with high-grade urothelial carcinoma. (B) Tumors derived from allografts that express human SOX2 demonstrated nuclear expression of the protein in a focal distribution. Both photomicrographs shown are 400X magnification.

tency through stem cell induction and renewal [13]. Models systems have shown SOX2 can induce fully mature fibroblasts to a pluripotent state, when combined with OCT4, MYC, and KLF4 [14].

Many cancer types express SOX2, and high expression is generally associated with worse outcomes [7]. A link has also been shown between SOX2 expression and epithelial-mesenchymal transition in several cancer types, including breast and prostate cancer [15].

We previously identified a transcriptional network of cell cycle dysregulation in noninvasive papillary urothelial carcinoma [6]. This analysis identified several transcription factors that likely drive cell cycle activity in this tumor type, a feature known to predict recurrence and progression [16]. These included transcription factors with a known role in regulating the cell cycle, such as E2F factors, as well as transcription factors active in development, including SOX2, SALL4, and several homeobox factors. The transcription factor SOX2 stood out as a transcription factor of interest, given its established role as a driver of aggressive disease in other tumor types.

The current study expands on this prior research. We first found that SOX2 is expressed focally in bladder cancer at the protein level, in both noninvasive papillary and invasive carcinoma. As expected based on this observation, the incidence of SOX2 expression was substantially lower using TMAs compared with whole tissue sections, the latter showing 40% of noninvasive papillary urothelial carcinomas express SOX2 to some degree. Despite being expressed focally, there was a significant correlation between SOX2 protein expression and *SOX2* mRNA expression, arguing that assays such as RNA sequencing may reasonably quantify SOX2 expression in bladder cancer. Using clinical cohorts with publicly available RNA sequencing data, we found *SOX2* is expressed at higher levels in muscle-invasive versus noninvasive cancers. We likewise found higher rates of stage progression in lamina propria-invasive cancers that express high levels of *SOX2*. These findings were supported by our *in vivo* studies, which demonstrated that SOX2 increases tumor size and locally aggressive behavior, indicating SOX2 enhances tumorigenicity and growth rate of bladder cancer. The overall findings suggest SOX2 drives aggressive behavior in bladder cancer, particularly progression from noninvasive to invasive carcinoma, and progression from lamina propria-invasive to muscle-invasive carcinoma.

Prior studies of SOX2 expression in bladder cancer support our findings [17-31]. Notably, Chui et al. showed SOX2 silencing slows bladder cancer growth in spheroid models and reduces spheroid formation [18]. Xie et al. found SOX2 promotes invasion of bladder cancer using cell lines [19]. Ruan et al. demonstrated inferior recurrence free survival in lamina propria-invasive bladder cancers that expres-

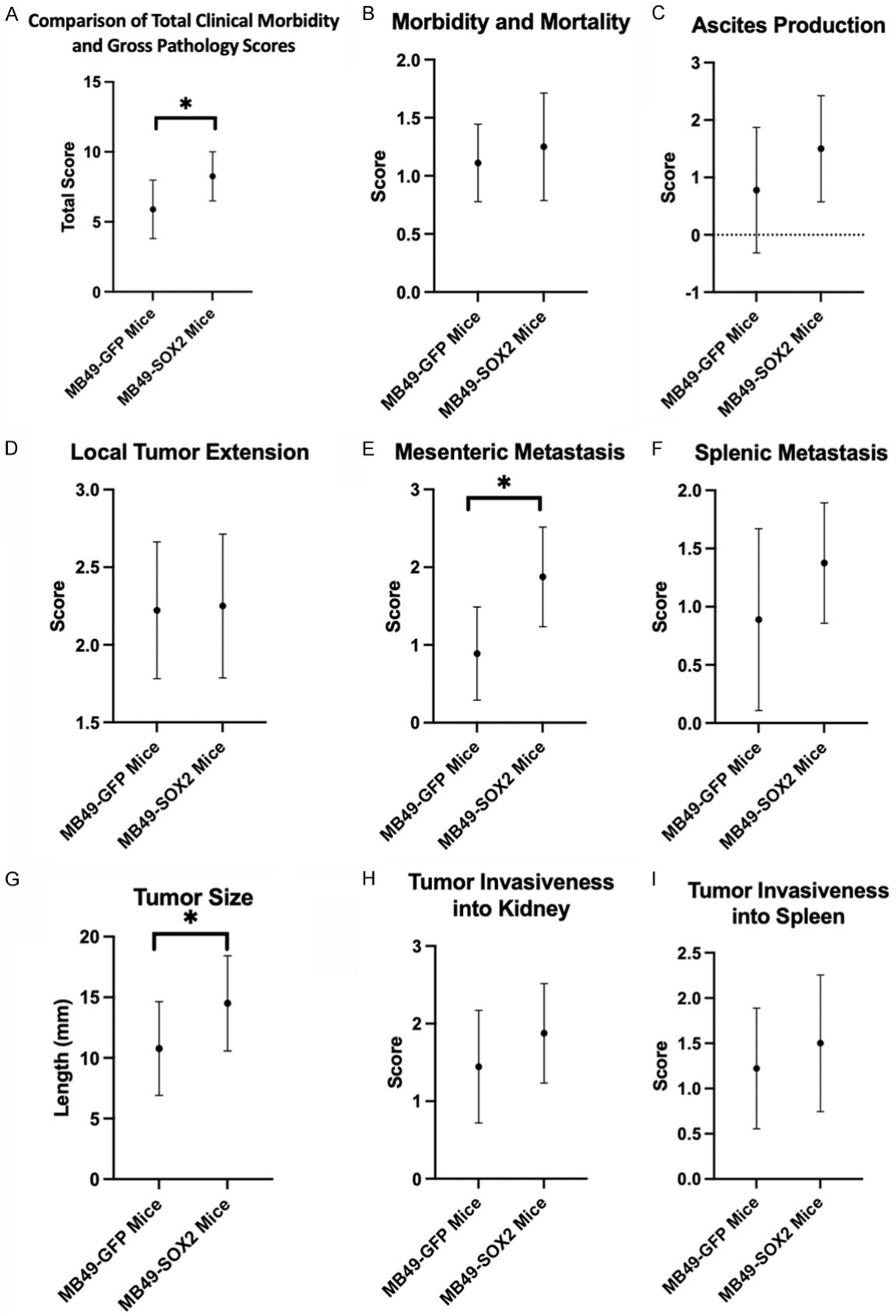

Figure 7. Tumor demographics and endpoints in murine model tumors. (A-F) Comparison of mice implanted with MB49-SOX2 allografts to MB49-GFP allografts by clinical morbidity and gross pathology scores. (A) The mice im-

planted with MB49-SOX2 allografts had significantly higher total gross pathologic scores than the mice implanted with the MB49-GFP allograft (*P=0.05, Wilcoxon rank sum test). The individual categories were scored as follows: (B) Morbidity and mortality (P=0.5, Wilcoxon rank sum test), (C) Ascites production (P=0.08, Wilcoxon rank sum test), (D) Local tumor growth (P=0.9, Wilcoxon rank sum test), (E) Mesenteric metastasis (*P=0.009, Wilcoxon rank sum test), (F) Splenic metastasis (P=0.2, Wilcoxon rank sum test). (G-I) Comparison of mice implanted with MB49-SOX2 allografts to MB49-GFP allografts in individual histologic scoring categories. (G) Tumor size (mm) (*P=0.025, Wilcoxon rank sum test), (H) Tumor invasiveness into kidney (P=0.2, Wilcoxon rank sum test), (I) Tumor invasiveness into spleen (P=0.8, Wilcoxon rank sum test).

sion high SOX2, similar to our findings [23]. Combined with our data, the totality of evidence indicates that SOX2 contributes to an aggressive phenotype in bladder cancer, and may drive progression from early-stage disease to muscle-invasive carcinoma.

The present study has several limitations. The tissue microarrays sampled only a small fraction of each tumor to evaluate a protein that is only focally expressed. The numbers, thus do not truly reflect the incidence of SOX2 expression in human disease. However, we mitigated this in part by studying whole tissue sections. *In vivo* models were also limited in the degree to which they reflect human disease. MB49 control tumors are aggressive, demonstrating fast tumor growth, locally aggressive invasion, and peritoneal metastasis. While our SOX2-expression cell lines were more aggressive, they were a more aggressive version of an already aggressive cell line.

Conclusion

SOX2 is focally expressed in a subset of bladder cancers, including at the noninvasive stage of growth. SOX2 may drive stage progression of early-stage bladder cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Joshua I Warrick, Penn State College of Medicine, 500 University Dr, Hershey, PA 17036, USA. Tel: 717-531-8351; Fax: 717-531-5021; E-mail: jwarrick@pennstatehealth.psu.edu

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Supplementary Materials

Supplementary materials and methods

Immunohistochemistry

We began by performing immunohistochemistry (IHC) for SOX2 and KI-67 on bladder cancers from a tissue microarray, which was created from a consecutive series of human cystectomy cases (n=303 patients) [1, 2]. These cases included noninvasive and invasive bladder cancers, the latter including several histologic variants. Immunohistochemistry was also performed on 25 whole tissue sections of noninvasive papillary urothelial carcinoma.

Slides were deparaffinized and rehydrated through a series of graded alcohols and washed in distilled water for 5 minutes. Antigen retrieval was performed by placing slides in 1% antigen unmasking solution (Vector Labs, Burlingame, CA) and heating slides for 25 minutes on high power in a pressure cooker (Cuisinart CPC-600FR). Steam was released in short bursts to prevent boiling and preserve tissue integrity. Slides were cooled to room temperature and washed 3 times for 10 minutes in PBS (pH 7.4). All incubations were performed at room temperature unless otherwise noted. Endogenous peroxidases were blocked by incubation in 1% hydrogen peroxide in methanol for 20 minutes, and slides were again washed 3 times for 10 minutes in phosphate-buffered saline (PBS). Sections were incubated in PBS containing horse serum (Vector Labs) for 1 hour to reduce nonspecific antibody binding and then incubated overnight with primary antibody at 4°C in a humidified chamber. The primary antibody used for IHC was anti-SOX2 (1:1000; D6D9; Cell Signaling Technology) and Ki-67 (1:1000; 8D5; Cell Signaling Technology). Following overnight incubation, slides were washed 3 times for 10 minutes in PBS and sections were incubated in biotinylated secondary antibody diluted in PBS containing horse serum (1:200; Vector Labs) for 1 hour. Specific antibody binding was visualized using Vectastain Elite ABC Peroxidase kit (Vector Labs) according to the manufacturer protocol with diaminobenzidine substrate buffer as the chromogen (Thermo Scientific). Expression of SOX2 was quantified by H-score, an approach that multiplies the percentage of positive cells by the intensity of expression (range 0-3), giving possible scores of 0-300. Ki-67 was quantified as the percentage of positive cells, termed Ki-67 index.

Gene expression analysis on clinical cases

RNA sequencing data were extracted from publicly available sources from the Uromol study of non-muscle invasive bladder cancer [3], and the study by The Cancer Genome Atlas (TCGA) Consortium [4]. Cases in the TCGA study were excluded if they appeared noninvasive on the digitally scanned slides, as previously described [5]. Expression by immunohistochemistry was compared with expression by RNA sequencing using cases previously published by our group [5].

Stable cell line generation

HEK293T cells were transfected with the lentiviral vector lv105-SOX2 (Genecopoeia, Rockville, MD). The lentivirus vector (4 ug) was added to the target cells (MB49) in a transfection media of 5 ml OptiMEM, 9 ul ViraPower (Thermo Fisher Scientific, Waltham, MA), and 36 ul Lipofectamine (Thermo Fisher Scientific). At 24 hours following transfection, the media was replaced with complete DMEM. At 24 hours post-media change, media was passed through a 0.45 um filter and diluted 1:1 in target cell media. Polybrene (Sigma-Aldrich, St. Louis, MO) was added to give a final concentration of 10 ug/mL, and target cells were incubated in this solution for 24 hours. Excess viral media was stored at 4°C to allow for 3 consecutive treatments, each 24 hours apart. Following viral infection, cells were treated with complete media containing 1 ug/mL puromycin for selection to stably express SOX2. Control cell lines were created in the same manner using pCMV6-AC-GFP vector (Origene, Rockville, MD). Genetic and protein expression was confirmed as described below using the prostate cell line (R1) as a positive control.

Cell culture

HEK293T cell lines and genetically modified MB49 cell lines (MB49-GFP and MB49-SOX2) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% FBS and penicillin/streptomycin. MB49 cell lines (MB49-GFP and MB49-SOX2) were grown in the presence of puromycin at the final concentration of 1 uM for selection. The cells were incubated at 37°C with 5% CO_2 in a VWR Air Jacketed CO_2 Incubator (Marshall Scientific, Hampton, NH). All cells were grown to 80% confluence prior to RNA or protein extraction.

RNA extraction and quantitative real-time polymerase chain reaction (Q-RT-PCR)

RNA extraction from bladder cancer cells was performed using RNeasy (Qiagen, Valencia, CA) as per manufacturer protocol. The cDNA was synthesized using M-MLV reverse transcriptase (Thermo Fisher Scientific). Q-RT-PCR was performed using QuantaStudio7 Real-Time PCR System (Applied Biosystems, Waltham, MA) using a 96 well format. Reactions consisted of 5 ul of cDNA per reaction, 10 ul of 2× Taqman Gene Expression Master Mix (Applied Biosystems), and 1 ul of 20× Taqman probe (Thermo Fisher Scientific), as well as nuclease-free water for a total reaction volume of 20 ul per well. The Taqman probe used for detecting SOX2 expression was SOX2 (Hs01053049_s1). Relative gene expression change was calculated by ΔΔCt method using 18S ribosomal RNA as an endogenous reference.

Western blotting

All cell lysates were prepared using radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Thermo Fisher Scientific). Sonication was performed using Sonic Dismembrator Model 500 (Fisher scientific) for 10 seconds at 30% amplification to break up the cellular and nuclear membranes. Protein concentrations following cell lysis were measured by using the PierceBCA Protein Assay Kit (Thermo Fisher Scientific) as per manufacturer's instructions. Following extraction, 40 ug of protein samples in 1× LDS sample buffer (Thermo Fisher Scientific) with 10% 2-mercaptoethanol (Sigma-Aldrich) were electrophoresed on 4-12% Bis-Tris NuPAGE gels (Thermo Fisher Scientific), and proteins were subsequently transferred to nitrocellulose Blotting Membrane (GE Healthcare Life science, Fairfield, CT) using a Pierce G2 Fast Blotter (Thermo Fisher Scientific), according to manufacturer's protocol. Following transfer, membranes were incubated at room temperature in 5% non-fat milk (NFDM) (Sigma-Aldrich) dissolved in Tris hydrochloride (Sigma-Aldrich) buffered saline containing 0.1% Tween-20 (TBST) (Sigma-Aldrich) for 1 hour. Additionally, all primary antibodies used in this study were diluted in TBST with 5% NFDM. Dilutions of primary antibodies were as follows: anti-SOX2 (1:1000; D6D9; Cell Signaling Technology, Danvers, MA), and anti-GAPDH (1:1000; 14C10; Cell Signaling Technology). After incubation with primary antibodies overnight at 4°C, all membranes were washed 5 times for 5 minutes with TBST. Secondary antibody (ECL anti-rabbit or mouse IgG, HRP-linked whole antibody; 1:2000; GE healthcare Life Science, Chicago, IL) was diluted in TBST containing 5% NFDM and incubated at room temperature for 1 hour. After incubation with secondary antibodies, membranes were washed 5 times for 5 minutes with TBST. Protein bands were visualized by exposing the membrane after addition of ECL Western Blotting Substrate (Pierce Biotechnology, Waltham, MA) to radiograph film (Thermo Fisher Scientific) via standard procedures. The total amount 1 mg protein of HEK293 cells overexpressing human SOX2 was used as a positive control.

Tissue recombination allografts

All animal experiments were performed in accordance with approved guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University College of Medicine. A total of 12 timed pregnant female HSD:Sprague Dawley outbred rats (Envigo, Indianapolis, IN) euthanized via bilateral thoracotomy under isoflurane administered via drop-method at embryonic day 16 (E16) (plug day =0) were used. After death was confirmed by cessation of heartbeat, the gravid uterus was removed from the female and embryos euthanized via decapitation. Urinary bladders were then microdissected under dissecting microscope (Olympus SZX7, Waltham, MA) from isolated embryos, and

embryonic bladders were separated from the urogenital sinus at the bladder neck and the attached ureters carefully dissected using Vanna spring scissors (Fine Science Tools, Foster City, CA). The mesenchyme and urothelium were separated manually under microscopic examination using two 25 gauge BD Precisionglinde syringe needles (Z192406, Sigma-Aldrich) connected to a 1cc syringe, leaving the mesenchyme behind as a bladder shell. MB49-GFP and MB49-SOX2 cells were trypsinized and neutralized, and washed with Dulbecco PBS (D-PBS, ThermoFisher Scientific) three times. After washing cells were re-suspended in 50 microliters of a 3:1 ratio of rat tail collagen I (354236, Corning) and setting solution (100 ml of 10× EBSS, 2.45 g of $NaHCO_3$, 7.5 ml of NaOH, 42.5 ml of Sterile distilled water) and were plated in 10 cm dishes.[11] Following the insertion of 1 eBLM per aliquot, tissue recombinants were placed at 37°C to promote solidification. McCoy's modified medium (ThermoFisher Scientific) containing 10% FBS was then applied to solidified grafts and incubated overnight.

The following day, eight-week-old male and female severe combined immunodeficiency C.B-17/IcrHsd-*Prkdc*^{scid} mice (SCID, Envigo) underwent allograft transplant of the tissue recombinants beneath the renal capsule of the left kidney. Male and female mice were implanted with tumor matrix for each genetically modified cell line (n=5 mice per group for each sex, total 10 mice per group): (1) MB49-SOX2, (2) MB49-GFP. A small hole was created in the renal capsule at the caudal end of the kidney using a stainless-steel Bonn micro probe (Fine Science Tools) or a heat-polished glass Pasteur pipette, after which the designated tissue recombinant graft was placed with forceps underneath the capsule. A secondary hole was made in the renal capsule at the cranial end of the kidney. Another graft was inserted with forceps for a total of two grafts per kidney. Two weeks following implantation, all mice were euthanized via CO_2 asphyxiation, followed by organ removal as a secondary form of euthanasia. Prior to euthanasia, a morbidity/mortality score was assigned to each mouse based on the behavior and attitude of the mouse at the experimental endpoint (**Table 1**). The mice were necropsied, and the spleen and both kidneys were collected for tissue preservation.

A scoring system was developed to evaluate the progression of the tumor. Scores were assigned for the following categories: ascites production, local tumor extension from the left kidney, tumor deposits in the mesentery, tumor deposits in the spleen, and tumor deposits in the liver. **Table 1** summarizes the scoring system and elaborates in the description for each score. Scoring was performed by the same individual who performed the surgeries and provided post-operative care to the mice. As such, the individual was not blinded to which mouse belonged to each cohort.

Histology

The spleen and left and right kidneys that were collected from each mouse were fixed in formalin for 48 hours and then rinsed and stored in 70% ethanol. Tissue sections were paraffin fixed and hematoxylin and eosin stained. The left kidney and spleen from each mouse were evaluated for tumor invasiveness. Tumor size, measured in millimeters, was also evaluated to quantify local tumor extension. **Table 2** gives a summary of this scoring system and details the description for each score. Scoring was performed by the same individual who performed the surgeries and provided post-operative care to the mice. As such, the individual was not blinded to which mouse belonged to each cohort.

Statistical analysis

The statistical approach for gene expression in clinical cases utilized nonparametric measures to compare continuous variables, specifically the Wilcoxon rank sum test for two-group comparisons and the Kruskal-Wallis for comparisons of greater than 2 groups. Cox models were utilized for survival analysis. SOX2 expression in the survival analysis was divided into high and low groups, defined as those with SOX2 expression above and below the median SOX2 expression for the whole group.

For the mouse allograft model, data are presented as the mean ± standard deviation of the mean. An unpaired, two-sided student t-test was used to evaluate relative expression of SOX2 in each cell line. The total gross pathology and histology score for each cell line overexpressing SOX2 was compared to the control group (GFP) using the Wilcoxon rank sum test. The individual categories were also compared

between cell lines overexpressing SOX2 and the control cell lines using the Wilcoxon rank sum test. Differences between sex were evaluated by a multivariable model with the total score as the dependent variable and sex and SOX2 status as independent variables. All values of significance were set to P=0.05. Statistical analysis was performed using SPSS IMB© Copyright IBM Corporation 1994, 2023 or R version 4.1.0.

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Table S1. Clinical and staging information for Tissue Microarray Cohort (n=303 patients)

Demographic and clinical data	
Patient age in years, median (range)	70 (39-93)
Male patients, number of cases (%)	217 (69%)
Invasive carcinoma, number of cases (%)	230 (76%)
Neoadjuvant chemotherapy, number of cases (%)	77 (25%)
AJCC Pathologic Staging (8th edition) information	
Tumor stage; number cases (%)	
pT0	34 (11%)
pTis/pTa	40 (13%)
pT1	25 (8.5%)
pT2	53 (17.5%)
pT3	116 (38%)
pT4	36 (12%)
Nodal Stage; number cases (%)	
pNx	13 (4%)
pN0	207 (68%)
pN1	30 (10%)
pN2	38 (13%)
pN3	15 (5%)

AJCC = American Joint Committee on Cancer.