Functional Neoangiogenesis Imaging of Genetically Engineered Mouse Prostate Cancer Using Three-Dimensional Power Doppler Ultrasound

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Abstract
We report the first application of high-frequency three-dimensional power Doppler ultrasound imaging in a genetically engineered mouse (GEM) prostate cancer model. We show that the technology sensitively and specifically depicts functional neoangiogenic blood flow because little or no flow is measurable in normal prostate tissue or tumors smaller than 2–3 mm diameter, the neoangiogenesis "switch-on" size. Vascular structures depicted by power Doppler were verified using Microfil-enhanced micro-computed tomography (micro-CT) and by correlation with microvessel distributions measured by immunohistochemistry and enhanced vascularity visualized by confocal microscopy in two GEM models (transgenic adenocarcinoma of the mouse prostate (TRAMP) and PSP94 gene-directed transgenic mouse adenocarcinoma of the prostate (PSP-TGMAP)). Four distinct phases of neoangiogenesis in cancer development were observed, specifically, (a) an early latent phase; (b) establishment of a peripheral capsular vascular structure as a neoangiogenesis initiation site; (c) a peak in tumor vascularity that occurs before aggressive tumor growth; and (d) rapid tumor growth accompanied by decreasing vascularity. Microsurgical interventions mimicking local delivery of antiangiogenesis drugs were done by ligating arteries upstream from feeder vessels branching to the prostate. Microsurgery produced an immediate reduction of tumor blood flow, and flow remained low from 1 h to 2 weeks or longer after treatment. Power Doppler, in conjunction with micro-CT, showed that the tumors recruit secondary blood supplies from nearby vessels, which likely accounts for the continued growth of the tumors after surgery. The microsurgical model represents an advanced angiogenic prostate cancer stage in GEM mice corresponding to clinically defined hormone-refractory prostate cancer. Three-dimensional power Doppler imaging is completely noninvasive and will facilitate basic and preclinical research on neoangiogenesis in live animal models.

Introduction
The formation of tumor-associated new blood vessels, neoangiogenesis, is the cardinal feature of virtually all malignant tumor growth and the initiation of metastasis. Because of this commonality, probing tumor-induced and associated angiogenesis is a viable approach to detect and treat a wide range of cancers. Recently, accumulated evidence has confirmed that the combination of chemotherapy with inhibition of neovascularization is a promising therapeutic strategy. This strategy is motivated by the fact that tumor growth beyond 2 to 3 mm diameter is strictly dependent on neoangiogenesis (for reviews, see refs. 1–4).

However, many potential antiangiogenic therapies have not lived up to the expectations of preclinical trials because those drugs are usually cytotoxic and lack the specificity to target angiogenic tissues in tumors. This occurs because normal vascular development or angiogenesis is required for many normal physiologic organ and tissue maintenance processes, and both endogenous angiogenic promoters and angiogenic inhibitors are required (2, 3). Tumor-associated angiogenesis is controlled only by a different balance between angiogenic promoters and inhibitors relative to normal tissues (3). Furthermore, maximum tolerated doses and responses to anticancer treatments vary widely among individuals (4). Advances in imaging are transforming our understanding of angiogenesis and the evaluation of drugs that stimulate or inhibit angiogenesis in preclinical models and human disease (for reviews, see refs. 2, 5–7).

The objective of this study is to image in vivo the process of neoangiogenesis using a genetically engineered mouse (GEM) prostate cancer model. Prostate cancer is the most common cancer and the third leading cause of cancer mortality in adult men in North America. Prostatectomy and radiation therapy are more widely used to control early-stage disease. Although most patients initially respond to hormone therapy, almost all prostate cancer patients eventually develop progressive disease, a condition called androgen-independent or hormone-refractory prostate cancer. Currently, there is no curative therapy for hormone-refractory prostate cancer.

Because prostate cancer does not occur naturally in rodents, and because mice are the most prominent model organisms for research, basic and preclinical studies frequently employ GEM
Autochthonous prostate cancer models (for reviews, see refs. 8, 9). These GEM prostate cancer models normally employ promoters from prostate tissue-specific genes (probasin, refs. 10, 11; PSP94, refs. 12–14), or prostate-specific antigen to drive prostate-tissue–specific targeting of oncogene SV40 T antigen (T and/or t). Transgenic models, in contrast to s.c. or orthotopic tumors implanted in immunodeficient nude mice, show salient aspects of prostate cancer, including a complete process of tumorigenesis, tumor progression, and metastasis, along with angiogenesis mimicking the human disease.

Our research team and collaborators have developed high-frequency (~20 MHz) three-dimensional ultrasound microimaging for measuring tumor progression in mouse cancer models (15–17) and showed the use of the technology in a GEM prostate cancer model (12, 13). The sensitivity of high-frequency power Doppler to changes in tumor blood flow produced by an antivascular agent was first shown in an orthotopic melanoma in an athymic nude mouse model (18). However, xenograft models are frequently not representative of the corresponding human tumor.

Not all blood vessels are functional in living organisms. Examination of the morphologic structure may not necessarily allow direct assessment of tumor microcirculatory function (for a review, see ref. 19). This paper reports the use of high-frequency power Doppler to obtain the first high-resolution three-dimensional images of functional neoangiogenesis in a live transgenic prostate cancer model, the PSP94 gene-directed transgenic mouse adenocarcinoma of the prostate (PSP-TGMAP) model.

Materials and Methods

Microscopic and macroscopic prostate cancer in genetically engineered transgenic mice, anatomy and histology and pathology analysis. PSP94 gene and rat probasin-directed SV40T/t antigen GEM-prostate cancer PSP-TGMAP (12–14) and transgenic adenocarcinoma of the mouse prostate (TRAMP) (10) models were used. Transgenic mice in both models develop rapidly growing tumors within 4 to 8 months of age. All animal experiments were conducted according to standard protocols approved by the University Council on Animal Care. Vascular anatomy was identified by following the text by Cook (20).

Three-dimensional ultrasound imaging. Three-dimensional gray-scale (anatomic) and power Doppler (blood flow) images showing the prostate and surrounding structures were obtained using a Vevo 770 (VisualSonics Inc., Toronto, ON) ultrasound microimaging system with a 30-MHz transducer and three-dimensional image visualization and analysis software described previously (21). When operated in power Doppler mode, the nominal in-plane spatial resolution 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 >3 mm/s.

Anatomic images were used to measure tumor volumes as described in ref. (16). 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 regional CPD values were obtained from each image using a tumor boundary determined from the three-dimensional gray-scale image: (a) an overall CPD value for the entire tumor; (b) a peripheral CPD value within a three-dimensional shell extending 1 mm inward from the tumor boundary, and (c) an internal CPD value corresponding to the volume in the interior of the tumor >1 mm from its boundary. Peripheral CPD measurements in wild-type mouse prostate were done within a 0.5-mm-thick shell around the perimeter of the gland.

Microfil-enhanced X-ray micro-computed tomography. Micro-CT images were obtained by sacrificing the mice, clearing the blood with heparinized saline, and infusing a radiopaque silicone polymer (Microfil MV-122, Flow Tech, Carver, MA). Each mouse was imaged using a GE eXplore Locus micro-CT scanner (GE Healthcare Biosciences, London, ON); 720 projection views were obtained at 0.5° intervals, and three-dimensional CT images were reconstructed with 45 × 45 × 45-μm3 voxels.

Entire-tumor, peripheral, and internal regions of interest, defined as in the Doppler CPD analysis, were manually outlined in the CT images using three-dimensional analysis software (MicroView, GE Healthcare Biosciences). Voxels with CT numbers greater than an automatically determined threshold value (22) were treated as vessels and used to compute the vascular volume. Division of the vascular 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 region.

For visualization of vascular anatomy, three-dimensional surface renderings of vessels were generated using the MicroView software. Different colors were assigned to anatomically identifiable features to highlight the prostate and specific branches of the vasculature.

Immunohistochemistry analysis of microvessel distribution and FITC-labeled lectin and CD31 high-resolution confocal microscopy. Large prostate tumors (2.5 cm in diameter, at 28–38 weeks of age) from PSP-TGMAP mice were dissected, and frozen sections were prepared for immunohistochemistry staining from areas of the outer ring and inner core of the freshly dissected sample. Immunohistochemistry protocols were followed as we reported previously (13, 14) using a 1:50 dilution of rat antimesenchymal cell adhesion molecule 1 (CD31, BD PharMingen, San Diego, CA). Vascular labeling with FITC-lectin, CD31 immunofluorescence, and high-resolution confocal microscopy were mostly done in TRAMP mice. Following several washes with PBS, slides were incubated with a 1:200 dilution of AlexaFluor594 goat anti-rat immunoglobulin G (Molecular Probes, Eugene, OR), washed several times with PBS, and coverslipped. Images were collected using a Zeiss LSM 510 confocal laser scanning microscope.

Microsurgical procedure. In normal mice, the prostate and bladder typically share blood supplied by the superior vesical artery, which branches from the internal iliac artery, and by the inferior vesical artery, which branches from either the inferior iliac or the superior vesical artery (for more detailed views of the anatomy, see Fig. 1C, Supplementary Fig. S4, and ref. 20). The majority of the blood supply to the prostate was blocked by ligation the branches of the inferior vesical artery and vein using 11-0 nylon sutures. 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. Animal surgical procedures followed university Animal Care and Veterinary Services standard operating procedures.

All experimental procedures are described in detail in the Supplementary material.

Results

High-resolution, three-dimensional power Doppler ultrasound imaging detects tumor-associated angiogenesis verified by Microfil-enhanced micro-CT in a GEM prostate cancer model. Three-dimensional power Doppler and Microfil-enhanced micro-CT images of five prostate tumors with volumes ranging from 7 to 1300 mm3 (2–15 mm diameter) were acquired to compare the vascular architecture at different stages of tumor growth. Each row in Fig. 1A, from left to right, consists of a three-dimensional power Doppler image, a three-dimensional micro-CT image aligned with the three-dimensional power Doppler, a two-dimensional plane through the power Doppler, a matched two-dimensional plane through the micro-CT, and an overlay comparing the two-dimensional images. The vessels labeled with arrows were used to register the images. Comparisons of vascularity depicted in the two modalities suggest that the

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8 http://www.informatics.jax.org/cookbook/
smallest vessels reliably detected by power Doppler were ~100 to 150 μm diameter.

A movie (Supplementary Fig. S1) shows user manipulation of registered power Doppler and micro-CT images of the 370-mm³ tumor shown in Fig. 1A using image visualization software developed in our laboratory (21). Rotation of the images enhances the viewer’s appreciation of the three-dimensional nature of the vascular structures and shows that the majority of the vasculature depicted in the images is concentrated in the peripheral regions of the tumor. This impression is confirmed by the regional vascularity data (Fig. 1B) obtained from the three-dimensional Doppler and micro-CT images of the tumors shown in Fig. 1A. Peripheral vascularity is greater than internal vascularity in five of the six images; the micro-CT image of the 370-mm³ tumor was the only exception to this pattern. Inspection of that CT image revealed that Microfil formed small pools in the inner core of that tumor. Those
Microfil pools seem to have increased the CT internal vascular density estimate for that tumor.

Figure 1C shows a power Doppler view of a typical control prostate and surrounding tissue. The few functional vessels with sufficient flow to be detected by power Doppler supply the bladder and surrounding tissues; power Doppler shows flow around the outside of the prostate, but is not sensitive to blood flow within the prostate in the control mice. Prostate vasculature is essentially normal at 12 wks of age. At 18 wks, the PIN phenotype or well- to moderately differentiated adenocarcinomas (18 wks – 2) were highly angiogenic. In 24-week-old TRAMP mice, in the interior of large poorly differentiated tumors, regions of hemorrhage (arrowheads) were shown by the pooling of FITC-lectin. Magnification, 40×; 325 × 325 μm of specimen.

Power Doppler imaging provides an effective neoangiogenesis functional assay: correlation of three-dimensional power Doppler imaging with microvessel determination in histopathologic and high-resolution confocal microscopy fluorescence staining. The differences in peripheral and internal vascularity in the power Doppler images of tumors and the differences in vascularity depicted in prostate tumors compared with normal mouse prostate suggest that power Doppler can be used to detect the functional consequences of neovascularization in GEM prostate cancer models. To support these interpretations, immunohistochemical measurement of microvessel density (MVD) was done with an antibody to the endothelial cell marker CD31, which serves as a surrogate marker for angiogenesis. Five large tumors (2–3 cm diameter) were dissected, and frozen sections of tumor specimens from the outer ring and inner core were studied separately. Figure 2A shows immunohistochemistry results. As shown in Fig. 2B, graph 1, total CD31 staining signals in outer ring are statistically significantly higher than in the inner core (t-test, \( P = 0.004 \)). Graph 2 (Fig. 2C) shows the CD31 vessel counts from different vessel size ranges and shows that most of the microvessels are small (10–30 μm diameter) and large (100 μm), with fewer medium-sized vessels.
vessels (12–24 μm diameter, i.e., the true MVD) were detected in the outer ring of the tumor mass. Vessels of 100 μm or larger were mostly located in the inner core. H&E staining of the tumor mass (Fig. 2A) showed that the inner core consisted primarily of necrosis, hemorrhage (seen at ×40), large blood vessels, and a lower density of poorly differentiated prostate cancer cells, which is a vascular tumor structure. The concentration of larger vessels in the core of the tumor could explain the CT vascular density results (Fig. 1B) for the large tumor, because large blood vessels would provide space for the observed pooling of the Microfil. Conversely, the outer ring is an active neuroendocrine tumor (13) structure with less necrosis and a high density of tumor cells. Control measurements on five normal prostate specimens showed no or few MVD.

Similar CD31 staining was observed in the TRAMP model. Figure 2D shows high-resolution confocal microscopy images of FITC-labeled lectin and CD31 staining in TRAMP mice (12–24 weeks of age), demonstrating that neoangiogenic vessels are much more disorganized in GEM-prostate cancer tissues than are vessels in the normal mouse prostate. In the FITC images in Fig. 2D, the vascular network has broken down and allowed the lectin to leak out of the vessels and aggregate into large bright-green globs. This leakiness corresponds to the appearance of hemorrhagic regions of blood pooling on the H&E-stained slides (which was also observed in PSP-TGMAP tumors). It is likely that the blood leaking out of these vessels was flowing too slowly to be detected by Doppler, but the vessel permeability may have contributed to the pooling of Microfil in the CT image of the large tumor in Fig. 1A. In addition, regional differences exist in the blood flow within the tumor, as shown by the fact that regions of necrosis as well as regions of live cells were observed within the tumor mass (age of 24 weeks).

**Longitudinal three-dimensional power Doppler imaging shows kinetics of tumor-associated angiogenesis, with a peak**
in vascularity development before rapid tumor volume growth. Longitudinal three-dimensional gray-scale and power Doppler imaging was done in 10 PSP-TGMAP mice. A four-phase description (Fig. 3A–H) of the entire neoangiogenesis process is summarized from an illustrative mouse (27 weeks of age). Growth and vascularity curves (Fig. 3N–P) show longitudinal relationships between the internal, peripheral, and overall vascularity (CPD) and tumor volume. Microfil-enhanced micro-CT images were also acquired from some of the mice after they were euthanized.

In a first, "latent" phase illustrated in Fig. 3A (designated day 0), tumor diameters are <2 to 3 mm. Power Doppler exhibited minimal blood flow, although gray-scale ultrasound is capable of detecting GEM-prostate cancer at this stage.

The second phase establishes a peripheral capsular vascular structure (Fig. 3B–I, which was confirmed by contrast agent enhanced micro-CT imaging (Fig. 3B–2). Although the tumor initially grew slowly (reaching 3.65 mm³ on day 4), the Doppler vascularity (CPD) increased to 3.6%. This increase in CPD reflects increased flow in the peripheral capsule of the tumor because the peripheral CPD increased from 0.9% to 4.6% from days 0 to 4, whereas the internal CPD remained similar to the vascularity measured in wild-type mice at 0.2% to 0.4% (graphs shown in Figs. 3B-3 and 3O). Similar observations were made in the other untreated mice with tumors smaller than 2 to 3 mm in diameter (n > 5; Fig. 3B and J).

The peripheral capsular vascular structure was observed consistently in tumors larger than 2 mm diameter, as illustrated by additional examples in Fig. 3I–M. Figure 3I is a two-dimensional Doppler image of a small tumor that has not yet developed a peripheral capsular structure. The vascularity of this tumor is visibly different from the subsequent examples. Figure 3J is a three-dimensional Doppler image suggesting the beginning of a capsular structure; the arrow indicates the spermatic vessel that was highlighted in Fig. 3A. That capsular structure became more prominent a few days later (Fig. 3B-1). Figure 3K is a three-dimensional Doppler image of a 4-mm-diameter tumor showing a well-developed vascular capsule encircling the perimeter of the tumor. Figure 3L is a gross pathology photograph of a very large tumor obtained after the mouse was infused with Microfil. The yellow Microfil highlights the tumor’s peripheral vessels.

The third phase is characterized by a dramatic increase in tumor vascularity. As shown in Fig. 3N (blue curve), from days 3 up to 17,
the tumor grew slowly, but the vascularity (CPD) increased dramatically to a maximum value of 30.9%. A transient appearance of a well-developed internal vascular network was seen in the days 8, 10, and 18 Doppler images (Fig. 3C–E). The peripheral vascularity remained greater than the internal vascularity at all time points (Fig. 3O, purple and black curves, and Fig. 3P, green curve). The peak in the overall CPD for the entire tumor was coincident with peaks in the internal CPD (Fig. 3O) and the ratio of the internal CPD to peripheral capsular CPD (Fig. 3P green curve).

The fourth phase is a period of rapid tumor growth accompanied by decreasing vascularity. The tumor volume increased by a factor of 30 from day 18 (Fig. 3E) to day 34 (Fig. 3F) after having changed by only a factor of 3 over the 18 preceding days. During this rapid growth, the overall CPD regressed to the 1–4% range measured on days 0 to 4 and remained around this level for the rest of the time the tumor was followed. The ratio of internal to peripheral CPD (Fig. 3H green curve) simultaneously decreased to values similar to the earliest measurements. This decrease in vascularity in the internal portion of the tumor is evident in the days 34, 43, and 51 (Fig. 3F–H) power Doppler images and supported by the lack of internal vessels in the postmortem image. Figure 3H shows the end point (day 51), with a large tumor volume (1250 mm³) and low vascularity. Note that after day 44, the tumor was too large to permit Doppler measurements over its entire volume (similar to the example in Supplementary Fig. S3), so only volume data were obtained for the last three time points. All three graphs (Fig. 3N–P) show that, by any of the measures of vascular development, tumor vascularity peaks and regresses before the tumor reaches its maximum volume. Similar examples of low vascularity in another three mice with four tumors are shown in Supplementary Fig. S3 with both two-dimensional and three-dimensional power Doppler and micro-CT.

The increased vascularity and progression of angiogenesis observed by longitudinal imaging of the PSP-TGMAP model was confirmed in the TRAMP model by in vivo FITC-lectin perfusion and postfixation immunohistochemistry to detect CD31 in mice at ages of 12, 18, and 24 weeks (Fig. 2D). At 18 weeks, some mice display moderate angiogenesis that is typical for the prostate intraepithelial neoplasia (PIN) phenotype, whereas other mice display well- to moderately differentiated adenocarcinomas. Tumors of 18 weeks ~2 were highly angiogenic. A compromised vascular structure, similar to that observed in PSP-TGMAP mice in the interior of large, poorly differentiated tumors, is shown in 24-week-old TRAMP mice by the regions of hemorrhage visualized by the pooling of FITC-lectin (arrowheads).

An antivascular microsurgery mimicking interventional therapy by ligation of feeder vessels branching from the iliac artery by power Doppler imaging. Figure 4 illustrates the vascular response of a PSP-TGMAP tumor to microsurgical ligation of feeder vessels branching from the iliac artery. The intervention temporarily eliminates the capsular and intratumor blood flow detected by power Doppler. Similar images were obtained from each of the eight treated mice. A sequence of photographs detailing the microsurgery procedure is shown in Supplementary Fig. S4.

The first two Doppler images (Fig. 4A and B) show coronal and transverse three-dimensional views acquired before surgery. This tumor possessed several prominent blood vessels in its periphery contributing to an overall vascularity (CPD) of 4.1% (Fig. 4I, dashed curve). One hour after microsurgery (Fig. 4C), the CPD was reduced to 0.85%, indicating that the intervention eliminated most of the blood flow to the tumor, or at least reduced it to levels too low to be detected by power Doppler. The branching vessel seen near the bottom of the tumor in Fig. 4C to F (open arrows) is a spermatocoele artery or vein passing below the prostate and outside the tumor. No short-term recovery of blood flow is evident in the images acquired 1 h (Fig. 4C) and 8 h (Fig. 4D) after surgery. Blood flow to the surface of the bladder, seminal vesicles, spermatic vessels, and coagulation gland (data not shown) remained detectable immediately after surgery (the pulsed Doppler spectrum of flow in the vesical artery supplying the bladder is shown in Supplementary Fig. S5). The residual blood flow to the bladder is apparently supplied by branches of the superior vesical artery (shown in Fig. 4E). Detectable capsular flow returned by the fourth and eighth days after surgery (Fig. 4E and F); by day 8, the CPD had partially recovered to 2.4%. The mouse was followed for 6 more days, during which time, the CPD remained in the 2.0% to 2.5% range (Fig. 4I, dashed curve).

The Microfil-enhanced micro-CT images in Fig. 4G and H show that the surgical treatment induced regression of the vascularity on the treated side of the prostate relative to the contralateral side. Lower vascular density was observed on the treated side (Fig. 4G and H, arrows) of each tumor-bearing mouse that was imaged with micro-CT.

Longitudinal measurements of tumor volume and vascular CPD for the tumor shown in Fig. 4A to F are plotted in Fig. 4I. After the immediate postsurgical drop, the CPD followed a gradually increasing trend until to about 10 days after surgery and then decreased slightly as the tumor became larger. The surgical intervention apparently prevented the dramatic peak in CPD observed in many untreated tumors.

Power Doppler measurements meaningfully discriminate between tumors with differing levels of active blood flow. Figure 4J shows cumulative distribution functions for CPD values measured from the entire tumor in untreated and treated mice. At the low end of the distribution, 15 out of 37 images (41%) acquired from treated mice, but only 1 of 47 images (2%) from untreated mice, possessed CPD of 0.5% or less. At the high end of the distribution, 20 out of 47 images (43%) acquired from untreated mice, but only 3 of 37 images (8%) from treated mice, possessed CPD >3.0%.

Power Doppler and Microfil-enhanced micro-CT show that PSP-TGMAP tumors recruit additional blood supplies from nearby vessels. To further study the mechanism of tumor growth refractory to microsurgical blocking of blood supplies to GEM-prostate cancer, we compared Doppler and micro-CT images for each mouse. In tumor-bearing mice, additional feeder vessels to the prostate were identified.

Figure 5A shows a coronal Microfil-enhanced micro-CT image of an untreated tumor. The top solid arrow identifies a feeder vessel arising from a vesicle artery running across the surface of the bladder, which presumably represents a portion of the prostate’s original blood supply. The bottom solid arrow highlights an abnormal feeder vessel from the spermatic artery to the tumor. Figure 5B and C are power Doppler images of the same tumor displayed in sagittal and coronal views, respectively. The vesical artery highlighted in the micro-CT image is also recognizable in both Doppler images.

Figure 5D and E show power Doppler and micro-CT images of an untreated tumor that possesses abnormal feeder vessels from the pudic-epigastric trunk or hypogastric vein. Those feeder vessels are highlighted by arrows in both images. The characteristic “U” shape formed by the left and right pudic-epigastric trunks and hypogastric veins and the anastomotic vein that bridges the
hypogastric veins is highlighted in blue and easily recognized in the CT image (see also Figs. 113 and 116 in ref. 20).

Figure 5F shows a power Doppler image of another untreated tumor with an abnormal feeder vessel (arrow) arising from the vicinity of the urethra, which suggests that the feeder originated from the pudic-epigastric trunk. The white dashed line delineates the approximate location of the segment of the urethra caudal to the prostate as determined by inspection of the gray-scale three-dimensional ultrasound image of the same tumor.

Figure 5G is a micro-CT image of a treated tumor, indicated by the pink vasculature, acquired 40 days after surgery. In this image, it is possible to trace the left and right spermatic arteries (light blue) from their origins on the abdominal aorta (Ao) and identify abnormal feeder vessels (rightmost arrow) from the right spermatic artery to the tumor. A second set of abnormal feeder vessels (leftmost arrow) arising from a rectal artery is also depicted dorsal to the tumor. Note that the normal blood supply from the vesical arteries to the prostate was eliminated by the surgical intervention.

Additional images demonstrating tumor-specific vessels branching from the internal iliac artery, spermatic artery, and pudic-epigastric trunk are shown in Supplementary Fig. S6A to C, respectively.

**Discussion**

The observations presented in this paper collectively indicate that high-frequency three-dimensional power Doppler ultrasound can be used to assess functional neoangiogenesis in GEM prostate cancer models. The power Doppler signal is proportional to the concentration of blood cells flowing at velocities high enough to be detected by the Doppler system (e.g., > 3 mm/s in this study). The CPD is therefore considered an estimate of the fraction of the tissue volume that is occupied by moving blood (23). Tumor CPD data reflect flow in intermediate-sized (e.g., >100 μm diameter) arterioles and venules and arteriovenous shunts. Vessels imaged by power Doppler are functional in the sense that they support active blood flow. This point is an important aspect of interpreting Doppler images of tumors because as few as 20% of the vessels in a tumor may be perfused at any given instant, and tumor perfusion varies both spatially and temporally (19). In addition, unlike many other imaging techniques, Doppler ultrasound is completely...
noninvasive, which permits imaging as soon as 1 h after microsurgery.

Power Doppler differentiated normal prostate tissue from prostate cancer, i.e., Doppler provides tumor-specific imaging because little or no flow was detected the normal prostate. Substantial vascularity was depicted only in tumors larger than 2 mm diameter. It is noteworthy that this size is generally recognized as the point by which all solid tumors switch on their own blood supplies. These observations indicate that power Doppler images with high CPD identify tumors that have undergone a substantial amount of functional neovascularization.

Doppler images do not directly show newly developed microvessels. However, comparison of the Doppler images with the immunohistochemistry data in Fig. 2 indicates that power Doppler can be used to distinguish regions of active tumor growth, which correspond to areas of high MVD measured by CD31 staining (a tumor angiogenesis marker), from regions of necrosis, hemorrhage, and low or static blood flow that normally occur near the center of a mass. Normal prostate tissues also show very low CD31 staining, which agrees with the low Doppler vascularity measured in the normal prostate.

Through longitudinal observations, we identified four distinct phases of neoangiogenesis in cancer development, specifically, (a) an early latent phase, (b) establishment of a peripheral capsular vascular structure, (c) a peak in tumor vascularity, and (d) rapid growth. The peripheral capsular vascular structure is the important neoangiogenesis initiation site. The capsular structure was not detectable in normal prostate tissue, which lacks the tumor-associated increased surface flow,9 and was only detected after tumor size exceeded 2 to 3 mm diameter, indicating that it plays an important role in tumor neoangiogenesis "switch-on". Internal tumor-associated angiogenesis was seen after the peripheral capsule formed, although longitudinal follow-up of vascular development showed that vascularization was mostly located in the periphery of the tumor. The peripheral vascular capsule may capture flow from existing vessels, including branches supplying normal prostate tissue, but not all of the peripheral blood vessels surrounding the tumor are expected to be functional at any one time. If this is correct, it will be interesting to determine how a tumor develops functioning vascular structures from mostly inactive vessels (for similar images, see also Supplementary Fig. S2). If we assume the peripheral capsule is the neoangiogenesis initiation site for all solid tumors, the capsule may be considered a new, important target for antiangiogenesis therapy of cancer (24).

The major principle underlying the four-phase hypothesis is that active angiogenesis is a necessary enabling factor for tumor growth, so neoangiogenesis or tumor-associated neovascularity must increase before rapid growth. Angiogenesis is essential for tumor growth and metastasis, although tumorigenesis and malignant transformation does not depend on angiogenesis. Growth of small tumors (<2–3 mm, the switch-on size) is restricted by the capacity of the original normal prostatic blood supply. Vascularity decreases from the level observed in the normal prostate during the initial stages of tumor growth, which triggers the second and third phases, resulting in the first cycle of neoangiogenesis and the first peak in tumor vascularity. The rapid growth (fourth) phase is also characterized by declining CPD and, because tumors possess highly permeable vessels and limited lymphatic drainage, accumulation of fluid in the center of the lesion. The resulting increase in interstitial fluid pressure constricts the vessels and decreases blood flow in the central region (24). The second to fourth phases can be expected to alternate cyclically as the tumor grows.

The microsurgical ligation procedure is proposed as a preclinical model for interventional therapy of advanced angiogenic prostate

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9 An interpretation supported by our unpublished CT perfusion data.
cancer because most mice undergoing surgery showed poorly differentiated and neuroendocrine prostate cancer structure (Fig. 2). As with most interventional therapies, which increase survival rates but do not produce cures, the surgical procedure effectively amplifies vessel recruitment in the PSP-TGMAP mice and thus, provides a means of modeling neoangiogenic prostate cancer. As illustrated in Fig. 5, additional blood supplies were typically recruited from the downstream branches of the internal iliac artery, the spermatic artery, or the pudic-epigastric trunk. The surgical procedure also provides a means to mimic local delivery of antivascular agents, which would be most effectively used in combination with cytotoxic chemotherapy (4).

In this study, Microfil-enhanced micro-CT images were used to confirm that power Doppler presents an accurate depiction of large- and intermediate-sized tumor vessels and to identify the origins of vessels feeding the tumors. The vascular casting technique used in this study provides a gold standard representation of the vascular anatomy at sizes down to 50 μm diameter because the Microfil enhances X-ray contrast between the vessels and surrounding tissue and because postmortem imaging permits acquisition of high-resolution images without concern for the risks to the animal from high levels of X-ray exposure or extended periods of anesthesia. Microfil-enhanced CT images are not functional in the sense employed in this paper, but several other high-resolution methods for functional microvascular imaging are available, including CT and positron emission tomography perfusion imaging (25, 26), dynamic contrast-enhanced MRI, blood oxygen-level–dependent MRI, diffusion-weighted MRI (27), and optical techniques such as intravital video microscopy (28).

The four-phase description of GEM prostate cancer may have direct preclinical and clinical applications to prostate cancer and other tumors. For example, the longitudinal trends in tumor growth and vasculature suggest that timing of interventional therapy to prevent large peaks in vascularity may be a critical factor in the effectiveness of antangiogenic agents. Second, detection of rapid changes in tumor-associated vascular flow using power Doppler ultrasound may indicate a worse prognosis for both preclinical and clinical cases. This study also emphasizes the potential importance of vascular imaging in clinical cancer diagnosis and therapeutic planning.

Acknowledgments
Received 10/25/2006; revised 1/2/2007; accepted 1/18/2007.
Grant support: Canadian Institute of Health Research (MOP-77684), NIH-National Cancer Institute (2 U01 CA084296-06), the Prostate Cancer Research Foundation of Canada, the Canada Foundation for Innovation, the Ontario Innovation Trust, and the Ontario Research and Development Challenge Fund-Ontario Consortium for Small Animal Imaging. Aaron Fenster is a Canada Research Chair in Medical Imaging supported by funding from the Canada Research Chairs Program.

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