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Supplemental Information

SOX2 Is a Marker for Stem-like Tumor Cells in Bladder Cancer

Fengyu Zhu, Weiqing Qian, Haojie Zhang, Yu Liang, Mingqing Wu, Yingyin Zhang, Xiuhong Zhang, Qian Gao, and Yang Li

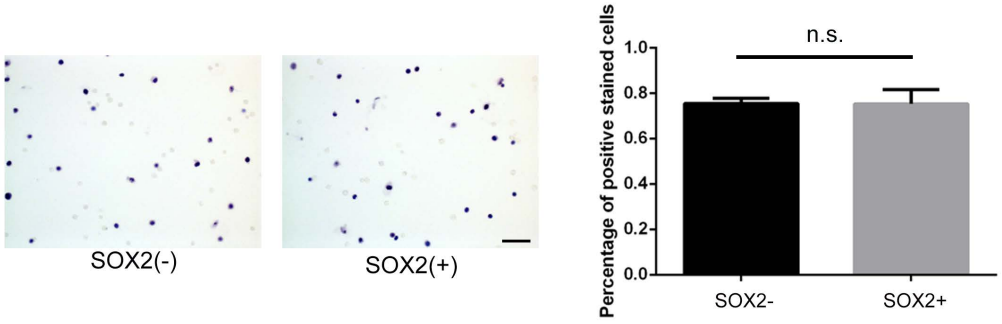
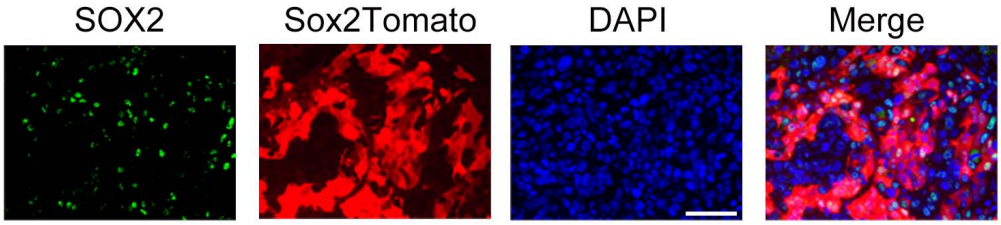
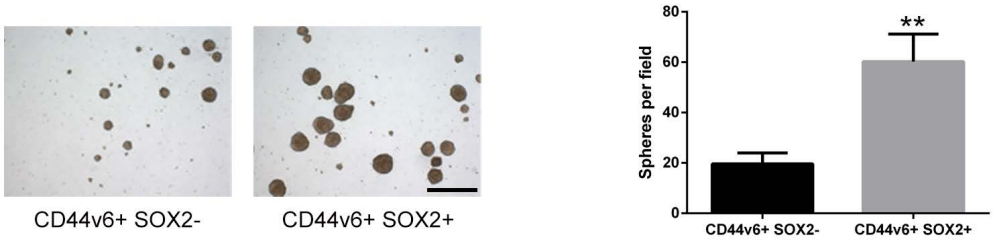


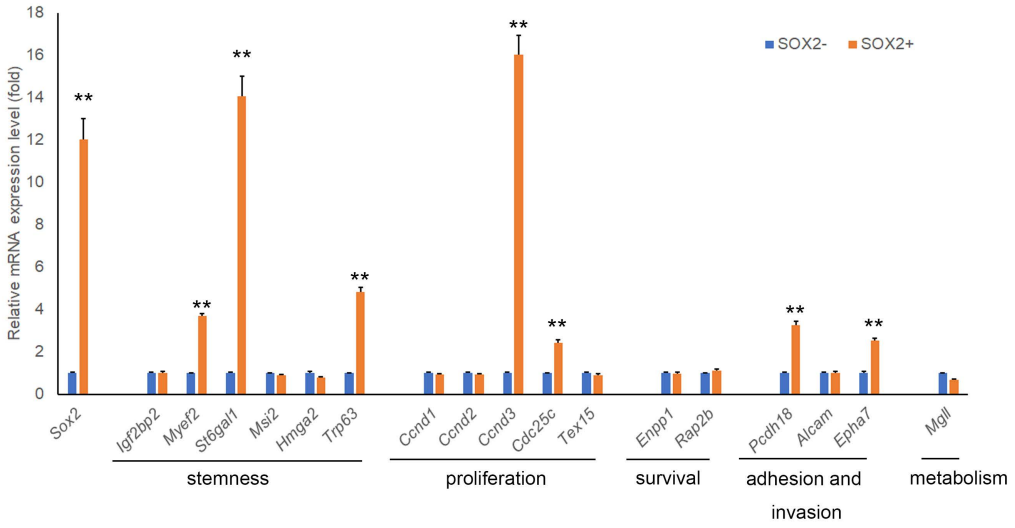
Figure S1 related to Figure 2. Validation of the cell viability after sorting. SOX2+/- cells were stained by typan blue after sorting and percentage of positive stained cells were calculated by counting 500 cells from randomly selected fields of each group, respectively. n.s.: no significant difference. Scale bars, 50 μm. Error bars represent the S.D. of three independent experiments.



Supplementary Figure S2, related to Figure 3, Immunofluorescent staining of SOX2 after tracing. Representative image shows immunostaining by SOX2 antibodies after tracing period. Scale bars, 50 μm.



Supplementary Figure S3, related to Figure 3, Sphere formation of CD44v6+Tomato+(SOX2+) and CD44v6+Tomato-(SOX2-) cells. CD44V6+Tom+(SOX2+) and CD44V6+Tom-(SOX2-) cells were FACS sorted and cultured in stem cell medium. Cultures at 10 days are shown and the sphere numbers are plotted. **, $p<0.01$, student t-test. Scale bars, 250 μm. Error bars represent the S.D. of three independent experiments.



Supplementary Figure S4, related to Figure 3, Expression pattern of SOX2 target genes. CD44v6+Tomato+(SOX2+) and CD44v6+Tomato-(SOX2-) cells were sorted by FACS, and mRNA level of SOX2 target genes were examined by quantitative RT-PCR. **, $p<0.01$, student t-test. Error bars represent the S.D. of three independent experiments.

Supplemental Experimental Procedures

Mice model

Sox2-Cre^{ERT2}, *R26*^{tdTomato} and *ROSA-DT*A mice were obtained from Jackson Laboratory. BBN(sc-486264, Santa Cruz) was dissolved in drinking water at a concentration of 0.05% (w/v) and provided to transgenic male mice for indicated times as previous described(Shin et al., 2014). Tamoxifen (T5648) were purchased from Sigma. For transplantation assays into immunodeficient mice, the different FACS-isolated cell populations (Sox2 Tomato+/-) from BBN-induced mice BCa tissue were collected in 4 ℃ medium. For limited dilution assay, different dilutions (64,000/16,000/4,000/1,000) of cells resuspended in 50 μl of Matrigel (Corning, 354230) were injected subcutaneously into BALB/cASlac-nu nude mice (Shanghai Laboratory Animals Center, SLAC). Secondary tumors were monitored weekly until mice presented signs of distress, and the mice were sacrificed. All animal procedures were performed under a protocol approved by the Laboratory Animal Center of Anhui Medical University and in accordance with National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Clinical sample

22 human BCa samples were obtained from Department of Urology, Huadong Hospital, Fudan University with patients' informed consent. The pathological condition of the BPH samples was determined by experienced urologists at Huadong Hospital.

Immunostaining

For frozen section, tissues were fixed in 4% paraformaldehyde at 4 ℃ for 48 h, followed with placing into 30% sucrose overnight and frozen in optimal cutting temperature (OCT) compound (Tissue Tek, Sakura). Frozen 10 mm sections were obtained and processed as previously described. For paraffin sections of bladder cancer tissue samples from mice and human patient were antigen retrieved, blocked and processed as described before(Liu et al., 2016). Hematoxylin–eosin stains were performed using standard histology procedures. The intensity of immunostaining was measured by Image-Pro Plus 6.0 image analysis software (Media Cybernetics). The intensity of each image was calculated by normalizing the average integrated optical density (IOD) with the total selected area of interest (AOI).

Primary antibodies					
Protein target	Company	Catalog Number	Host species	Clonality	Dilution
SOX2	Abcam	ab97959	Rabbit	polyclonal	1:100
KRT14	ProteinTech	60320-1-Ig	Mouse	monoclonal	1:150
Human CD44v6	Abcam	ab78960	Mouse	monoclonal	1:150
Mouse CD44v6	Thermo Fisher	33-6700	Mouse	monoclonal	1:150
Ki67	Thermo Fisher	PA5-19462	Rabbit	polyclonal	1:200
LAMININ	Abcam	ab11575	Rabbit	polyclonal	1:200
UroplakinIII	Abcam	ab78196	Mouse	monoclonal	1:200
Phospho-Stat3	Cell signaling	#9134	Rabbit	polyclonal	1:150

CD3	Abgent	AP52283	Rabbit	polyclonal	1:150
Secondary antibodies					
FITC conjugate	Proteintech	SA00003-2	Goat anti-rabbit IgG		1:200
FITC conjugate	Proteintech	SA00003-1	Goat anti-mouse IgG		1:200
TRITC conjugate	Proteintech	SA00007-2	Goat anti-rabbit IgG		1:200

FACS isolation and acquisition

Tumours were digested and blocked as previously described(Boumahdi et al., 2014). Immunostaining was performed using APC-conjugated anti-CD44v6 (1:100, BDPharmingen, 559250) by incubation for 30 min at room temperature. Cells were selected based on the expression of Sox2-tdTomato(PE) and/or CD44v6(APC). Gates for fluorescence fractionations were established using unstained and isotype controls. FACS analysis was performed using FACSAria and FACSDiva software (BD Biosciences). Sorted cells were collected either in culture medium for *in vivo* transplantation experiments or into lysis buffer for RNA extraction.

Sphere formation

FACS-sorted cells were cultured in ultra-low attachment plates (Corning Inc., Corning, NY, USA) at a density of 1,000 cells per well. Cells were cultured in modified serum-free culture mediums reported previously(Zhu et al., 2013). Spheres with a diameter over 20 μm were counted 3 days after plating.

Examine of gene expression level

Bladder samples were snap frozen in liquid nitrogen, homogenized with a mortar and pestle, and RNA extracted with the TRIzol (Invitrogen). Quantitative RT-PCR and western blot was performed as previously described(Li et al., 2015; Li et al., 2011). The primers used for RT-PCR is listed below.

Primer Names	Sequence
Sox2 F	5’-ACAGATGCAACCGATGCACC-3’
Sox2 R	5’-TGGAGTTGTACTGCAGGGCG-3
CD44v6 F	5'- CCTTGGCCACCACTCCTAATAG -3'
CD44v6 R	5'- CAGTTGTCCCTTCTGTCACATG -3'
Krt14 F	5'- AGCGGCAAGAGTGAGATTTCT -3'
Krt14 R	5'- CCTCCAGGTTATTCTCCAGGG -3'
Igf2bp2 F	5'- GTCCTACTCAAGTCCGGCTAC-3'
Igf2bp2 R	5'- CATATTCAGCCAACAGCCCAT-3'
Myef2 F	5'- GGGTCCAAGTGGAGTTGGG-3'
Myef2 R	5'- GCACTGCCAAGTCTACCAAAG-3'
St6gal1 F	5'- CTCCTGTTTGCCATCATCTGC-3'
St6gal1 R	5'- GGGTCTTGTTTGCTGTTTGAGA-3'
Msi2 F	5'- CTACAGTGCTCAACCGAATTTTG-3'
Msi2 R	5'- CTGGCCGCGCTTATGTAAT-3'
Hmga2 F	5'- GAGCCCTCTCCTAAGAGACCC-3'

Hmga2 R	5'- TTGGCCGTTTTTCTCCAATGG-3'
Trp63 F	5'- TACTGCCCCGACCCTTACAT-3'
Trp63 R	5'- GCTGAGGAACTCGCTTGTCTG-3'
Ccnd1 F	5'- GCGTACCCTGACACCAATCTC-3'
Ccnd1 R	5'- CTCCTCTTCGCACTTCTGCTC-3'
Ccnd2 F	5'- GAGTGGGAACTGGTAGTGTTG-3'
Ccnd2 R	5'- CGCACAGAGCGATGAAGGT-3'
Ccnd3 F	5'- CGAGCCTCCTACTTCCAGTG-3'
Ccnd3 R	5'- GGACAGGTAGCGATCCAGGT-3'
Cdc25c F	5'- AAAATGCAGCGTTCCTGCTTC-3'
Cdc25c R	5'- CTTGGGGTCCTAGTGCCTC-3'
Tex15 F	5'- TCATACCCACTGGTAACACAGC-3'
Tex15 R	5'- GGCAAAACATCACTCAAACCTG-3'
Enpp1 F	5'- CTGGTTTTGTCAGTATGTGTGCT-3'
Enpp1 R	5'- CTCACCGCACCTGAATTTGTT-3'
Rap2b F	5'- GCTCACCGTGCAGTTCGTAA-3'
Rap2b R	5'- GCTGTAGACGAGAATGAAGCC-3'
Pcdh18 F	5'- ATGCACTTTAGATTTGCACTTGC-3'
Pcdh18 R	5'- CAATTACCGATCCGACCCTCT-3'
Alcam F	5'- GGCAGTGGGTTCGTCATAAAC-3'
Alcam R	5'- ATCGCAGAGACATTCAGGGAG-3'
Epha7 F	5'- TGACCCTGAAACCTATGAGGAC-3'
Epha7 R	5'- ATTCTCCTGCACCAATCACAC-3'
Mgll F	5'-ACCATGCTGTGATGCTCTCTG-3'
Mgll R	5'-CAAACGCCTCGGGGATAACC-3'
Gapdh F	5'- AGGTCGGTGTGAACGGATTTG -3'
Gapdh R	5'- GGGGTCGTTGATGGCAACA -3'

Invasion assay

BioCoat™ Matrigel invasion chamber was used according to the manufacturer's instruction (BD Biosciences). Briefly, 4×10^4 isolated cells by the flow cytometry were resuspended in 100 μl of DMEM medium, and seeded in the upper portion of the invasion chamber. The lower portion of the chamber contained 500 μl of medium supplemented with 2% FBS and glutamine, which served as a chemoattractant. After 36 h, non-invasive cells were removed from the upper surface of the membrane with a cotton swab. The invasive cells on the lower surface of the membrane were stained with crystal violet, and counted in four separate areas with an inverted microscope(Chen et al., 2011).

Statistics

Data are presented as the means±standard deviation (S.D.) or standard error (S.E.). All of the statistical analyses were performed using Excel (Microsoft, Redmond, WA) or Prism (GraphPad Software Inc., La Jolla, CA). The two-tailed Student's t-test or one way ANOVA were used and a P-value of <0.05 was considered significant.

Supplemental References

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