**Supplementary Information**

**Paternally Expressed Gene 10 (PEG10) Promotes Growth, Invasion and Survival of Bladder Cancer**

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**Supplementary Materials and Methods**

**TMA construction and Immunohistochemistry (IHC)**

The VPC Bladder Cancer TMA was constructed by punching duplicate cores of 1 mm in diameter for each sample using a semi-automated tissue microarrayer from Pathology Devices TMArrayer attached to Leica M50 stereo microscope. IHC staining was performed using the Ventana Discover XT Autostainer (Ventana Medical Systems, Tucson, AZ) with a biotin streptavidin system and solvent-resistant DAB Map kit. The antibodies used in IHC were listed in Supplementary Table 1. PEG10 IHC staining in the TMA was scored as follows: 0 = no staining, 1 = faint or focal staining, 2 = convincing intensity in a minority of cells, and 3 = convincing intensity in a majority of cells.

**NE markers used for the analysis of the TCGA dataset**

NE markers used in the data analysis are: *Musashi-1 (MSI1), Pleckstrin Homology And RhoGEF Domain Containing G4B (PLEKHG4B), Amyloid Beta Precursor Like Protein 1 (APLP1), SRY-Box2 (SOX2), Rho Family GTPase 2 (RND2), Tubulin Beta 2B Class IIb (TUBB2B), enolase 2 (ENO2), G Protein Subunit Gamma 4 (GNG4), chromogranin A (CGA), synaptophysin (SYP), MYCN Proto-Oncogene, BHLH Transcription Factor (MYCN) and POU Class 3 Homeobox 2 (POU3F2).*

**Analysis of the Neo Adjuvant Chemotherapy (NAC) dataset**

The NAC cohort has been published before (1). RNA was isolated from pre-NAC transurethral resection (TURBT) specimens from 343 patients with MIBC. In addition, tissue from cystectomy specimens of invasive bladder cancers of patients receiving at least three cycles of cisplatin-based NAC were sampled and RNA was isolated similarly. Whole transcriptome profiling was performed with the Decipher assay (based on the Human Exon 1.0 ST oligonucleotide microarray) in a CAP/CLIA clinical laboratory (GenomeDx, Inc, San Diego CA). After quality control, 305 TURBT, 133 RC and 116 matched pairs were available.

**Western blot analysis**

Protein was isolated with RIPA buffer containing proteinase and phosphatase inhibitors (cOmplete, phosSTOP, Roche, Mississauga, Canada) for 30 minutes on ice. Samples were centrifuged at 13,000 rpm for 10 minutes, and then the supernatant was transferred into an empty eppendorf tube. The protein concentration was measured using a BCA protein assay (Thermo Scientific, Waltham, MA). Protein (30µg) was separated electrophoretically on 10% SDS polyacrylamide gels and then transferred to a polyvinylidene difluoride or a nitrocellulose membrane. After blocking, membranes were incubated with a primary antibody at 4°C overnight. Blots were developed either by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) or by fluorescence using the Odyssey Imaging System (LI-COR Biosciences). Where the whole-protein lysates were collected, the cells were collected with 1x sample buffer (50mM Tris-HCl, pH6.8, 2% SDS, 10% glycerol, 0.1M DTT and 0.02% bromophenol blue) and then boiled to denature the proteins before loading to the polyacrylamide gels. Vinculin, actin or tubulin was used as loading control. Primary antibodies are shown in Supplementary Table 1.

**Quantitative real-time qPCR**

Real-time qPCR were performed as described previously (2, 3). RNA was extracted from cells using an RNEasy mini kit (Qiagen) according to the manufacturer’s instructions, followed by DNase I (Life Technologies) treatment for 15 minutes at room temperature. Then, RNA was subjected to reverse transcription using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time qPCR amplification of cDNA was carriedout using probes (listed in the Supplementary Table 2) in the Viia7 Real Time PCR System (Applied Biosystems) withUniversal Probe Master Mix (Roche) in a 10 μL final volume. Relativetarget gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level using the comparative **ΔΔ**Ct method. Results are representative of at least three independent experiments with each sample run in triplicate.

**Proliferation assay and cell cycle analysis**

Cell growth was assessed with a Cell Counting Kit 8 (Dojindo) according to manufacturer’s protocol. The cell cycle distribution was analyzed by double staining with bromodeoxyuridine (BrdU) and 7-Aminoactinomycin D (7-AAD) using a FITC BrdU Flow Kit (BD Biosciences) according to the manufacturer’s protocol.

**Cell migration and invasion assays**

For migration assays, scratches were made using a pipet tip, and cells were maintained in serum-free medium containing TGF-β (0.1 ng/ml) and Mitomycin C (0.3 µg/ml). Images were taken right after the scratch and also 24h after the scratch. Cell invasion was assessed using Biocoat Matrigel invasion chambers (BD Biosciences). Cells were seeded in the upper chamber with serum-free medium, and the medium in the lower chamber was supplemented with 20% serum. Eighteen hours after seeding, polycarbohydrate membranes from the bottom of the upper chambers were stained with crystal violate to visualize the invaded cells.

**Orthotopic bladder cancer xenograft model**

Six-week-old female nude mice (Harlan Laboratories, Indianapolis, IN) were anesthetized with 2% isoflurane. Analgesia was provided by a subcutaneous injection of meloxicam (Boehringer Ingelheim Vetmedica, St. Joseph, MO). Six-week-old female nude mice (Harlan Laboratories, Indianapolis, IN) were anesthetized with 2% isoflurane. Analgesia was provided by a subcutaneous injection of meloxicam (Boehringer Ingelheim Vetmedica, St. Joseph, MO). Cell suspension (50 μL) in Matrigel containing 4.0×105 cells were inoculated into the bladder wall of nude mice with a 30 G needle by percutaneous injection under ultrasound guidance. For *in vivo* photoimaging (IVIS Lumina, PerkinElmer), cells underwent transduction with a lentiviral construct containing a firefly luciferase gene under Blasticidin selection (Life Technologies). The direct relationship between the cell number, tumor size, luciferase activity, and bioluminescence was monitored and controlled (R>0.99; data not shown) using the Xenogen IVIS Spectrum imager. Bioluminescence was used to quantify tumor burden and measured by the Xenogen IVIS Spectrum imaging system (Perkin Elmer, Waltham, MA). Images were recorded at 10 and 15 minutes after luciferin injection. Average counts were used for statistical analysis.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.**

Freshly cut tissue sections were analyzed for immunoexpression using Ventana Discovery Ultra autostainer (Ventana Medical Systems, Tucson, Arizona). For apoptosis assay, Terminal transferase (Roche, Cat# 03333566001, 1:20 dilution), Digoxigenin-11-dUTP, (Roche, Cat# 11093088910) and dATP (Sigma, Cat# D6920) were used. In brief, tissue sections were treated with protease 2 (Ventana, Roche, Cat# 760-2019 ) for 8 minutes at 37°C to retrieve antigenicity, followed by incubation with a TUNEL reaction mixture at 37°C for 60 min. The exposed apoptotic DNA fragments were tagged with Biotinylated anti-DIG (Sigma, Cat# B7405, 1:500) at 37°C for 32 min and visualized using Ventana DAB Map detection kit (Cat# 760-124).

**Supplementary References**

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