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Canine Prostate Cancer Cell Line (Probasco) Produces Osteoblastic Metastases In Vivo

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Abstract

BACKGROUND—In 2012, over 240,000 men were diagnosed with prostate cancer and over 28,000 died from the disease. Animal models of prostate cancer are vital to understanding its pathogenesis and developing therapeutics. Canine models in particular are useful due to their similarities to late-stage, castration-resistant human disease with osteoblastic bone metastases. This study established and characterized a novel canine prostate cancer cell line that will contribute to the understanding of prostate cancer pathogenesis.

METHODS—A novel cell line (Probasco) was derived from a mixed breed dog that had spontaneous prostate cancer. Cell proliferation and motility were analyzed in vitro. Tumor growth in vivo was studied by subcutaneous, intratibial, and intracardiac injection of Probasco cells into nude mice. Tumors were evaluated by bioluminescent imaging, Faxitron radiography, μ CT, and histology. RT-PCR and genome-wide DNA copy number profiling were used to characterize the cell line.

RESULTS—The Probasco cells grew in vitro (over 75 passages) and were tumorigenic in nude mice. Probasco cells expressed high levels of BMP2, CDH1, MYOF, FOLH1, RUNX2, and SMAD5 modest CXCL12, SLUG, and BMP, and no PTHrP mRNA. Following intracardiac injection, Probasco cells metastasized primarily to the appendicular skeleton, and both intratibial and intracardiac injections produced osteoblastic tumors in bone. Comparative genomic

hybridization demonstrated numerous DNA copy number aberrations throughout the genome, including large losses and gains in multiple chromosomes.

CONCLUSIONS—The Probasco prostate cancer cell line will be a valuable model to investigate the mechanisms of prostate cancer pathogenesis and osteoblastic bone metastases.

Keywords

prostate cancer; osteoblastic metastases; canine; dog

INTRODUCTION

The American Cancer Society estimates that approximately 1,660,290 Americans will be diagnosed with cancer in 2013. Approximately 238,590 of those cases will be prostate cancer [1]. Prostate cancer affects 1 in 6 men in the western world, and 17% of affected men will die as a direct result of metastatic disease [2]. The most common site of metastasis is bone, and approximately 90% of patients with metastatic disease will develop bone metastases that are predominantly osteoblastic or bone-forming [3,4]. Even though the pathogenesis of prostate cancer has been studied for over 70 years, few experimental models of osteoblastic skeletal metastasis have been developed [5]. Animal models of prostate cancer that mimic disease in men are vital for translational research and developing new diagnostics and therapeutics to improve patient quality of life.

To understand the pathogenesis of prostate cancer, animal models must reflect one or more stages of the human disease. This includes slow growth, increased incidence with age, prostatic intraepithelial neoplasia (PIN), initial androgen sensitivity followed by castration resistance, and development of predominantly osteoblastic bone metastases in late-stage disease. At this time, there is no animal model that encompasses all of these features; however, dogs are the best available model for spontaneous disease. Aside from humans, dogs are the only animal to naturally develop prostate cancer; however, the incidence is lower than in men [6]. As with men, dogs develop prostate cancer late in life [7]. By the time prostate cancer is diagnosed in dogs, as many as 80% will have metastases and, similar to men, the metastases are often in bone and are typically osteoblastic [8]. For this reason, canine prostate cancer is a relevant model of late-stage human disease.

An important difference to note between men and dogs is the role of androgens in prostate cancer pathogenesis. The androgen signaling pathway is considered essential to the development of prostate cancer in men [9]. In men the androgen signaling pathway is the therapeutic target in both early and late-stage disease, even after progression to androgen-resistance [10]. In dogs the androgen receptor (AR) is present in normal prostatic tissue and is necessary for the development of BPH in adult intact male dogs. However, most dogs with prostate cancer lack expression of the AR [11]. It is possible that androgen signaling does not play a central role in the pathogenesis of canine prostate cancer as it does in men. Interestingly, while the secretory cells of the prostate are androgen dependent, the prostatic ducts and urothelium of the prostatic urethra are maintained independent of androgen signaling [12]. This suggests that canine prostate cancer may originate from prostatic ductular epithelium rather than the acinar secretory cells as in men.

There are few canine prostate cancer cell lines used in research [13–17]. We report the formation and characterization of a novel canine prostate adenocarcinoma cell line originating spontaneously from a mixed-breed dog. The Probasco cells have unique morphologic features that include robust growth in vitro, slow but consistent growth in vivo and dramatic osteoblastic bone metastases in nude mice. These characteristics make the Probasco cells a valuable resource in prostate cancer research.

MATERIALS AND METHODS

Establishment of Canine Prostate Carcinoma Cell Line (Probasco)

A section of tissue was aseptically removed from a primary prostate carcinoma of a 10.5-year-old castrated mixed-breed dog that had been previously treated with palliative radiation therapy and metronomic chemotherapy (piroxicam, cyclophosphamide, toceranib phosphate, and chlorambucil). The clinical disease was approximately 9 months, and at the time of tumor sampling there was a suspected right hind limb metastasis that was not confirmed due to elective euthanasia. The owner declined a post-mortem examination. The tumor specimen was rinsed three times with sterile Dulbecco's phosphate-buffered saline (DPBS) and minced to approximately 1 mm³ fragments with scalpels. The minced tumor was then cultured in DMEM/F12 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a 25 cm² flask. Medium was replaced every 4 days. Differential trypsinization with 0.25% trypsin ethylenediamine-tetraacetic acid (EDTA) was used to remove stromal cells, which released from the culture flasks earlier than the epithelial cells. Detachment of the cells was monitored every 2 min to remove the stromal cells. Once this population was removed, the remaining epithelial cells were collected and replated. The epithelial cells were allowed to become 70% confluent before passaging.

Lentiviral Luciferase Transduction

The Probasco cells were grown in a 6-well culture plate with DMEM/F12, 10% FBS and 1% penicillin/streptomycin. Once the cells had reached approximately 95% confluence, the media was removed and replaced with 500µl of luciferase-containing virus (VC 2192 ConcpLuc (VSV-G)), 1.5 ml of culture medium, and 1.6µl of polybrene stock (8 µg/ml). YFP-Luc lentiviral particles were produced by transient co-transfection of 293T cells with 10 µg packaging plasmid pCMV R8.2, 2µg envelope plasmid pMD.G (obtained from Dr. Kathleen Boris-Lawrie, Ohio State Univ.) and 10 µg transfer plasmid pHIVSIN-YFP-Luc using calcium phosphate (Sigma–Aldrich, St. Louis, MO) as previously described [18]. Following centrifugation at 2,700 rpm for 60 min at 30°C, the plate was placed in a cell culture incubator at 37°C and 5% CO₂ for 24 hr, after which the virus-containing media was aspirated and replaced with fresh culture media without virus. After 48 hr the cells were detached with trypsin and grown in 25 cm² flasks as described above.

Subcutaneous, Intratibial, and Intracardiac Injection of Probasco Cells Into Nude Mice

All animal experimental procedures were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

Subcutaneous—Five nude mice were anesthetized in an induction chamber with 3% isoflurane. The dorsal thoracic skin was scrubbed with alcohol-soaked gauze to remove superficial contamination. A suspension of 500,000 luciferase/YFP-transduced Probasco cells (Probasco-Luc) in 0.25 ml of sterile DPBS was injected subcutaneously between the shoulder blades using a 25-ga needle. Tumor volume was measured weekly using calipers. Measurements were taken in three dimensions and tumor volume was calculated using the formula $\text{length} \times \text{width} \times \text{height} \times 1/2$. Tumor growth was also monitored weekly using bioluminescent imaging.

Intratribial—Fifteen nude mice were anesthetized in an induction chamber with 3% isoflurane and maintained with 2.5% isoflurane. The right rear limb was scrubbed with alcohol-soaked gauze. The leg was held so the knee joint was at a 90° angle and a 27-ga needle was introduced through the patellar ligament and into the tibial marrow space through the articular cartilage. Using a Hamilton syringe, 0.025 ml of sterile DPBS containing a suspension of 200,000 Probasco-Luc cells was introduced into the marrow cavity. Tumor growth was monitored weekly using bioluminescent imaging.

Intracardiac—Eight nude mice were induced at 3% isoflurane gas anesthesia and maintained at 2.5%. The mice were placed in dorsal recumbency and their front limbs were placed perpendicular to their thorax and restrained to the procedure table with surgical tape. The ventral thorax was scrubbed with alcohol. A 1 ml syringe with a 25-ga needle was loaded with 0.1 ml of DPBS containing a suspension of 300,000 and was introduced into the thoracic cavity through the third intercostal space, approximately 1 mm to the left and lateral from the sternum. Once a pulsatile jet of blood was present in the hub of the needle, the cell suspension was slowly injected into the left ventricle of the heart for 30 sec. Tumor growth was monitored weekly using bioluminescent imaging.

Bioluminescent Imaging

Mice were anesthetized in an induction chamber with 3% isoflurane and maintained with 2% isoflurane. A 1 ml syringe was used to inject 0.15 ml of sterile DPBS containing 4.5 mg α -Luciferin (Caliper Life Sciences, Hopkinton, MA) into the peritoneal cavity. Bioluminescent in vivo imaging was performed using the IVIS 100 (Caliper Life Sciences) and photon signal intensity was quantified using LivingImage software version 2.50 (Caliper Life Sciences). Imaging was performed every 2 min until peak photon signal was achieved (approximately 10 min post-injection). The IVIS 100 was set to 1 min exposures with small binning.

In Vitro Growth Rate

Probasco-Luc cells from passage 25 were plated in a six-well cell culture plate in triplicate at a density of 200,000 cells per well. Cells were cultured in DMEM/F12 with 10% FBS and 1% penicillin/streptomycin and incubated in 37°C and 5% CO₂. The cells were collected using 0.25% trypsin at 24, 48, and 72 hr after the initial plating and counted with an automated cell counter (Nexcelom Bioscience, Lawrence, MA) using trypan-blue dye exclusion to differentiate between live and dead cells. The doubling time was calculated

using the formula: $(t_2 - t_1) \times \log(n_2)/\log(n_2/n_1)$, where n is the cell number at time points (t).

Histopathology and Immunohistochemistry

Complete necropsies were performed on the mice, subcutaneous tumors were weighed, and tissues were fixed in 10% neutral-buffered formalin at 4°C for 24 hr. Bones were decalcified in 10% EDTA (pH 7.4) for 2 weeks at 4°C and embedded in paraffin. The specimens were sectioned (5µm) and were either stained with hematoxylin and eosin (H&E) or evaluated by immunohistochemistry against cytokeratins AE1/AE3, 5/6, 7, and 20 (Table I).

RNA Extraction and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from Probasco cells at passages 4, 5, and 6 and Ace-1 canine prostate carcinoma cells at passages 22, 23, and 24 using the Absolutely RNA Miniprep Kit (Stratagene, Cat. No. 400800) and the manufacturer's protocol for adherent tissue culture cells. Total RNA (0.5 µg) was reverse transcribed using the Superscript II First Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) and RT-PCR was performed for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as the following genes related to bone microenvironment and prostate cancer progression and metastasis: bone morphogenic protein-2 (BMP2), cadherin-1 (CDH1), chemokine ligand 12 (CXCL12), folate hydrolase 1 (FOLH1), myoferlin (MYOF), runt-related transcription factor 2 (RUNX2), snail homolog 2 (SLUG), parathyroid hormone-related peptide (PTHrP), AR, and mothers against decapentaplegic homolog 5 (SMAD5) using canine specific primers (Table II). Each primer pair was chosen from three to four different primer pairs designed for each gene. These primers were compared side-by-side and the primer pair that had the best amplification and qRT-PCR product melting characteristics was chosen for each gene. All primer pairs were designed using Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). In order to confirm primer specificity, qRT-PCR products for each gene were verified by electrophoresis on a 2% agarose gel and stained with ethidium bromide to confirm a single amplification product of the expected size. The entire PCR reactions were then purified using the QIAquick PCR Purification Kit (Qiagen, cat. No. 28106) and sequenced by the Plant-Microbe Genomics Facility at The Ohio State University using a 3730 DNA Analyzer (Applied Biosystems, Grand Island, NY) and BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems). Sequences were verified by a BLAST search using the NCBI website.

DNA Extraction and Polymerase Chain Reaction (PCR)

Total DNA was extracted from Probasco cells at passage 6 using the DNeasy Blood & Tissue Kit (Qiagen, Cat. No. 69581) and the manufacturer's protocol for adherent tissue culture cells. PCR was performed for the AR using canine specific primers (Table II).

Radiography

Radiography was performed postmortem on the mice that received intratibial and intracardiac injections. Formalin-fixed tissues were placed centrally on a Faxitron laboratory

radiography system LX-60 (Faxitron X-ray Corp., Wheeling, IL) imaging platform and high-resolution radiographs were taken at 25KV and 5 sec exposures.

Micro-Computed Tomography (μ CT)

The tibias were removed postmortem from mice injected intratibially and imaged using microcomputed tomography (μ CT) (Siemens, Inveon Preclinical CT scanner, Siemens AG, Munich, Germany). Images were acquired in 400 projections over 360° at 100 kVp, 200 MA, 900 ms exposure, Bin 2, and a medium-high system magnification with a pixel width of 19 μ m. Image data were reconstructed using Cobra software (Exxim, Pleasanton, CA) and analyzed using 3D analysis software (Inveon Research Workplace 3-Dimensional Image Software, Siemens PreClinical). Segmentation thresholds were kept constant for all bones. Five mice were euthanized at 3 weeks post-injection to characterize early lesions and other mice were euthanized at 6 weeks (late lesions).

Analysis of Genomic Imbalances by Comparative Genomic Hybridization (CGH) and Fluorescence In Situ Hybridization (FISH)

Metaphase chromosome preparations and genomic DNA were obtained simultaneously from the same flask of cells. The cells in the flask were grown to 80% confluence and exposed to 50 ng/ml of Karyomax (Invitrogen) for 4 hr prior to routine recovery from the flask with conventional trypsin-EDTA. Pelleted cells were divided equally into two tubes, one used to isolate genomic DNA for array comparative genomic hybridization (aCGH) and the second harvested for chromosome preparations by routine procedures [19,20].

Chromosome preparations were isolated following conventional procedures of hypotonic treatment and fixation in 3:1 methanol:glacial acetic acid. To identify the position of the centromeres of the chromosomes, two BAC clones (330E21 and 326K03) from the CHORI-82 BAC library, which have been shown previously to hybridize to all autosomal centromeres of the canine genome, were both labeled with SpectrumGreen-dUTP and hybridized to chromosome preparations of this cell line according to routine procedures [21]. Chromosome preparations were counterstained with 80ng/ml 4',6-diaminidino-2-phenylindole (DAPI) and mounted in antifade solution (Vectashield, Vector Laboratories, Burlingame, CA). Images were acquired with a fluorescence microscope (Axioplan 2ie, Zeiss) equipped with a DAPI filter set and a cooled CCD camera (CoolSnapHQ, Photometrics, Tuscon, AZ) both driven by dedicated software (SmartCapture 3, Digital Scientific, Cambridge, UK).

CGH was performed using a commercial ~180,000-feature microarray comprising repeat-masked ~60-mer oligonucleotides distributed at approximately 13 kb intervals throughout the dog genome sequence assembly (canFam version 2.0, May 2005 [22]) (Agilent Technologies, Santa Clara, CA). All steps were performed as described previously [20]. Briefly, DNA isolated from the cell line was labeled with cyanine-3-dUTP and hybridized against a reference DNA sample (comprising equimolar quantities of constitutional DNA from 10 clinically healthy male dogs) labeled with Cyanine-5-dUTP. Following hybridization and washes the array image file was processed using Feature Extraction version 10.10 (Agilent Technologies) and imported into Nexus Copy Number version 7

(BioDiscovery, El Segundo, CA). DNA copy number calling was performed using the FASST2 segmentation algorithm in Nexus Copy Number, using at least three consecutive probes with $\log_2$ tumor: reference values of 0.201 or -0.234 to indicate copy number gain or loss. Genomic recoding of the canine genome coordinates to represent the corresponding human genome locations was performed as described previously [20]. The resulting 'humanized' data were output simultaneously to facilitate direct comparison with CGH data already available for human prostate specimens.

Statistical Analysis

Results are displayed as means $\pm$ SD. Normalized gene expression data were analyzed using one-way ANOVA followed by Sidak's multiple comparisons test to perform comparisons using GraphPad Prism version 6.03 (La Jolla, CA). Data with P values <0.05 were considered statistically significant.

RESULTS

Histopathologic Characterization of the Primary Carcinoma and Subcutaneous Xenograft

The primary prostate carcinoma was a moderately differentiated alveolar-type prostatic carcinoma based on the light microscopic morphology. The carcinoma was composed of numerous ducts filled with polygonal epithelial cells arranged in nests and anastomosing cords (Fig. 1A). The cancer cells contained moderate homogenous eosinophilic cytoplasm and a single, round to oval nucleus with finely stippled chromatin and 1–2 nucleoli. Desmoplasia was present throughout the tumor, as well as multifocal areas of necrosis and lymphocytic inflammation.

Probasco xenografts were grossly visible in the subcutis of all mice between 2 and 4 weeks after implantation. Mice were euthanized 7 weeks after implantation, and tumors ranged in size from 170 to 430 mm³. The histologic appearance of the xenograft was similar to the primary carcinoma and was comprised of polygonal epithelial cells forming nests and anastomosing cords in a fine fibrovascular stroma with moderate to severe multifocal coagulation necrosis (Fig. 1B). Pseudo-cyst formation was common in the tumor secondary to necrosis, and the neoplastic cells lined cavities containing sloughed cells. Occasionally, small foci of mineralization and ectopic bone formation were present. Subcutaneous tumors were not highly invasive and were easily dissectible from the subcutaneous space.

Immunohistochemistry

The Probasco cells were positive for pan-cytokeratins AE1/AE3 and 5/6 and negative for cytokeratins 7 and 20 (data not shown).

In Vitro and In Vivo Growth

The Probasco cells grew in an anchorage-dependent manner in vitro and formed monolayer sheets with polygonal cells exhibiting a tightly packed cobblestone growth pattern (Fig. 1C). Probasco cells grew exponentially in vitro with a doubling time of approximately 18 hr under standard culture conditions (Fig. 2A). In vivo, the doubling time of the viable cells in

subcutaneous xenografts was approximately 2.5 weeks as estimated by BLI (Fig. 2B). The subcutaneous xenograft volume doubled approximately every 1.4 weeks (Fig. 2C).

qRT-PCR and PCR of Probasco In Vitro

The Probasco cells expressed a high level of CDH1, MYOF, FOLH1, SMAD5, and RUNX2 and a modest level of BMP2, SLUG, and CXCL12 (Fig. 3A and B). There was little to no expression of PTHrP. Probasco cells expressed significantly greater BMP2, CDH1, MYOF, RUNX2, FOLH1, and SMAD5 mRNA compared to a second canine prostate carcinoma cell line, Ace-1 (Fig. 3B). The expression of CXCL12, PTHrP and SLUG were significantly lower in Probasco cells. Neither cell line expressed AR mRNA, though the gene was present by PCR.

Intratibial Injections of Probasco in Nude Mice

Fourteen of 15 mice developed successful xenografts in the tibias. Radiographs and μ CT imaging demonstrated extensive new bone formation on both the endosteal and periosteal surfaces of the tibias (Fig. 4A and B). The new bone formed a radio-opaque starburst pattern of woven bone emanating from the periosteal surface, while the endosteal new bone presented as sclerosis of the marrow cavity with loss of distinct trabeculae. In the early lesions (3 weeks post-injection), the new woven bone was confined to the endosteal surfaces and marrow spaces. At 6 weeks post-injection the Probasco cells penetrated the cortical bone and induced marked periosteal new bone formation. The overall effect on the bone was markedly osteoblastic, with mild osteolysis. Histology of the tibia demonstrated large multifocal to coalescing islands of woven bone lined by both numerous cuboidal osteoblasts and polygonal neoplastic cells (Fig. 5). Large intratibial tumors formed pseudocystic cavities that contained necrotic cell and proteinaceous (eosinophilic) fluid.

Intracardiac Injections (IC) and In Vivo Bioluminescent Imaging (BLI) of Probasco in Nude Mice

Six of eight mice developed bone metastases (ranging from 1 to 5 metastases per mouse) after IC injection with Probasco cells. At 5 min after injection of Probasco cells and IP luciferin administration, the bioluminescence was diffusely distributed throughout the body, with the highest signal within the thoracic cavity. Two mice had bioluminescence within the forelimbs at day seven. By 2 weeks, six of the eight mice had bioluminescence within in the appendicular skeleton. The intensity of the BLI increased throughout weeks 3 and 4, reflecting increased tumor burden. At the end of week 4, bioluminescence demonstrated metastases in the femurs of two mice, tibias in six mice, radius/ulnar metastases in five mice, and vertebrae in four mice. One mouse with vertebral tumor growth had bilateral hind limb paralysis at the time of euthanasia. Faxitron radiography demonstrated numerous radio-opaque radial spicules of periosteal new bone formation and medullary sclerosis in both the metaphysis and diaphysis of long bones, with minimal osteolysis.

Analysis of Genomic Imbalances by CGH and FISH

Analysis of >50 DAPI stained metaphase preparations indicated that while the cell line was not clonal, all of the cells evaluated were hyperdiploid, containing between 88 and 108

chromosomes, with a mean of 92. Hybridization of BAC clones targeting the centromeres of all canine autosomes [23] revealed that while most of the chromosomes were single-armed, approximately 20% were bi-armed, indicative of centric fusions (Fig. 6). Centromeres were not detected on either of two grossly different bi-armed chromosomes, suggesting that these two aberrant structures may contain centromeres from the sex chromosomes, even though neither resembled a “normal” canine X or Y chromosome.

The genome wide profile of DNA isolated from passage four of the Probasco line revealed numerous copy number aberrations, representing whole (or almost whole) chromosomes (e.g., gain of dog chromosome (CFA) 4, 11, 20, 24, and 26 and loss of CFA 7, 19, 27, 33, 34, and 36), large chromosome segments (e.g., gain of large regions of CFA 9, 13, 14, 18 and loss of regions of CFA 1, 3, 12, 13, 16, 22, 31, and Xq) (Fig. 7). In addition there were numerous focal copy number changes involving gains of small regions of CFA 2, 13, 20, 34, and 36, and losses of small regions of CFA 8 and 28 (Fig. 7). The complex pattern of adjacent DNA copy number gain and loss along CFA 13 and Xq is suggestive that these chromosomes may be involved in complex translocation events, which would merit further investigation. Recoding of the canine data to output the profile in the context of the human genome is presented in Figure 8.

DISCUSSION

The creation of cell lines and animal models that closely recapitulate prostate cancer progression in men is essential for understanding prostate cancer pathogenesis and the development of novel therapeutics [24,25]. Dog are unique in the animal kingdom because they develop spontaneous prostate cancer with an increasing incidence with age and frequently develop osteoblastic bone metastases [8]. In this study, we describe the development and characterization of a novel canine prostate cancer cell line (Probasco).

There are six canine prostate cancer cell lines that have been reported (DPC-1, CPA-1, Ace-1, Leo, CT-1258, and Probasco), each with unique characteristics that contribute to prostate cancer research. The Leo cell line produces osteolytic bone metastases and, more importantly, consistently metastasizes to the brain and spinal cord after intracardiac injection [13]. The Ace-1 cell line demonstrates marked epithelial-mesenchymal transition and produces mixed osteoblastic and osteolytic metastases [14]. The Ace-1 cells have been used to develop an experimental model of prostate cancer in immunosuppressed dogs to investigate molecular imaging, focal therapy of prostate cancer, and metastasis to lymph nodes, lungs and bone [7,26]. The DPC-1 cell line is tumorigenic in both mice and dogs and produces undifferentiated prostate carcinoma xenografts [16,27]. In immunosuppressed dogs, DPC-1 metastasizes to lung, lymph node, and produced mixed osteoblastic and osteolytic bone metastases [27]. The CPA-1 cell line, while not extensively characterized, is tumorigenic in nude mice and produces well differentiated prostate carcinoma xenografts [17]. The CT-1258 cell line produces tumors when injected subcutaneously or intraperitoneally in immunodeficient mice and does not metastasize [15]. Of all the canine prostate cancer cell lines, the Probasco cell line is the only one to produce predominantly osteoblastic bone metastases. As men with prostate cancer bone metastases most commonly

develop osteoblastic lesions, so the Probasco cells represent a uniquely valuable tool for evaluating this particular aspect of prostate cancer pathogenesis.

There are few animal models of prostate cancer bone metastasis that closely mimic human disease. Most currently available genetically modified mouse models of prostate cancer do not develop bone metastases, and xenograft models of prostate cancer bone metastasis often result in the formation of mixed or osteolytic metastases [28]. The two most commonly used human lines that stimulate new bone formation include the human LNCap subline C4-2B and the LuCaP 23.1 tumor line; however, both of these form mixed metastases as they induce significant bone destruction as well as formation [29,30]. A less commonly used human cell line, the MDA PCA 2b cells, forms predominantly osteoblastic lesions when injected into mouse long bones, however, it takes significantly longer (60 days) than the Probasco cells (21 days) for osseous lesions to become evident [31]. It is expected that ability of the Probasco cells to mimic the osteoblastic nature of human prostate cancer bone metastases will further the understanding of the pathogenesis of osteoblastic metastases in prostate cancer, creating the potential for novel therapeutics, and imaging modalities.

The pathogenesis of bone metastases has been intensely investigated over the last decade, but there is still much to understand. Cell lines that actively metastasize and grow in bone are vital to advancing our understanding of this untreatable disease. Comparing cell lines that produce osteoblastic metastases to cell lines that produce mixed or osteolytic lesions may help identify important pathways in the molecular pathogenesis of osteoblastic prostate cancer bone metastases. As previously mentioned, the Ace-1 canine prostate cancer cell line (developed by our lab) metastasizes to bone and forms mixed osteoblastic/osteolytic lesions [14]. This is in contrast to the osteoblastic lesions induced by the Probasco cells. When comparing gene expression between these two cell lines, several genes were identified that may provide insight into the reasons for their different metastatic phenotypes. One such gene is BMP2. Interestingly, Probasco has a much higher expression of BMP2 compared to Ace-1. Bone morphogenic proteins are growth factors of the transforming growth factor-beta (TGF- β) superfamily that transduce signals through SMADs and have a critical role in bone formation [32]. BMP signaling activates SMAD1/5/8, resulting in upregulation of transcription factor and coactivators that are important in osteoblast differentiation (i.e., Osterix and RUNX2) and new bone formation [33,34]. The elevated expression of BMP2 may play a role in the osteoblastic nature of Probasco bone metastases. Another gene is CXCL12, a chemokine ligand that regulates proteases such as MMP9. The decreased expression of CXCL12 in the Probasco cells may in part account for the reduced osteolysis in bone metastases compared to the Ace-1 cells [35].

Osteomimicry has been implemented as an important feature in bone metastasis for over a decade, and has been found to be a feature of prostate cancer bone metastasis in men [36]. The theory of osteomimicry proposes that neoplastic cells express bone matrix proteins or osteoblast-related factors to enhance their survival and proliferation in bone. One such factor is the osteoblast transcription factor, RUNX2 [37]. RUNX2 targets pathways such as Wnt, Src, BMP, and TGF- β and is considered a key regulator of metastasis-related genes in human prostate cancer [34]. Elevated RUNX2 expression has been documented in advanced human prostate cancer compared to early lesions [38]. The elevated expression of RUNX2

in the Probasco cells supports its use as a model of late-stage human prostate cancer and potentially provides a model to target osteomimicry in the development of therapeutics.

Both Probasco and Ace-1 have similar levels of expression of FOLH1. This gene, also known as prostate-specific membrane antigen, has been under intense investigation in recent years for its use as a target for prostate cancer imaging by positron emission tomography (PET), single photon emission computed tomography (SPECT), and transrectal gamma imaging (TRGI) [39–42]. FOLH1 is overexpressed in most human prostate cancers, and a similar overexpression in canine prostatic carcinoma cell lines makes them a useful tool in validating such imaging studies.

The role of parathyroid hormone-related peptide in prostate cancer is not clearly defined. In other tumors, such as breast and lung cancer, PTHrP plays significant role osteolytic metastases. In these tumors, PTHrP is thought to be one of the main drivers of the “vicious cycle” from its ability to enhance osteoclastogenesis and bone lyses [43]. The function of PTHrP in prostate cancer is less clear, though the predominantly osteoblastic nature of metastases suggests it plays a different role in the progression of bone metastases. One study has found that PTHrP overexpression in the Ace-1 cell line is linked to increased osteoblastogenesis, osteoclastogenesis, and angiogenesis as well as increased bone turnover [44]. Interestingly, reports of PTHrP expression in prostate cancer bone metastases are conflicting, with several reports claiming its presence in 100% of human prostate cancer bone metastases and others reporting a less universal expression [45,46]. The Probasco cell line may be useful as a model of bone metastases that have little to no expression of PTHrP, perhaps highlighting the importance of other pathways in disease progression.

Epithelial-mesenchymal transition (EMT) is a well-known characteristic of invasive tumors. EMT is often characterized by loss of expression of cell–cell adhesion molecules like E-cadherin and increased expression of mesenchymal proteins, such as vimentin [47]. The relatively high expression of CDH1 in Probasco cells compared to Ace-1 suggests that it has not undergone extensive EMT. This is supported by its polygonal cell shape both in culture and in vivo. The lack of prominent EMT may explain why the subcutaneous Probasco xenografts are minimally invasive and easy to dissect from the mouse when compared to Ace-1 xenografts, which are reported to invade into the underlying epaxial musculature, ribs and vertebrae [14]. Interestingly, the Probasco cells express higher levels of myoferlin (MYOF) compared to Ace-1. MYOF has not been investigated in prostate cancer, however, several studies have indicated a role in breast cancer. Knockdown of MYOF in breast cancer cells resulted in reduced invasion and mesenchymal to epithelial transition (MET) [48,49]. The elevated expression of MYOF in the Probasco cell line is suggestive that some EMT is present, and that the Probasco cell line may be a suitable model for testing MYOF-based therapeutics.

AR signaling is known to be a strong driver of prostate cancer progression in men; however, this does not seem to be the case in dogs. The lack of expression of the AR by Probasco is consistent with previous data stating that AR is not present in most canine prostate carcinomas [12]. Both the lack of AR expression and the frequent development of prostate cancer in neutered dogs suggest prostate cancer in dogs rapidly progresses to androgen

independence or is derived from cells not maintained by androgen signaling, such as cells of the prostatic ducts. It is interesting to note that prostate basal and stem cells are also androgen independent and are present in the prostate gland of neutered dogs [6]. Investigations into this divergence of pathogenesis of prostate cancer in dogs will be valuable in developing novel therapeutics for the disease in dogs.

The genomic determinants for aggressiveness of prostate cancer are still poorly understood. Copy number variants in the genome are known to modulate cell function and disease. In recent years, several copy number aberrations have been shown to be associated with aggressive prostate cancer and poorer prognosis, including deletions in 1p36.13, 3p25, 3p14.2, 14q32.33, 15p11, and 20p13 and duplications in 12p13, 16q23.3, and 17p13.3 [50,51]. Array CGH analysis revealed that the Probasco cell line has numerous copy number aberrations including deletions in 1p36.13 and duplications in 17p13.3. These findings highlights the usefulness of the Probasco cell line as a model of human disease, as even on the genomic level there are similarities between the tumors in the two species.

Previous studies of high-resolution aCGH of human prostate specimens revealed numerous genome copy number aberrations associated with high-risk disease [52,53]. Among these aberrations the most frequent DNA copy number aberrations involve HSA 8, with gains of 8q (generally involving 8q21.3–24.3 (containing c-MYC) and deletions of 8p (generally involving 8p21). Recoded CGH data for Probasco revealed that humanized data did not reveal copy number changes in the regions that are evolutionarily conserved with HSA 8p, but did reveal complex copy number changes in the region of 8q23–24. The region of HSA 1 at 1q42.12–q42.3 demonstrated DNA copy gain in a third of human prostate cancers [52]. It is interesting to note that not only is this region of the genome also gained in Probasco, but that it is flanked by adjacent regions of DNA copy number loss (Fig. 8), perhaps suggestive of a region that has retained a critical association with prostate cancer in both species. Further consideration of such conserved aberrations, shared between human and dog prostate cancers, may provide a means to reduce the regions of significance in the human genome as we move toward better refining key genetic pathways and improved treatments.

CONCLUSIONS

Bone metastases are a devastatingly common complication of late state prostate cancer. We have developed a canine prostate carcinoma cell line and xenograft model that preferentially metastasizes to bone and results in marked new bone formation. This model system will be vital in the investigations of the pathogenesis of osteoblastic metastases in prostate cancer.

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Fig. 1. Photomicrographs of the primary prostatic carcinoma, the subcutaneous xenograft, and the in vitro cell line (Probasco). (A) Photomicrograph of hematoxylin and eosin (H&E)-stained primary prostatic carcinoma. The polygonal neoplastic cells form nests and cords separated by fibrous connective tissue. (B) Photomicrograph of H&E-stained subcutaneous Probasco xenografts. Tumors were multilobular and contained multifocal necrosis and pseudo-cyst formation. (C) Phase contrast microscopy of Probasco cells (passage 48). The polygonal cells have a tightly packed cobblestone growth pattern.

Fig. 2.

In vitro and in vivo growth patterns of Probasco cells. (A) In vitro growth curve of Probasco cells. Data presented as mean with a standard deviation of three replicates. (B) Graph represents the average bioluminescence measured from subcutaneous xenografts at the indicated time points. Data presented as a mean with a standard deviation of 5 mice. (C) Graph represents the average tumor volume of the subcutaneous xenografts. Data presented as a mean with a standard deviation of 5 mice.

Fig. 3.

Expression of select genes in the Probasco prostate cancer cells. (A) Graph represents the relative mRNA expression of PTHrP, CXCL12, SLUG, BMP2, RUNX2, SMAD5, FOLH1, MYOF, and CDH1 in the Probasco cell line. (B) Graph represents the relative mRNA expression of the same genes and the androgen receptor (AR) compared to another canine prostate carcinoma cell line, Ace-1 (Student's *t*-test; * $P < 0.05$).

Fig. 4. μ CT and radiographic analysis of a nude mouse tibia injected with Probasco cells. **(A)** μ CT of Probasco injected tibia and control tibia at 3 weeks. Tibias in the Probasco-injected group had increased radio-opacity in the diaphyseal region compared to the control bones. **(B)** Radiograph of a control, early (3 weeks) and late (6 weeks) Probasco-injected tibia. The early radiographs show increased diaphyseal intramedullary radio-opacity, while the radiograph taken at 6 weeks demonstrate the marked periosteal reaction that occurs as neoplastic cells exit through the cortex.

Fig. 5.
Photomicrograph of H&E-stained tibia 6 weeks post-injection with Probasco cells.
Neoplastic cells and osteoblasts line new woven bone present within the medullary cavity of
the diaphysis and along the endosteal surfaces.

Fig. 6. Hybridization of canine autosomal centromeric BAC clones to an example metaphase spread of Probasco. In this example the chromosome number was 108. The chromosomes are shown with DAPI counterstain (**A**) and after application of a linear DAPI filter to reveal chromosome bands (**B**). In both cases the Spectrum Green labeled BAC probe reveals the presence and location of the centromeres. In this example there are 86 single-armed and 22 bi-armed structures. The asterisk in A and B indicate two clearly bi-armed chromosomes

that did not exhibit autosomal centromere hybridization. This suggests that these two structures had sex chromosome-derived centromeres.

Fig. 7.

DNA copy number profiling of Probasco. **(A)** Whole genome copy number profile: X-axis shows the 180,000 probes on the CGH array as log₂ ratio organized according to chromosomal order from the top of CFA I to bottom of the X chromosome. The Y chromosome is not represented since the canfam2 genome assembly is female. The y-axis shows the log₂ ratio of the fluorescence intensity of Cy3-labeled Probasco DNA and Cy5-labeled "reference" DNA (y-axis). **(B)** 460 band ideogram representation of the dog [19] with the FASST2 called aberrations from A indicated as copy number gain and loss by shading in blue and red, respectively. The data indicate the presence of numerous DNA copy aberrations involving gains and losses of small to large, contiguous regions of the canine genome as well a complex series of alternating copy number status of CFA13.

Fig. 8. DNA copy number profiling of Probasco recoded to human genome coordinates and output as a human data (A) Recoded whole genome copy number data of Probasco as a pseudo-human profile, with the X-axis shows the 180,000 probes on the CGH array as log2 ratio organized according to chromosomal order from the top of human chromosome (HSA) I to the bottom of the HSA X. The y-axis shows the log2 ratio of the fluorescence intensity of Cy3-labeled Probasco DNA and Cy5-labeled “reference” DNA (y-axis). (B) Human850

band ideogram representation of the data in A with the FASST2 called aberrations indicated as copy number gain and loss by shading in blue and red, respectively.

TABLE I

Antibodies Used for Immunohistochemistry of Probasco Cells

Antibody	Species	Clone	Company	Dilution
CK AE1/AE3	Mouse	M3515	Dako Corporation, Carpinteria, CA	1:100
CK5/6	Mouse	D5/16 B4	Dako Corporation, Carpinteria, CA	1:50
CK7	Mouse	OV-TL 12/30	Dako Corporation, Carpinteria, CA	1:150
CK20	Mouse	Ks20.8	Dako Corporation, Carpinteria, CA	1:250

TABLE II

Primers Used for qRT-PCR and PCR of Probasco Cells

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
qRT-PCR primers		
BMP2	TGCGCAGCTTCCACCACGAAGAA	CAAAGGTTCTGCATCTGTTCCCGA
CDH1	GCTGCTGACCTGCAAGGCGA	GGCCGGGGTATCGGGGACAT
CXCL12	ACTGTGCCCTTCAGATCGTGGCA	CCACCTGCGCCTCTCACATCTTG
FOLH1	GCAGGGGACCCTCTCACACCTG	CTCGGAAGACCAACAGCCTCTGTGA
GAPDH	CCCACTCTTCCACCTTCGAC	AGCCAAATTCATTGTCATACCAGG
MYOF	TGCCCCGAAAGGCTGGGAAT	ACTCCGTGTGCCCTGCGTCT
RUNX2	TGCCTCTGGCCTTCCACTCTCAG	TGCATTCTGTGGGTTGGAGAAGCG
SLUG	GGCAAGGCGTTTTCCAGACCCT	GGGCAAGAAAAAGGCTTCTCCCCAG
SMAD5	CCCAGCCAATGGATACAAGC	ATGCATGAAAAGCCTCCCCA
PTHrP	AGCTCGGCCGCCGGCTCAA	GGAAGAATCGTCGCCGTAAG
AR	CACTCGGCCACCTCAGGGATTGG	AACATGGTCCCTGGCAGTCTCCA
PCR primers		
AR Exon 1-1	CCTTTCCAGGAGCTTCCCGCTG	CAGCGGAGCGCAAGGACGTA
AR Exon 1-2	GACAGCGCCAAGGAGTTGTGTAAGG	GACAGCGCCAAGGAGTTGTGTAAGG
AR Exon 2	TAGCCCATGAGAGTGGCTGGTTCC	AGGACTGCCCCAGGCTCTAAGC
AR Exon 3	TGGCCATTCTCTGTTCACTTCTGTC	AGCTGGGTGATGGCCACATAGGA
AR Exon 4	GCTTAGGCAACCTTCTCACAGGG	AGTGGGCATTATACCAATCCCTCCC
AR Exon 5	TGCTCCTCAGCATTAATGGCCCCT	CCAATCCCCCATTGCTTCCCACC
AR Exon 6	GAGGGCACAGGGACCTGGAAATTC	AGGAGCTGGCTTCTCCCCGAT
AR Exon 7	TGACTGAAGGACCCAGGCGTACA	CACCACCAACAAGAGGTGCCA
AR Exon 8	TGCATGCGCAAACACAAACACGT	TCCCCAAGGCACTGCAGAGTAGT