Supplemental Experimental procedures

Microscopic and macroscopic prostate cancer in genetically engineered transgenic mice, dissection, and histology and pathology analysis:

In a previous study, we used a 3.84 kb promoter/enhancer region of the PSP94 gene directing SV40T/t antigen targeted specifically to mouse prostate tissue (strain F1[C57BL/6 x CBA]) to establish the PSP-TGMAP model \(^1\)\(^-\)\(^3\). Similar to the TRAMP model, most PSP94 transgenic mice develop fast-growing tumors in lobes of the ventral prostate (VP), dorsolateral prostate (DLP), and anterior prostate (AP, the coagulation gland) within 4 to 8 months of age. Transgenic mice were identified by a quick PCR genotyping protocol \(^1\),\(^2\),\(^3\). All animal experiments were conducted according to standard protocols approved by the University of Western Ontario Council on Animal Care.

After imaging, mice with tumors were euthanized. A dissection procedure was followed as per \(^1\),\(^2\),\(^3\). In brief, the prostate tissue including VP, DLP, and AP, the male accessory gland (SV, seminal vesicles), and the whole bladder were removed for gross examination and serial sectioning. Histopathological characterization and standard definitions of various degrees of mPIN (mouse prostatic intraepithelial neoplasia) and well, moderately and poorly differentiated CaPs were classified as we previously reported \(^1\),\(^2\),\(^3\).

3-D power Doppler ultrasound imaging: Three-dimensional gray scale (anatomical) images of a 17 × 17 × 17 mm\(^3\) field of view containing the prostate and surrounding structures were obtained using a Vevo 770 (VisualSonics Inc., Toronto, ON) ultrasound micro-imaging system with a 30 MHz transducer. The ultrasound system was equipped with 3-D image visualization and analysis software described previously \(^4\). Imaging studies were performed using procedures similar to those detailed in Ref \(^5\). Tumor volumes were measured by manually outlining lesions in 3-D images as described in \(^5\). Tumor volume doubling times were estimated from longitudinal sequences of images by fitting exponential growth curves to the measured volumes using nonlinear least-squares curve fitting.
Three-dimensional blood flow images were acquired using the ultrasound system’s power Doppler mode. The system employs a “continuous scan” power Doppler data acquisition strategy similar to that described by Goertz et al. 6. When operated in power Doppler mode, the nominal in-plane spatial resolution of the system was 140 µm (lateral) by 130 µm (depth), the out-of-plane resolution was 140 µm, and the system parameters were set to provide sensitivity to vessels with flow velocities greater than 3 mm/s.

Tumor vascularity was quantified in power Doppler images by computing the color pixel density (CPD), which is equal to the percentage of image voxels within a region of interest that exhibit detectable flow. Three CPD values were obtained from each image using the boundary defined when measuring the tumor volume in the 3-D gray scale image: (1) an overall CPD value for the entire tumor, (2) a peripheral CPD value within a 3-D shell extending 1 mm inward from the tumor boundary, and (3) an internal CPD value corresponding to the volume in the interior of the tumor greater than 1 mm from its boundary.

Microfil-enhanced x-ray micro-CT images were obtained by sacrificing the mice, clearing the blood with retrograde aortic perfusion of heparinized saline, and infusing a radiopaque silicone polymer (Microfil MV-122, Flow Tech, Carver, MA), which increases x-ray attenuation and allows for visualization of the vasculature. The abdomen and perineum of each mouse (75 mm field of view) were imaged using a GE eXplore Locus micro-CT scanner (GE Healthcare Biosciences, London, ON); 720 x-ray projection views (80 kVp, 450 mAs) were obtained at 0.5° intervals. Three-dimensional CT images were reconstructed with isotropic voxels of 45 × 45 × 45 µm³.

Tumor vascularity was quantified in the micro-CT images by computing the vascular density as follows. Entire-tumor, peripheral, and internal regions of interest were manually outlined in the CT images using 3-D analysis software (MicroView, GE Healthcare Biosciences, London ON). The regions of interest were defined relative to the tumor boundary in a manner identical to the power Doppler CPD analysis. Within each region of interest, a global threshold was determined using an automatic technique28 to differentiate vessels from surrounding tissue. The number of voxels with CT numbers
greater than the threshold value was counted and that result was used to compute the volume occupied by vessels within that region. Division of the vessel volume by the total volume of the region of interest yielded the vascular density, which has the same units (\%) as, and hence can be compared with, the power Doppler CPD of the corresponding region.

For visualization of vascular anatomy, 3-D surface renderings of the vasculature were generated using the isosurface feature of the MicroView software. This technique allows surfaces defined by a specified gray level to be extracted from a 3-D image. Different arbitrary colors were assigned to anatomically identifiable surfaces to highlight the prostate and specific branches of the vasculature. The color coding facilitates visual differentiation between vascular structures. In this study, consistent colors were applied to identifiable features in each of the rendered CT images (e.g., vessels supplying the prostate or prostate tumor are displayed in pink and vessels supplying the bladder are displayed in yellow).

**FITC-labeled lectin and CD31 high-resolution confocal microscopy in TRAMP mice:**

Vascular labeling with FITC-lectin, CD31 immunofluorescence, and high resolution confocal microscopy were mostly performed in TRAMP mice. Mice were anesthetized using Avertin (240 mg/kg) and immobilized. Vasculature was labeled by injecting 50 mg of FITC conjugated Lycopersicon esculentum lectin (Vector Laboratories, Burlingame, CA) into the left ventricle. Mice were then euthanized and perfused with a fixative containing 1% paraformaldehyde and 0.5% glutaraldehyde in PBS, and subsequently rinsed with PBS, and the urogenital tracts were removed. The dorsal, lateral, and ventral lobes of the prostate were microdissected, flash frozen in OCT (frozen tissue matrix) using a liquid nitrogen and isopentane bath, and stored at -80°C. Frozen sections were cut to a thickness of 100 \( \mu \text{m} \) using a Leica CM1850 cryostat. Sections were dried and post-fixed in 4% paraformaldehyde for 15 minutes at room temperature. After washing several times with PBS, sections were blocked for one hour with PowerBlock and then incubated overnight with a 1:200 dilution of rat antimouse CD31 (BD Pharmingen, San Diego, CA). Following several washes with
PBS, slides were incubated with a 1:200 dilution of AlexaFluor594 goat anti-rat IgG (Molecular Probes, Eugene, OR), washed several times with PBS, and coverslipped. Images were collected using a Zeiss LSM 510 confocal laser scanning microscope.

**Immunohistochemistry (IHC) analysis of microvessel distribution in PSP-TGMAP mice:** Large prostate tumors (2.5 cm in diameter, at 28-38 weeks of age) from either the dorsolateral or ventral lobes of the prostate were dissected and flash frozen in liquid nitrogen. Frozen sections were prepared for IHC staining from areas of the outer ring and inner core of the freshly dissected sample. IHC protocols were followed as previously described. Sections were incubated with a 1:50 dilution of rat antimouse PECAM-1 (CD31, BD Pharmingen, San Diego, CA) for two hours, and the second antibody used was biotin conjugated polyclonal antibody against rat IgG (1:100 dilution, from Pharmingen). IHC staining signals (from small or large microvessels) in all slides were counted within an area of approximately 1 cm² for each field observed in a microscope to compare the microvessel distribution. H&E staining was used for pathological determinations. Normal mouse (age of 14 weeks) prostate tissues were used as controls and processed using the same procedures.

**Microsurgical procedure:** All animal surgical operation procedures followed the University Animal Care Veterinary Services Standard Operating Procedure for approved protocols on rodent surgical prep and recovery surgery including post-operative care. Mice were anesthetized in an induction chamber using 3-4% isoflurane in oxygen, and then anesthesia was maintained by supplying 1-1.5% isoflurane through a breathing mask. A median abdominal incision was performed. The mouse’s prostate gland and branches of the internal iliac artery supplying the ventral and dorsolateral prostate were identified visually under the operating microscope. The majority of the blood supply to the prostate was blocked by ligating the branches of the inferior vesical artery and vein using 11-0 nylon suture. If the tumor was small and localized to one side of the prostate, then only the vessels on that side were ligated; otherwise, both the left and right vesical arteries and veins were blocked. The ventral body wall was sutured closed and the mouse was
allowed to recover. Fig. 4 included in the online supplementary information illustrates
the procedure in more detail. Gray-scale and power Doppler imaging was performed
immediately before surgery and longitudinally over several days following surgery to
monitor to the tumor’s response to the intervention. This procedure was performed on 8
PSP-TGMAP mice that were confirmed by gray-scale ultrasound to possess small to
medium-sized tumors, and on 5 wildtype Balb/c mice as controls.

Reference List