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Structure and Function of a Prostate Cancer Dissemination Permissive Extracellular Matrix

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Abstract

Purpose—The poor prognosis of metastatic prostate cancer continues to present a major challenge in prostate cancer treatment. The tumor extracellular matrix (ECM) plays an important role in facilitating metastasis. Here we investigated the structure and function of an ECM that facilitates prostate cancer metastasis by comparing orthotopic tumors that frequently metastasize, to poorly metastatic subcutaneous tumors.

Experimental Design—Both tumors were derived from a human prostate cancer PC3 cell line engineered to fluoresce under hypoxia. Second harmonic generation (SHG) microscopy was used to characterize collagen 1 (Col1) fiber patterns in the xenografts as well as in human samples. Magnetic resonance imaging (MRI) was used to determine albumin-Gd-diethylenetriaminepentaacetate (alb-GdDTPA) transport through the ECM using a saturation recovery MR method combined with fast T₁ SNAPSHOT-FLASH imaging. Cancer associated fibroblasts (CAFs) were also quantified in these tumors.

Results—Significant structural and functional differences were identified in the prometastatic orthotopic tumor ECM compared to the less metastatic subcutaneous tumor ECM. The significantly higher number of CAFs in orthotopic tumors may explain the higher Col1 fiber volumes in these tumors. *In vivo*, alb-GdDTPA pooling was significantly elevated in metastatic orthotopic tumors, consistent with the increased Col1 fibers.

Conclusions—Developing noninvasive MRI indices of macromolecular transport, together with characterization of Col1 fiber patterns and CAFs can assist in stratifying prostate cancers for aggressive treatments or active surveillance. These results highlight the role of CAFs in supporting or creating aggressive cancers, and the importance of depleting CAFs to prevent metastatic dissemination in prostate cancer.

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Keywords

Metastases; collagen 1 fibers; cancer associated fibroblasts; extracellular matrix; hypoxia; prostate cancer; macromolecular transport

INTRODUCTION

Prostate cancer is the second leading cause of death from cancer in men in the U.S. (1). The majority of men dying of prostate cancer succumb to metastatic androgen refractory disease. There is therefore a compelling need to understand the metastatic cascade in prostate cancer to design effective interventions to prevent metastasis. Implantation of human prostate tumors in the orthotopic site of the prostate frequently results in metastasis to the lymph nodes, lungs and liver (2–6). In contrast, implantation of the same cells in a heterotopic site such as the flank results in fewer metastases to the lymph nodes and virtually none in the lungs or liver. These metastasis-permissive orthotopic and metastasis-preventive subcutaneous environments provide an opportunity to understand and identify tumor microenvironments and extracellular matrix (ECM) parameters that characterize metastatic potential.

Tumors display abnormal microenvironments such as hypoxia that mediates a more aggressive phenotype (7), and an ECM that is shaped by cancer cells, with structural and functional features that can facilitate metastasis (8). Collagen 1 (Col1) fibers form a major component of the ECM and have been associated with breast tumor formation, and with metastasis in breast cancer (9). Col1 fibers can be detected using intrinsic signal from second harmonic generation (SHG) microscopy (10). Col1 fibers can also influence the movement of macromolecules in the tumor ECM as detected by *in vivo* magnetic resonance imaging (MRI)-derived ECM transport parameters (11), and the stiffness and porosity of these fibers can facilitate or oppose cancer cell migration (12).

Here, for the first time, we performed a structure-function characterization of the ECM of human prostate cancer xenografts that expressed enhanced green fluorescence protein (EGFP) under hypoxia (2, 13). ECM structure was characterized by SHG microscopy of Col1 fibers, and ECM function by *in vivo* MRI derived indices of transport of the macromolecular contrast agent alb-GdDTA. By implanting these tumors orthotopically in the prostate or subcutaneously in the flank we derived tumors from the same cell line that displayed significantly different metastases formation and frequency. This approach allowed us to quantify and relate macromolecular transport and Col1 fiber patterns in hypoxic and oxygenated regions, in metastasis-permissive orthotopic and metastasis-restrictive subcutaneous environments.

Since cancer associated fibroblasts (CAFs), commonly identified by expression of alpha-smooth muscle actin (α -SMA) (14), are a major source of Col1 fibers in tumors and promote tumor progression (15), we characterized α -SMA in orthotopic and subcutaneous tumors. Transforming growth factor (TGF)- β plays an important role in Col1 fiber production (16). We therefore investigated the expression of TGF- β in orthotopic and subcutaneous tumors.

A microarray gene expression assay was performed on orthotopic and subcutaneous tumors to understand the molecular mechanisms underlying the structural changes in the ECM.

To further confirm findings made in the xenografts, we analyzed Col1 fiber patterns in a human prostate cancer tissue microarray that included core biopsies from normal tissue, normal adjacent tissue, primary prostate cancer tissue from tumors without metastasis at time of biopsy, and primary prostate cancer tissue from tumors with metastasis at time of biopsy.

Collectively, our data identified significant differences in Col1 fiber patterns in tumors that metastasized, together with significant differences in the number of CAFs, as well as macromolecular transport that provide new insights into the structure and function of an ECM that facilitates prostate cancer metastasis.

MATERIALS AND METHODS

Cell line and xenografts

Tumors were derived from PC3 cells stably transfected with the hypoxia response element (HRE) of human vascular endothelium growth factor (VEGF)-A ligated to the EGFP gene (HRE-EGFP-PC3 cells). Expression of EGFP in these cells was used to detect hypoxia (13). PC3 cells are androgen-independent human cancer cells, derived from a metastatic lesion of prostate adenocarcinoma in a lumbar vertebra. Solid tumors were derived from HRE-EGFP-PC3 cells by inoculating 2×10^6 cells in 0.05 ml of Hanks Balanced Salt solution (Sigma, St. Louis, MO) subcutaneously in the right flank of male severe combined immunodeficient (SCID) mice. Intact tumor tissue obtained from subcutaneous tumors was then used for both orthotopic and subcutaneous implantations. For orthotopic implantation, we used a microsurgical method that avoids disseminating cancer cells during inoculation in the prostate (17). A piece of viable tumor tissue of approximately 1 mm^3 was implanted in the prostate of anesthetized SCID male mice by suturing it into the lobes of the gland under a surgical microscope. By implanting tissues rather than injecting cells, the stromal tissue and the three dimensional cytoarchitecture, believed to play a critical role in tumor progression and metastasis, were maintained, and the leakage and dissemination that might occur from inoculating a cell suspension in the gland were avoided. Mice were implanted subcutaneously with a similar sized piece of tumor tissue for comparison with the orthotopic xenografts. Mice were scanned when tumor volumes were approximately $300\text{--}400 \text{ mm}^3$. Animal studies were performed in compliance with approved animal protocol from the Institutional Care and Use Committee at Johns Hopkins Medical Institutions.

MR acquisitions

All imaging studies were done on a 4.7 T Bruker Avance (Bruker, Billerica, MA) spectrometer using a home-built solenoid coil placed around the subcutaneous tumors, and a home-built volume coil placed around the lower torso of the mouse, for the orthotopic tumors. Mice were anesthetized with an intraperitoneal injection of ketamine (25 mg/kg; Phoenix Scientific, Inc., St Joseph, MO) and acepromazine (2.5 mg/kg; Aveco, Phoenix Scientific, Inc., St Joseph, MO) diluted in saline. Multi-slice diffusion weighted images were

acquired with an in-plane spatial resolution of $250\ \mu\text{m} \times 250\ \mu\text{m}$ (128×128 matrix, 32 mm field of view and a b-value of 100 mT/m) to localize the orthotopic tumors that appeared hyperintense in these images. Diffusion-weighted images were used to identify orthotopic tumor regions. These regions were mapped to the alb-GdDTPA contrast-enhanced images from the same 1 mm thick slices to analyze the vascular and transport parameters. Vascular and interstitial fluid transport parameters were measured as previously described (18), from quantitative T_1 maps obtained before and after intravenous administration of the contrast agent alb-GdDTPA (500 mg/kg dose). The tail vein was catheterized before placing the animal in the magnet. Briefly, multislice relaxation rate ($1/T_1$) maps were obtained by a saturation recovery method combined with fast T_1 SNAPSHOT-FLASH imaging (flip angle of 10° , echo time of 2 ms). First, M_0 maps of 4 slices (1 mm thick) with a recovery delay of 7 s were acquired following which images, acquired with an in-plane spatial resolution of 125 or 250 μm respectively (128×128 matrix, 16 mm field of view for the subcutaneous tumors and 32 mm for the orthotopic tumors, 8 averages), were obtained for three relaxation delays (100 ms, 500 ms, and 1 s). These T_1 recovery maps were obtained before i.v. administration of alb-GdDTPA in saline and repeated over a 2 hour period, starting 3 min after i.v. injection of alb-GdDTPA. Alb-GdDTPA was synthesized as previously described (19). Images were acquired in two “phases” corresponding to the biphasic kinetics of the macromolecular contrast agent (18). The “early phase” images obtained over the initial 30 minutes were used to characterize the tumor vasculature, i.e. vascular volume and permeability surface area product (PSP). The second block of MR data was acquired up to 123 minutes after the injection of the contrast agent to characterize interstitial transport parameters, i.e. number of draining and pooling voxels, draining and pooling rates and exudate volumes (18). At the end of the imaging studies, the T_1 of blood was measured. Relaxation maps were reconstructed from data sets for three different relaxation times and the M_0 dataset on a pixel-by-pixel basis. Data were processed with an operator-independent computer program that enabled selection, mapping, and display of the regions with a routine written using Interactive Data Language (IDL, Research Systems, Boulder, CO).

SHG microscopy of xenografts

After scanning, mice were euthanized and the tumors removed for *ex vivo* fluorescence microscopy. Three to five serial 1mm thick fresh tissue slices were cut using an acrylic adjustable tissue slicer and blades (Braintree Scientific, Inc, Braintree, MA) and maintained on ice to avoid tissue degradation. EGFP expression in the tumor slices was detected by fluorescence microscopy using a 1x objective attached to a Nikon inverted microscope, equipped with a filter set for 450 to 490 nm excitation and 500 to 550 nm emission, and a Nikon Coolpix digital camera (Nikon Instruments, Inc, Melville, NY). These 1x images were used to identify hypoxic and normoxic tumor regions in subsequent SHG microscopy acquisitions. SHG microscopy was performed on a Zeiss LSM 710 NLO Meta multiphoton microscopy system (Carl Zeiss MicroImaging, Inc, Thornwood, NY) equipped with a 680–1080 nm tunable Chameleon Vision II laser (Coherent, Inc, Santa Clara, CA). For Col1 SHG microscopy, incident laser light at 880 nm was used, and the SHG signal was detected at 410–470 nm. Simultaneously, EGFP was excited with the 488 nm laser line and detected at 500 to 560 nm. A 25x lens was used to acquire z-stacks of $\sim 100\ \mu\text{m}$ thickness with a z-interval of 5 μm . The Col1 fiber volume within the entire slice was quantified using 6 to 14

slices for each tumor with in-house software written in MATLAB 7.4.0 (The MathWorks, Natick, MA) (10). A two-tailed unpaired student t-test was used to detect significant differences in Col1 fiber parameters between orthotopic and subcutaneous tumors. Regions of interest containing defined fractions of hypoxic EGFP-fluorescing cells were drawn and analyzed. A two-tailed paired student's t-test was used to detect significant differences in Col1 fiber volume between hypoxic and normoxic ROI in orthotopic and subcutaneous tumors using Microsoft Office Excel 2007 (Microsoft, Redmond, WA). *P* values < 0.05 were considered to be significant.

Histological analyses of tumors and spontaneous metastasis

Tissues (tumor, liver, axillary lymph nodes and lungs) were excised and fixed in 10% formalin for sectioning and staining. Lungs were inflated before fixing with a 0.5% agarose solution. Adjacent 5 μ m thick histological sections were stained with H&E. Tumor positive liver, lymph nodes and lungs from all animals were scored by microscopic examination of H&E stained sections. Significant differences ($\alpha = 0.05$) between the cancer cell positive (+ve) fractions for subcutaneous ($n = 7$) and orthotopic ($n = 10$) tumors were evaluated using a two-tailed Fisher's Exact test.

Immunoblot of tumor extract

Expression levels of α -SMA and TGF- β were determined by immunoblot analysis. Proteins were extracted from freeze-clamped tumors using radioimmunoprecipitation lysis buffer fortified with a protease inhibitor cocktail, dithiothreitol, phenylmethylsulfonyl fluoride, sodium orthovanadate, and sodium fluoride (Sigma Chemical Co., St Louis, MO). Protein concentration was estimated using the Bradford Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). About 50 μ g of total protein was resolved on 10% SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies directed against α -SMA (dilution 1:200) or against TGF- β (dilution 1:1000). Anti- α -SMA antibody was purchased from Sigma-Aldrich (St. Louis, MO), and anti-TGF- β antibody from Cell Signaling (Danvers, MA). GAPDH was used as a loading control and detected with a mouse monoclonal antibody (dilution 1:10,000). Immunoblots were developed using SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific, Rockford, IL).

Immunohistochemistry analysis of tumor sections

Immunohistochemistry (IHC) was performed as previously described (20) using the streptavidin-peroxidase technique and the DAKO EnVision System (Dako Cytomation, Hamburg, Germany). Consecutive paraffin-embedded tissue sections (5 μ m thick) were deparaffinized and rehydrated. For antigen retrieval, slides were pretreated in citrate buffer (pH 6.0) in a microwave oven for 12 minutes. Slides were then cooled to room temperature in deionized water for 5 minutes. Endogenous peroxidase activity was quenched by incubation of the slides in methanol containing 0.3% hydrogen peroxide, followed by washing with deionized water for 3 minutes, after which the sections were incubated for 1 h at room temperature with normal goat serum, and subsequently incubated at room temperature overnight with the primary anti- α -SMA (1:200 dilution) antibody from Sigma-Aldrich (St. Louis, MO). Next, the sections were rinsed with washing buffer (PBS containing 0.5% Tween 20) and incubated with biotinylated anti-mouse IgG and

streptavidin-peroxidase complex, followed by reaction with diaminobenzidine (DAB) and counterstaining with H&E. High-resolution digital scans of the immunostained sections were obtained using ScanScope (Aperio, Vista, CA). Images were processed and nuclei and membrane intensity quantified using ImageScope software and the algorithm supplied by the manufacturer. α -SMA-positive fractional areas were computed using this software.

GeneChip microarray gene expression assay

Total RNA was isolated from subcutaneous and orthotopic tumors using the RNeasy Mini Kit (Qiagen, Inc, Valencia, CA) and QIAshredder homogenizer spin columns (Qiagen) as previously described (21). The microarray hybridization was performed at the Johns Hopkins Medical Institution Microarray Core Facility (Johns Hopkins University School of Medicine) using the Human Genome U133 Plus 2.0 GeneChip array (Affymetrix, Inc, Santa Clara, CA) and the Affymetrix GeneChip platform, Agilent GeneArray Scanner, and Micro Array Suite 5.0 software by Affymetrix as previously described (22).

Human samples source and analysis

A prostatic carcinoma tissue microarray containing cases of prostate adenocarcinoma, adjacent prostate normal tissue and normal tissue, with triplicate cores per case was obtained from US Biomax (Rockville, MD). The H&E stained tissue cores were 1.5 mm in diameter and 5 μ m thick. Prostate adenocarcinoma ranged from grade 1 to 4 with and without metastasis. SHG confocal microscopy was used to detect the intrinsic Col1 fiber signal from the H&E stained tissue microarray. Tile scan SHG microscopy of the entire core was performed to acquire Col1 fiber maps in 3D using an Olympus Laser Scanning FV1000 MPE multiphoton microscope (Olympus Corp., Center Valley, PA), with excitation wavelength = 860 nm and detection wavelength = 430 nm, using a 25 $\times$ lens, acquiring field of view of 390 μ m $\times$ 390 μ m, and z-intervals = 3 μ m. Col1 fiber quantification analysis was done in MATLAB (Mathworks Inc., Natick, MA). Hotspot analysis was performed as previously described (23). Briefly, regions with the highest Col1 fiber SHG signal intensity, based on the signal histogram for each core, were analyzed for spatial distribution of Col1 fibers. The percent Col1 fiber volume, inter-fiber distance, and fiber alignment were computed from the Col1 SHG images (8). Fourier analysis was used to quantify Col1 fiber alignment. The Fourier transform of randomly distributed Col1 fibers is approximately a circle, while that of preferentially aligned Col1 fibers is an ellipse. The alignment of Col1 fibers was further analyzed by fitting an ellipse to the Fourier transform of each image, to calculate the aspect ratio, orientation and eccentricity (8). These parameters characterized Col1 fibers as random or aligned. An unpaired two-tailed t-test was used to detect significant differences in the Col1 fiber density and alignment for normal, normal adjacent, malignant without metastasis, and malignant with metastasis cores from the tissue microarray using Excel 2010. *P* values of < 0.05 were considered significant. An hierarchical clustering method using the Euclidean distance metric and average linkage was used to cluster patient data based on the aspect ratio and eccentricity of Col1 fibers as previously described (8).

RESULTS

Metastases to liver, lymph nodes, and lungs were routinely observed following orthotopic implantation, but rarely occurred after subcutaneous implantation (Figure 1). Subcutaneous tumor growth resulted in lymph node metastases in 4 out of 7 mice, but none in the lungs or liver. In contrast, orthotopic tumor growth resulted in lymph node metastases in 8 out of 10 mice, liver metastasis in 8 out of 10 mice, and lung metastasis in 3 out of 10 mice. The data confirmed that orthotopic PC3 tumors metastasized more readily than subcutaneous PC3 tumors.

We applied *in vivo* MRI to characterize tumor vascularization and macromolecular transport parameters in orthotopic and subcutaneous tumors. Representative images from subcutaneous (top) and orthotopic (bottom) tumors are shown in Figures 2A–C. Orthotopic tumors were identified from their higher signal intensity in diffusion-weighted images, as shown in Figure 2A (bottom). Representative maps of vascular volume (Figure 2B) and pooling voxels (Figure 2C) demonstrate the higher vascular volume and number of pooling voxels in orthotopic compared to subcutaneous tumors. Data summarized in Figures 2D–G demonstrate the significantly higher vascular volume (Figure 2D), % pooling voxels (Figure 2E), total pooling volume (Figure 2F), and significantly lower efflux and influx rates (Figure 2G) in orthotopic compared to subcutaneous tumors. There were no significant differences in vascular permeability (data not shown).

We next performed *ex vivo* analyses of fresh tumor slices to characterize Col1 fibers in subcutaneous and orthotopic tumors. Representative images of Col1 fibers overlaid with EGFP expression in a randomly picked field of view from a subcutaneous (Figure 3A) and an orthotopic tumor (Figure 3B) demonstrate the difference in Col1 fibers between subcutaneous and orthotopic tumors. The overall volume of Col1 fibers in orthotopic and subcutaneous tumors, regardless of the normoxic or hypoxic status, was obtained from entire tumor slices.

We further analyzed the Col1 fiber content within normoxic or hypoxic fields of view. Hypoxic fields of view were identified as containing more than 75% of EGFP expressing cells, while normoxic fields of view were identified as containing less than 1% of EGFP expressing cells. Representative images of Col1 fibers overlaid with EGFP expression in a normoxic field of view from a subcutaneous tumor (Figure 3C) and an orthotopic tumor (Figure 3D) confirmed the differences in Col1 fiber geometry between subcutaneous and orthotopic tumors. Hypoxic tumor regions, on the other hand, displayed a low Col1 fiber volume in subcutaneous (Figure 3E) and orthotopic tumors (Figure 3F). These data are summarized in Figure 3G and demonstrate the significantly higher overall fiber volume and significantly higher normoxic region fiber volume in orthotopic compared to subcutaneous tumors. There were no significant differences in the hypoxic tumor areas, as detected by EGFP expression, between orthotopic and subcutaneous tumors, or between Col1 fiber volumes in hypoxic regions identified in orthotopic or subcutaneous tumors.

Since CAFs are a principal source of Col1 fibers in tumors, we investigated their presence in the orthotopic and subcutaneous tumors by IHC of α -SMA in tumor sections and

immunoblot analysis of α -SMA in tumor extracts. Representative IHC stained sections from subcutaneous (Figure 4A) and orthotopic (Figure 4B) tumors identified a higher number of CAFs in orthotopic tumors. Quantitative analysis of the IHC sections confirmed a significantly higher fraction of α -SMA positive pixels in orthotopic tumors (Figure 4C). Data obtained with IHC staining was further validated by the immunoblot analysis that detected higher α -SMA expression in the orthotopic tumors (Figure 4D). We observed higher levels of TGF- β , a growth factor that increases Col1 production, in orthotopic tumors compared to subcutaneous tumors (Figure 4E).

Consistent with the Col1 fiber data, microarray data demonstrated a 5–7-fold increase of COL1A1 gene expression in the orthotopic compared to subcutaneous tumors (Table 1). Since COL1A1 encodes for the pro- α 1 chain, a major component of Col1, the several-fold increase of COLA1 gene expression was consistent with the higher Col1 fiber volume observed in the orthotopic tumors observed with SHG. Orthotopic tumors also exhibited a 2-fold increase of COL4A1, COL4A2, COL6A3, COL12A1, SPON1, and SPARC compared to subcutaneous tumors.

SHG microscopy of a human tissue microarray confirmed the presence of Col1 fibers in human prostate cancers. Col1 fibers of comparable volumes were detected in biopsies from normal, normal adjacent, and malignant tissues obtained from prostate cancers that had not metastasized and from prostate cancers that had metastasized at the time of biopsy. Visually, however, the fiber patterns were very different between nonmalignant and malignant tissue as shown in the representative images in Figure 5A. This difference was confirmed by the differences in aspect ratios obtained from the Fourier analysis. As shown in Figure 5B, the aspect ratio was significantly different in biopsies obtained from normal tissue, normal adjacent tissue, and malignant tissue with and without metastasis, and between malignant tissue without metastasis and malignant tissue with metastasis. Biopsy tissue from malignant tumors that had metastasized, contained Col1 fibers that had a preferential alignment compared to biopsy tissue from malignant tumors that had not metastasized, and to normal adjacent and normal tissue Col1 fibers. Normal and normal adjacent core Col1 fibers were more randomly oriented (Figure 5B). Figure 5C shows a dendrogram of patient data clustered using the aspect ratio and eccentricity as clustering variables. Only two tumors out of 5 without metastasis were not classified within the dendrogram.

DISCUSSION

The structure and function of the metastasis-permissive orthotopic tumor ECM was significantly different from that of the metastasis-preventive subcutaneous tumor ECM, in a human prostate cancer xenograft model. Since identical cells were used for the two sites, the differences in metastasis were most likely due to differences in the ECM and the tumor microenvironment (TME) at the two locations. The orthotopic tumor ECM was characterized by significantly higher Col1 fiber volume, higher vascular volume, a higher number of pooling voxels and pooling volumes, and a lower efflux and influx rate compared to subcutaneous tumors. Orthotopic tumors contained a higher number of CAFs and showed increased levels of TGF- β . Gene expression of COLA1, COL4A1, COL4A2, COL6A3,

COL12A1, SPON1, and SPARC was significantly higher in metastatic orthotopic tumors compared to poorly metastatic subcutaneous tumors.

Col1 fiber characterization of a human tissue microarray containing normal, normal adjacent, primary prostate cancers that had not metastasized, and primary prostate cancers that had metastasized revealed significant differences in the Col1 fiber patterns between normal and malignant tissues, as well as between primary cancers that had metastasized and those that had not. Malignant prostate cancer tissue contained Col1 fibers that were more aligned and linear than those in normal tissues.

Much of the work identifying an association between Col1 fibers and cancer progression and metastasis has been performed in the breast cancer setting. Overexpression of Col1 in MMTV transgenic mice was found to accelerate mammary tumor initiation and progression (9). Increased stromal collagen in mouse mammary tissue facilitated tumor formation, resulting in a significantly more invasive phenotype with more lung metastasis (9). An environment rich in collagen can contribute to tumor initiation and metastasis. Increased Col1 fibers have been associated with poor prognosis and metastases in breast cancer patients (24). High Col1 fiber density in primary tumors can facilitate invasion by providing avenues along which cancer cells migrate to establish distant metastases. Invasion of breast cancer cells at the tumor-stromal interface was shown to occur along radially aligned Col1 fibers (25). Since lymphatic vessels are attached to collagen through anchoring filaments, Col1 fibers may guide migrating cancer cells into tumor-associated lymphatics (8). While several studies showed an association between increased expression of collagen and elevated incidence of metastases (9, 26), Col1 fibers have also been shown to present a structural barrier against tumor invasion (27), although these fibers were found to be necessary for maintaining the invasive phenotype and supporting migration and proliferation of cancer cells (27).

Here, for the first time, we identified significant differences in Col1 fiber patterns in a tissue microarray of human prostate cancers. In a human prostate cancer xenograft, we identified increased Col1 fiber volume in metastatic orthotopic tumors compared to poorly metastatic subcutaneous tumors. Unlike the xenografts where the fields of view were chosen over the entire tumor, the microarray represented tissue obtained from a minute biopsy sample. Nevertheless, clear differences in Col1 fiber patterns were identified in these human prostate cancers underscoring the importance of Col1 fibers in prostate cancer metastasis. To form metastases, cancer cells must interact with their extracellular environment to migrate toward the underlying lymphatic or blood vessel. The higher vascular volume observed in orthotopic tumors was consistent with our earlier observations (2), and may have provided more vessels for the cells to disseminate from. Cancer cells can adhere to Col1 fibers through the $\alpha 2\beta 1$ integrin; adherence to the fibers promotes attachment and motility of the cells, resulting in a more invasive phenotype, as observed in prostate cancer cells where $\alpha 2\beta 1$ integrin was required for efficient metastasis of the prostate cancer cells to the skeleton. The Col1 receptor, $\alpha 2\beta 1$, was identified as necessary but not sufficient for the establishment of prostate cancer metastases within the bone in intratibial and intracardiac experimental tumor models (28). Prostate cancer cells derived from bone metastasis displayed changes in adhesion to Col1 fibers compared to the parental LNCaP prostate cell

line from which they were derived (29). E-cadherin was shown to be down-regulated in response to Col1 fibers in PC3 cells (30). A reduction of E-cadherin is a key feature of the epithelial-mesenchymal transition, known to be associated with metastases (31). Col1 fibers can increase cell invasiveness by reducing E-cadherin mediated cell-cell adhesion (32). Our data support evaluating Col1 fiber patterns to predict for prostate cancer aggressiveness.

The gene microarray data detected several fold higher COL1A1 in the orthotopic tumors. COL1A1 is strongly expressed by activated stromal cells in human cancers but is not usually observed in cancer cells (33). Since we used a Human Genome U133 Plus 2.0 GeneChip array, the COL1A1 detected was most probably from the human cancer cells and not the mouse CAFs suggesting that cancer cells in orthotopic tumors may also contribute to the increased Col1 fibers.

The increase of SPARC that codes for osteonectin and SPON1 that codes for spondin 1 in orthotopic tumors support the more metastatic phenotype of the orthotopic tumors (34–36). SPARC has been shown to be expressed in metastatic prostate primary tumors (37), and evaluation of SPARC in primary prostate cancer could be used as a prognostic marker of metastatic progression (36). Spondin 1 contributes to osteosarcoma metastatic progression (38). Our data also showed upregulation of COL12A1 and COL6A3. Both have been described as upregulated in colorectal cancer (39, 40). In a small cohort of colon cancer patients, Col12 was highly expressed in desmoplastic stroma by and around α -SMA positive CAFs, as well as in cancer cells lining the invasion front (39).

Our data are consistent with previous studies demonstrating that prostate cancer-derived primary cultures contained more myofibroblasts, characterized by the presence of α -SMA, than cultures obtained from benign prostatic hyperplasia. Additionally, the percentage of myofibroblasts in high-grade prostate cancer derived primary cultures was higher than in moderate and low grade cancer (41), confirming the importance of CAFs in tumor formation and progression. Prostate cancer is characterized by a reactive stroma that is enriched with fibroblasts and myoblasts (42). Our data suggest that the presence of CAFs in prostate cancers may provide an additional marker of tumor aggressiveness, and support the development of treatments that reduce the presence of CAFs in prostate cancers to reduce metastatic dissemination. Higher levels of TGF- β , observed in orthotopic tumors, can provide one explanation for the higher Col1 fibers in orthotopic tumors compared to subcutaneous tumors. TGF- β is produced by several cells including cancer cells, fibroblasts, endothelial cells and immune cells (43). Since the antibody was cross-reactive with mouse and human TGF- β , it was not possible to determine if the TGF- β was from cancer cells or stromal cells or both. Inactive TGF- β is stored in the ECM and activated by mechanical force, proteases, integrins, reactive oxygen species and pH (43, 44). An increase of Col 1 fiber formation induced by TGF- β was previously reported in human prostatic stromal cells (45). In tumors, TGF- β activates fibroblast-to-myofibroblast transition, promotes angiogenesis, and promotes the secretion of both matrix proteins and proteases. It also increases the potential for metastasis by driving the epithelial-to-mesenchymal transition (46).

Functional differences in the prometastatic ECM included a higher number of pooling voxels and pooling volumes, and a lower efflux and influx rate compared to subcutaneous tumors. We have previously observed an association between Col1 fibers and macromolecular transport (11). In those studies, performed in an MDA-MB-231 human breast cancer xenograft that expressed red fluorescence under hypoxic conditions, we found that frankly hypoxic areas contained significantly fewer Col1 fibers (11). These areas with few Col1 fibers were like ‘silent zones’ with minimal macromolecular transport. The significant reduction of Col1 fibers observed here in hypoxic regions is consistent with our earlier observations (10, 11). Within this context, the higher Col1 fibers in the orthotopic tumors may have mediated the increased movement of the macromolecular contrast agent within the tumor interstitium following extravasation, compared to the subcutaneous tumors. The absence of functional lymphatics may have resulted in an overall increase of the pooling volume in these tumors. It is also possible that the higher number of Col1 fibers in the orthotopic prostate tumors may have acted as a barrier to contain the macromolecular contrast agent in the tumor. A previous study has described the ECM as a dispersive filter controlling the composition of extracellular fluid and the rate of molecular trafficking (47). Another study explored the coefficients of diffusion of proteins, dextrans and liposomes by fluorescence recovery after photobleaching technique in human melanoma and glioma xenograft models implanted in mice, in a dorsal chamber as an orthotopic model for melanoma Mu89 cells, or in a cranial window, on the pial surface, as an orthotopic model for glioma U87 cells (48). Diffusion coefficients were related to the tumor collagen content and organization as estimated by transmission electron microscopy. Diffusion of larger molecules was faster in the cranial window compared to the dorsal chamber tumor. The tumor in the dorsal chamber was characterized by a higher density of fibroblast-like cells and by high levels of Col1 fibers and fibrillar collagen (48).

Irrespective of the mechanism underlying the differences in macromolecular transport, our data support further characterization of macromolecular transport parameters as noninvasive *in vivo* MRI indices to identify aggressive prostate cancers.

Our data identified significant structural and functional differences in the ECM of metastatic orthotopic prostate cancer xenografts compared to subcutaneous prostate cancer xenografts derived from the same cells. Col1 fiber geometry and volume were significantly altered in more aggressive tumors that are likely to metastasize. These tumors also contained a higher number of CAFs, highlighting their role in supporting or creating more aggressive cancers, and the importance of developing strategies to deplete them in prostate cancers. With noninvasive MRI we detected significant differences in *in vivo* macromolecular transport parameters that, together with characterization of Col1 fiber patterns and CAFs, merit further development for prostate cancer prognosis.

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ABBREVIATIONS

alb-GdDTPA	Albumin-Gd-diethylenetriaminepentaacetate
CAF	cancer associated fibroblast
Col1	collagen 1
ECM	extracellular matrix
EGFP	enhanced green fluorescent protein
Gd-DTPA	gadolinium diethyltriaminepentaacetic acid
HRE	hypoxia response element
IHC	Immunohistochemistry
MRI	magnetic resonance imaging
PSP	permeability surface area product
SCID	severe combined immunodeficient
SHG	second harmonic generation
SMA	smooth muscle actin

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STATEMENT OF TRANSLATIONAL RELEVANCE

We identified significant differences in collagen 1 (Col1) fiber patterns, number of cancer associated fibroblasts (CAFs) and *in vivo* macromolecular transport in tumors that metastasized, providing new insights into the structure and function of an extracellular matrix that facilitates prostate cancer metastasis. The results highlight the role of CAFs in supporting or creating more aggressive cancers, and the importance of developing strategies to deplete CAFs in prostate cancer. Noninvasive MRI identified significant differences in macromolecular transport that, together with characterization of Col1 fiber patterns and CAFs, merit further development as biomarkers for prostate cancer prognosis.

Figure 1.

Representative hematoxylin and eosin stained sections of (A) liver (B) lymph nodes and (C) lungs from mice bearing subcutaneous (left) or orthotopic (right) tumors at x20. Scale bar: 100 μ m (D) Cancer cell positive liver, lymph nodes and lungs from all animals were scored by microscopic examination of H&E stained sections, and significant differences ($\alpha = 0.05$) between the cancer cell positive (CA +ve) fractions for subcutaneous ($n = 7$) and orthotopic ($n = 10$) tumors were evaluated using a two-tailed Fisher's Exact test (P -values: liver: 0.0023, lymph nodes: 0.5928, lungs: 0.1923).

Figure 2.

(A) Representative anatomic images of a subcutaneous PC3-HRE-EGFP tumor (top row) and an orthotopic PC3-HRE-EGFP tumor (bottom row). Representative maps of (B) vascular volume and (C) draining (red) and pooling (green) voxels from a subcutaneous tumor (top row) and an orthotopic tumor (bottom row). The orthotopic tumor is delineated by a white line. Bar graphs showing (D) total vascular volume, (E) percentage of draining and pooling voxels, (F) pooling and draining volumes, and (G) efflux and influx rates in subcutaneous and orthotopic tumors. Values represent Mean $\pm$ SEM. (n = 7 and n = 8 respectively, * $P < 0.05$; ** $P < 0.001$).

Figure 3.

Representative images of Col1 fibers overlaid with EGFP expression in a randomly picked field of view from (A) a subcutaneous and (B) an orthotopic tumor. Representative images of Col1 fibers overlaid with EGFP expression in a normoxic field of view from (C) a subcutaneous tumor and (D) an orthotopic tumor. Hypoxic tumor regions from (E) a subcutaneous tumor and (F) an orthotopic tumor. (G) Bar graphs of fiber volume in subcutaneous and orthotopic PC3-HRE-EGFP tumors. Col1 fibers of the entire slice from 6 to 14 slices per tumor were analyzed in nine subcutaneous and twelve orthotopic tumors; a subset of four subcutaneous and four orthotopic tumors were analyzed for the hypoxic

versus normoxic comparison with 5 to 13 fields of view analyzed per tumor for hypoxia, and 8 to 20 for normoxia. Values represent Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Figure 4. Representative α-SMA IHC stained sections from an (A) subcutaneous and (B) orthotopic tumor. Positively-stained fibroblasts are indicated by arrows. (C) Percentage of positive pixels occupied by fibroblasts in the tumor sections. Values represent Mean $\pm$ SEM. * $P < 0.05$. (D) Immunoblots showing a high expression of α-SMA in 3 orthotopic tumors and undetectable level in 3 subcutaneous tumors. (E) Immunoblots showing TGF-β expression levels in 3 subcutaneous and 3 orthotopic tumors.

Figure 5. Representative fiber patterns observed in human samples by SHG (A) in normal, normal adjacent, malignant stage II without metastasis, malignant stage II with metastasis, malignant stage II/III without metastasis, and malignant stage III with metastasis. (B) Aspect ratio quantification (n = 3, normal, normal adjacent) (n = 5, malignant without metastasis, malignant with metastasis). Values represent Mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. (C) Dendrogram and heat map of clustered patient data from Fourier analysis for parameters aspect ratio and eccentricity. The color scale ranges from green to red, where black represents the mean, green represents values below the mean and red represents values above the mean values for each parameter across all patient data.

Table 1

List of ECM genes of interest upregulated in orthotopic tumors compared to subcutaneous tumors

Gene Group	Gene Title	Gene Symbol	Probe set ID	Fold Change
Extracellular matrix	collagen, type I, alpha 1	COLLAGEN 1A1	1556499_s_at	6.91
Extracellular matrix	collagen, type I, alpha 1	COLLAGEN 1A1	202310_s_at	5.08
Extracellular matrix	collagen, type IV, alpha 1	COL4A1	211980_at	2.54
Extracellular matrix	collagen, type XII, alpha 1	COLLAGEN 12A1	231879_at	2.41
Extracellular matrix	collagen, type XII, alpha 1	COLLAGEN 12A1	225664_at	2.40
Extracellular matrix	collagen, type XII, alpha 1	COLLAGEN 12A1	231766_s_at	2.37
Extracellular matrix	collagen, type I, alpha 1	COLLAGEN 1A1	202311_s_at	2.25
Extracellular matrix	collagen, type IV, alpha 2	COL4A2	211964_at	2.23
Extracellular matrix	collagen, type IV, alpha 1	COL4A1	211981_at	2.22
Extracellular matrix	collagen, type IV, alpha 2	COL4A2	211966_at	2.21
Extracellular matrix	collagen, type VI, alpha 3	COL6A3	201438_at	2.18
Extracellular matrix	spodin 1, extracellular matrix protein	SPON1	213994_s_at	2.10
Extracellular matrix	secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	212667_at	2.03