PATHOGENESIS OF OSTEOBLASTIC METASTASIS IN PROSTATE CANCER: ROLE OF ANIMAL MODELS

DISSERTATION

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ABSTRACT

Prostate cancer (PCa) is the most commonly diagnosed cancer in men accounting for 25% of the new cases every year in the USA. PCa is the second leading cause of cancer-related deaths due to the development of serious skeletal complications in advanced stages of the disease. Bone metastases in PCa are predominantly osteoblastic characterized by formation of excessive low quality woven bone leading to spinal cord compression, paralysis, pathological fractures and severe pain eventually resulting in a poor quality of life. However, a few cases of predominant by osteolytic bone metastases also were reported in PCa patients that destroy bone and cause morbidity. Therefore, bone metastasis represents a major clinical and research interest to investigate and develop therapeutic strategies.

The pathogenesis of bone metastasis is a complex process that is poorly understood due to the lack of relevant animal models. Therefore, recapitulation of PCa osteoblastic bone metastasis in animal models is necessary to discover the mechanisms involved in bone metastasis. Other than men, dogs are the only non-mammalian species that develop spontaneous prostate cancer (though not as common as in men) and have similar clinical features of the disease such as multifocal bone metastases.
Previously, our lab established a canine PCa cell line (Ace-1) that metastasized to bone and caused mixed osteoblastic and osteolytic lesions in nude mice similar to that observed in human patients. We utilized this animal model to investigate the molecular pathogenesis of bone metastasis.

First, we investigated the role of osteolytic activity in the development of bone metastasis by inhibiting bone resorption with the bisphosphonate, zoledronic acid. We showed that zoledronic acid significantly inhibited osteolysis in this model, but did not affect the PCa growth, incidence and the osteoblastic phenotype of the metastases. These data demonstrated that PCa bone metastases were independent of osteolysis and that PCa secreted factors that directly regulated bone metastasis. To explore the molecular mechanisms involved in metastasis, we investigated the role of Wnt signaling that plays an important role in oncogenesis and osteogenesis in the progression of prostate cancer growth, and incidence and osteoblastic phenotype of bone metastases. Antagonizing Wnt signaling using Dickkopf-1 (Dkk-1) in our model resulted in increased tumor growth although it significantly decreased new woven bone formation in the metastases.

Although bone metastasis accounts for the majority of the metastases that occur in prostate cancer patients, soft tissue metastases also occur. To investigate the differences in the mechanisms involved in soft tissue and bone
metastases, we established a novel dog prostate carcinoma cell line (Leo) that is tumorigenic in nude mice and metastasized predominantly to brain as well to other organs including bones, adrenal gland and spinal cord.

In conclusion, these investigations demonstrated that osteolysis did not have a significant role in the development of PCa bone metastasis and PCa secrete factors that induce bone metastasis. Importantly, Wnt signaling in PCa was important for the induction of the osteoblastic phenotype in bone metastases which was inhibited by Dkk-1. Therefore, our study demonstrated that prostate directly induced the osteoblastic bone metastases and investigating the factors responsible for prostate cancer-induced bone induction in bone metastases will be useful to design novel therapeutics.
Dedicated to my parents Thudi Venkat Ranga Reddy, and Jyothi, my wife
Praneetha and my sister Gayathri and brother-in-law Sudershan Reddy
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Prostate cancer is a major health concern in men associated with high morbidity and mortality. Despite the high incidence of prostate cancer in men, the pathogenesis of prostate cancer and its metastases are incompletely understood. Therefore, animal models of prostate cancer are essential to investigate this disease in vivo and to characterize the fundamental biological abnormalities contributing to its development. Although spontaneous, xenograft, and transgenic animal models have become indispensable tools to advance investigations on the basic mechanisms underlying prostate cancer progression, genetic and interspecies differences have impaired an accurate recapitulation of the human disease.

The usefulness of animal models has improved in recent years due to novel biological and technological approaches. Advances in small animal
imaging, for example, permit the precise analysis of real time physiological, anatomical, and molecular modifications. Some studies have demonstrated similarities in molecular profiles and mechanisms in prostate cancer pathogenesis between humans and animals. The present review will focus on describing the usefulness, challenges, and fundamental differences in prostate cancer pathobiology between man and animal models in dog, mice, and rats.

**INTRODUCTION**

Prostate cancer is the most commonly diagnosed cancer in men accounting for 25% of newly diagnosed cancers each year and is the second leading cause of cancer related mortality in men (1). Morbidity and mortality associated with prostate cancer are due primarily to the development of bone metastasis in advanced stages of the disease (2~4). Despite advances in the early diagnosis and treatment, 50% of patients with localized prostate cancer at time of diagnosis develop bone metastasis due to the development of increasingly aggressive, androgen-independent cancer, and fail to respond to treatment (5).

Bone metastases in prostate cancer are unique. Most bone metastases are 'osteoblastic' and characterized by excessive new woven bone formation with a modest osteolytic component. Few patients develop predominantly osteolytic bone metastases (6,7). Pathological and molecular aspects of the pathogenesis of prostate cancer and osteoblastic bone metastases are not well understood.
There is a lack of relevant animal models to recapitulate the pathogenesis of the human clinical disease, particularly osteoblastic bone metastases (8).

Dogs and a few strains of rats are the only species other than man that develop spontaneous prostate cancer (9). Bone metastases occur in dogs with prostate cancer, but not in rats (10). Multiple xenograft models of human and animal prostate cancer and genetically engineered mice that develop prostate carcinoma have been established to investigate the pathogenesis and treatment of prostate cancer in vivo (11~16). While there are differences in the anatomical structure and pathophysiology of prostate cancer between species, animal models are useful to investigate the molecular pathogenesis, prevention and treatment of prostate cancer (10). Animal models are instrumental in validating therapeutic targets and evaluating the response of prostatic cancer to novel therapies.

**COMPARATIVE PROSTATE ANATOMY AND BIOLOGY**

McNeal first systematically described the structure of the prostate gland in men. There are four distinct anatomical zones including the peripheral zone, central zone, transitional zone, and fibromuscular zone (Fig 1) (17). Peripheral, transitional and central zones constitute the glandular portion that forms two-thirds of the prostate gland. The peripheral zone is boat-shaped and composed
of small spherical glands with loose stroma and it immediately surrounds the posterolateral central zone (17,18).

The peripheral zone constitutes 70% of the glandular prostate and is the primary site of origin of carcinoma in about 70% of patients (19). The central zone is a cone-shaped glandular structure consisting of large glands and dense stroma (Fig 2) and is distinct compared to the peripheral zone. It constitutes 25% of the glandular prostate, but uncommonly (about 10% develops carcinoma) (20). The transitional zone consists of two paraurethral lobes located anterior to the urethra and peripheral zone. The glands of the transitional zone are histologically similar to the peripheral zone except that it has few glands and a dense stroma (18,21). Although the transitional zone constitutes a small portion (5%) of the glandular prostate in young adults, it continues to increase in size with age and eventually becomes the major site of benign prostate hyperplasia. Twenty percent of prostate carcinomas originate in the transitional zone and they are typically well-differentiated with a better prognosis compared to those originating from the peripheral zone (22).

The glandular prostate tissue is covered by a smooth muscle capsule and connective tissue, which thickens at the anterior region to become fibromuscular stroma. This anterior fibromuscular zone constitutes 33% of the prostate gland and tightly binds to all three glandular zones (21).

The prostate gland in dogs is a spherical-shaped, bilobed (Fig 1), homogenous gland divided by a median septum into right and left lobes and
covered by a thin fibrous capsule (Fig 1) (23). It surrounds the cranial urethra and neck of the urinary bladder. Each lobe consists of lobules that are comprised of tubuloalveolar glands (Fig 2) radiating from a periurethral region to the periphery of the gland with alveoli separated by dense stroma (24). Prostate carcinoma in dogs originates from alveolar and ductal regions of the prostate gland and is highly invasive and metastatic (Fig 3) (25,26). Dogs are the only species known to develop spontaneous, clinically relevant, prostate cancer similar to men, but prostate cancer is less common in dogs than men (9).

In nonhuman primates, the prostate gland lies on the dorsal urethra, is bilobed and is contained within a capsule (Fig 1) (27). The gland is divided into cranial and caudal lobes peripherally, which become merged anteromedially. In contrast to the human prostate which surrounds the entire urethra, the prostate of nonhuman primates does not cover the ventral aspect of the urethra. The cranial and caudal prostate lobes are approximately equal in size in nonhuman primates, except for baboons in which the cranial lobe is smaller than the caudal lobe (27). The cranial and caudal lobes have distinct gross and microscopic features. The surface of cranial lobe is lobulated and resembles seminal vesicles in appearance, while the surface of the caudal lobe is smooth. Histologically, the cranial lobe is composed of large acini with irregularly branched tubules compared to small acini and simple tubules in the caudal lobes. Cranial lobes contain a dense stroma that separates individual acini, whereas caudal lobes contain a scanty and diffuse stroma (27).
The rodent prostate gland is divided into four lobes (Fig 1 and 4) based on differences in the morphological organization and includes the anterior, dorsal, ventral, and lateral lobes, with respect to their spatial relationship to the urethra (28). The anterior lobe is also called the coagulatory gland. The histological structure of the prostate glands is a tubulo-alveolar type. The dorsal and lateral prostate lobes are called the dorsolateral lobes (DL) since they are closely associated, but the lobes have morphological and functional heterogeneity (29). The dorsolateral prostate shares similarities with the human peripheral zone including histology, site of origin of carcinoma, and gene expression profile. However, there are no clear data to support the direct correlation of human prostate zones with mouse prostate lobes (30,31).

Rodent prostate lobes have differences in histology, biochemistry, gene expression profiles, and incidence of neoplasia in transgenic models (32). Different ductal branching patterns occur in various lobes (28). Stromal organization in the rodent prostate gland has regional variations along the ductal system and expression of the androgen receptor (AR) by stromal cells may explain how androgens regulate epithelial-mediated ductal branching (33,34). Unlike humans and dogs, the prostatic stroma in rodents is sparse and epithelial cells are loosely arranged (35).

**Epithelial cells of the prostate gland:** Alveoli and ducts in the prostate consist of three types of epithelial cells including basal cells, luminal/secretory cells, and
neuroendocrine cells. In addition, intermediate cell types are found that partially express both basal and secretory characteristics.

**Basal epithelial cells**: In men, basal cells typically form a continuous single layer of cells lining the basement membrane in both alveolar and ductular regions (25,36). The dog prostate, in contrast to men, has a basal cell layer which is continuous in the ductal region and discontinuous in acini since the secretory cells interpose between basal cells to directly contact the basement membrane (25). Basal cells have the capacity to proliferate, are undifferentiated, and are precursors of the secretory cells. Basal cells in men and mice express high molecular weight cytokeratins (CK) 5 and 14, whereas in dogs CK5 is abundantly expressed and CK14 expression is scattered and scarce (26,37,38). Basal cells also express other markers such as ETS-2, Maspin, CD44, p63, Ki67 and glutathione S-transferase P (37,39).

Human and mouse basal epithelial cells that expressed high levels of trophoblast cell-surface antigen (Trop2) demonstrated characteristics of functional progenitor activity including the ability to regeneration of prostatic tubules *in vivo* as well as colony and sphere formation *in vitro* (40). These cells also give rise to basal, luminal, and neuroendocrine cells. The Trop2-expressing cells constitute 30-40% of the basal cells and possess stem cell characteristics. The remaining basal cells that do not express Trop2 have minimal functional activity demonstrating that that basal cells are divided into two functionally distinct subpopulations (40).
Secretory/luminal cells: These are the most abundant epithelial cells in the prostate and are the most common cell type in human prostate carcinoma (41). The secretory cells are tall columnar, terminally differentiated cells that line ducts and alveolar lumens, express low molecular weight cytokeratins 8 & 18, produce secretory proteins such as kallikreins, (e.g., prostate specific antigen (PSA) in men), prostatic acid phosphatase (PAP), and 15-lipoxygenase (LOX)-2 (37). Dogs and mice do not express prostate specific antigen (PSA) in their prostate glands, but dogs do have a related kallikrein enzyme, arginine esterase which shares 58% homology with PSA (42,43). PSA is expressed and secreted by nonhuman primate prostate glands. Serum PSA concentrations are positively correlated with increasing age, but vary depending on the species; 0.04 to 6.2 ng/ml in cynomolgus monkeys, 0.07 to 2.4 ng/ml in rhesus monkeys and 0.05 to 0.08 ng/ml in baboons (44,45).

In the mouse, expression of CK8 is heterogenous between different lobes with weaker expression in the anterior prostate (46). It has been reported that 15-LOX-2 has tumor suppressive activity, reduced expression in prostate cancer, and its metabolites have antiproliferative action in normal epithelial cells (47). Luminal cells express the androgen receptor (AR) and are androgen-dependent for survival (48). These cells have a low proliferative capacity and a high apoptotic index (49).
Neuroendocrine (NE) cells: Neuroendocrine cells have neural and epithelial characteristics, are few in number, and are present between the basal cell layer in ducts and acini of the human and dog prostate glands (50~52). Based on morphology, neuroendocrine cells are classified into two subsets: the open type in which cells open into the lumen through long slender processes, and the closed type, which is embedded in the adjacent epithelial cells without luminal extensions (53). The neuroendocrine cells produce hormones and prohormones such as thyroid-stimulating hormone, somatostatin, parathyroid hormone-related protein (PTHrP), calcitonin, chromogranin A, serotonin, and synaptophysin (53,54).

Although the exact function of neuroendocrine cells is not clear, secretion of neuroendocrine hormones and growth factors and direct contact of NE cells with the surrounding prostate cells suggests that they regulate growth and maintenance of the prostate gland in an androgen-independent manner (55). Two different theories for the origin for NE cells have been suggested; (1) NE cells are neurogenic in origin and represent a distinct cell lineage and (2) NE cells originate from prostatic pluripotent progenitor cells (56,57).

The NE cells express basal and luminal cell-specific cytokeratins 5, 14, 8, and 18, and the neuroendocrine marker, chromogranin A (58,59). NE cells in prostate cancer express neuroendocrine markers and luminal cell cytokeratins but not basal cell cytokeratins (60). In addition, NE cells in prostate cancer can
be distinguished from normal NE cells by their absence of dendrite processes and expression of the anti-apoptotic protein, bcl-2, and the α-methylacyl-CoA racemase enzyme, which distinguishes them from normal NE cells (59). Prostate cancer cells can undergo transdifferentiation to acquire NE-like characteristics which are associated with improved cancer cell survival and promote of cancer progression (61). NE-like cells can be found in most human prostate cancers (62).

The androgen receptor is absent in NE cells and androgen ablation enhances the differentiation of NE cells in both men and dogs (51). Androgen depletion induced NE characteristics in human prostate cancer cells. Androgen depletion increased receptor protein tyrosine phosphatase α (RPTP α), protein tyrosine phosphatase, and protocadherin-PC (PCDH-PC) and induced NE marker expression in human LNCaP prostate cancer cells (61,63,64). Synaptophysin-expressing cells were present in the prostate glands of TRAMP transgenic mice, but not in non-transgenic prostate tissue. Synaptophysin was co-localized with the androgen receptor in prostate carcinoma originating in TRAMP mice (65). In addition, expression of neuroendocrine markers in poorly differentiated prostate cancers in TRAMP mice, but not in PIN lesions and well differentiated cancers suggesting that there is acquisition of neuroendocrine characteristics as a result of a phenotypic switch in TRAMP mice with prostate cancer progression similar to that observed in men (46).
Intermediate cell type: Intermediate cells express markers that are between basal and secretory epithelial cell types such as CK 5, 14, 8, and 18, but they do not express AR and p63, and they do not secrete PSA (57,66). This shows that intermediate cells are not yet fully differentiated into secretory cells suggesting they are in transition between basal and secretory cells.

Prostate intraepithelial neoplasia (PIN): In men prostate intraepithelial neoplasia is considered the precursor of prostate carcinoma (67,68). There are three different types of PIN: low grade, medium grade and high grade PIN. Low grade PIN consists of irregularly crowded epithelial cells with variation in nuclear size (67). Whereas, in mid grade and high grade PIN, the alveolar gland basal cell layer is disrupted with increased proliferation of cells and increased nuclear and nucleolar size (Fig 5) (19,68). Androgen ablation can cause regression of high grade PIN suggesting its androgen dependency (69).

The TRAMP model of prostate carcinoma in mice develop low grade PIN at 5-9 weeks of age, high grade PIN from 9-12 weeks of age, and invasive carcinoma after 12 weeks (67). Waters et al described spontaneous high grade PIN in dogs similar to men characterized by disruption of the basal cell layer, crowding of secretory cells in acini and ducts with variations in nuclear size and shape, and nucleolar enlargement. The basal cell layer was absent in dog prostate cancer (70). Histologically, the tufting pattern of PIN was predominant.
although the micropapillary form was observed in a few cases. Comparison of the occurrence of high grade PIN in dogs aged 7-14 years demonstrated a lesser incidence of PIN (9%) in castrated dogs compared to intact dogs (55%) (68,70,71). Dogs less than 4 years of age have a very low incidence (8%) of high grade PIN. Therefore, the occurrence of high grade PIN in dogs is age and neutered status dependent (70). In addition, high grade PIN was observed in dogs without prostate carcinoma (72); however, the role of PIN as a precursor of prostate cancer in dogs has not been confirmed.

**Role of androgens in prostate cancer and mechanisms for development of hormone refractory prostate cancer:** Testosterone is converted to its more active form, 5α-dihydrotestosterone, by 5α-reductase type 2 in the prostate gland. Both testosterone and 5α-dihydrotestosterone act through the androgen receptor (AR) (73). In men, the AR is primarily expressed by secretory epithelial cells, although some stromal cells are positive for AR and play an important role in the development and progression of prostate cancer through paracrine action of stromal secreted growth factors, such as fibroblast growth factor, transforming growth factor-α, epidermal growth factor and keratinocyte growth factor (31,74).

Despite the heterogeneous nature of AR expression in prostate cancer, its expression is greater in well differentiated prostate carcinoma compared to normal epithelia and moderately to poorly differentiated carcinoma (75). AR
exerts its action by phosphorylation and dimerization of AR, which binds to the promoter regions of target genes including PSA and survival proteins.

Initially androgen deprivation therapy is used to treat prostate cancer patients to cause cancer regression and inhibition of cancer growth (76). However, prostate cancer eventually fails androgen ablation therapy and progresses to hormone refractory prostate cancer with few treatment options (77). Androgen independence of prostate cancer results from diverse mechanisms, such as AR amplification, AR mutations and abnormal AR co-regulatory proteins that make AR sensitive to low concentrations of androgens (74,78,79). Higher levels of AR may increase the sensitivity of the receptor to low levels of ligand or the AR might remain constitutively active in the absence of ligand due to intrinsic androgen–independent transcriptional activity (80).

High AR levels can recruit the RNA polymerase II enzyme and decrease the nuclear receptor corepressor (NCoR) to gain androgen-independent transcriptional activity (81). In some cases, the regulatory function of AR on prostate growth is circumvented by other factors such as Bcl2 to confer survival mechanisms by resisting the response to apoptotic stimuli (82). Increased AR expression can convert AR antagonists, such as bicalutamide, cyproterone acetate and flutamide to agonists causing an increase in androgen-sensitive genes resulting in androgen-independent prostate growth (81).

Cancer progression and growth of specific clonal populations also account for the loss of androgen sensitivity (83–85). In some androgen-
independent prostate cancers, AR expression is downregulated due to hypermethylation of the AR gene (86). The androgen receptor also can be activated by IL-6 in androgen-independent prostate cancer (87). Overexpression of Her-2/neu transactivates AR function by promoting the interaction between AR and AR co-activators (88). Akt-kinase-mediated phosphorylation of the AR promotes ubiquitination of the AR suggesting that cells with increased Akt levels have decreased AR expression (89). Rb and BRCA1 and 2 enhance the expression of AR in prostate cancer cells (90). PTEN inhibits AR transcriptional activity and induces apoptosis of prostate cancer cells (91).

The AR contains two types of trinucleotide repeats, CAG and GGC. A decrease in the number of these polymorphic microsatellite repeats has been linked to prostate cancer (92). In androgen deprivation therapy, prostate cancer cells developed altered morphology and decreased proliferation, but did not undergo apoptosis (82). This suggests that resistance to apoptosis in androgen deprivation therapy plays an important role in the progression of prostate cancer. Overexpression of the antiapoptotic protein, bcl-2, promoted the survival of the human LNCaP prostate cancer cells during androgen ablation. However, when supplemented with androgens, bcl-2 expression was decreased suggesting that the AR mediated bcl-2 regulation (82). Therefore, multiple mechanisms potentially promote the development of hormone refractory prostate cancer.
**Prostate specific membrane antigen (PSMA):** Prostate-specific membrane antigen is present in human epithelial cells and prostate carcinoma but was not found in primates, dogs and mice (93,94). PSMA was demonstrated in the human prostate cancer cell line, LNCaP, but was not found in other prostate cancer cell lines, such as PC3 and DU145 (95). Although PSMA was thought to be confined to the prostate, it also occurred in other malignancies such as colon, bladder and renal cancer (96). In addition to malignancies, PSMA was detected in normal tissues, such as kidney, duodenum, and colon (96).

**GENETICS OF PROSTATE CANCER**

Genetic alterations can be involved with the development of prostate cancer. Approximately, 25% of prostate cancer cases in men are associated with familial history (97). The first identified locus that was shown to be responsible for hereditary prostate cancer was linked to chromosome 1q24-q25 (98). Prostate cancer susceptibility was also demonstrated to be linked to alterations in loci 1q42-43, Xq27-q28, and 1p36 (99–101). The 8p22-p23 locus on chromosome 8 was shown to have tumor suppressor gene deletions at loci NEFL, D8787 and ANK1 (101–103). Chanh et al. showed that chromosome 8p alterations are present in 80% of prostate cancer patients (104).
Nupponen et al reported that prostate cancer chromosome losses are most often observed in 1p, 6q, whereas chromosomal gains occurred at 1q, 2p, 7, 8q, 18q, and Xq (105). On chromosome 17, presence of frame shift mutations and two missense mutations on the ELAC2 gene were associated with prostate cancer (106). Allelic imbalance on chromosome 16q23.2 leads to the increased susceptibility of prostate cancer (107). Prostate cancer susceptibility was linked to amplification of chromosome 20q13 (108). Loss of heterozygosity (LOH) was detected in chromosome 5q31-q33 in prostate cancer patients demonstrating its potential relationship to cancer aggressiveness (109).

Loss of heterozygosity or homozygous deletion of PTEN on chromosome 10q23 was detected in 10-15% of prostate cancer patients (110). p53 mutations are commonly found in early prostate cancer, advanced stages, recurrent stages and metastatic disease (111). Loss of function of P27 due to loss of expression or aberrant proteosomal degradation was often present in prostate cancer patients (112). Loss of chromosome 13q, including functional loss of retinoblastoma (Rb) gene expression or mutations, was present in approximately 50% of prostate cancer patients (113).

Epigenetic hypo- and hypermethylation mechanisms contribute to the development of prostate cancer (114). The glutathione S-transferase P1 (GSTP1) gene, which is important for DNA damage repair, was commonly hypermethylated in prostate cancer (115). Hypermethylation of tumor suppressor gene, Rb, and Ras association domain family 1 gene (RASSF1A) were reported
in human prostate cancer patients. (116). In contrast, hypomethylation of the urokinase plasminogen activator gene in prostate cancer has been correlated to increased invasiveness and tumorigenesis (117).

Loss of function of genes by deletions, mutations hypermethylation of tumor suppressor genes and gain of function of proto-oncogenes contribute to prostate cancer in men. In addition, gene fusions and chromosomal translocations occur and cause structural rearrangement of genes that can be important in prostate cancer (118). Erythroblastosis virus E26 transforming sequence (ETS) variant gene 1 (ETV1) and v-ETS erythroblastosis virus E2 oncogene-like (ERG) genes were found to be translocated in prostate cancer (119). Chromosomal relocations of transmembrane protease serine 2 (TMPRSS2)-ERG and TMPRSS2-ETV1 gene fusion were detected in 50% of prostate cancer patients (120). TMPRSS2 regulates ETS and has been shown to play a role in the development of prostate carcinoma (119). Therefore, multiple structural and functional alterations in genes and chromosomes contribute to the development and progression of prostate cancer.

PROSTATE CANCER ANGIOGENESIS

Angiogenesis in prostate cancer is regulated primarily by the VEGF signaling axis (121). In prostate cancer, tumor-associated capillaries are tortuous with increased density and are more dilated than normal vessels (122). The
transgenic adenocarcinoma of mouse prostate (TRAMP) model was used to study the temporal and spatial changes of angiogenesis (123). Factors responsible for angiogenesis correlated well in human prostate cancer patients and in the TRAMP model (123). VEGFR1 played an important role progression of prostate hyperplasia into dysplasia of initiated epithelial cells (123). VEGFR1 is abundant in prostatic intraepithelial neoplasia (PIN) and well differentiated carcinomas but absent in normal prostate and poorly differentiated prostate cancers, which suggest a role for VEGFR1 in the angiogenic switch early in the process of multi-stage carcinogenesis (123).

Increased amounts of the VEGF 165 isoform and VEGFR2 occur in poorly differentiated and advanced prostate cancer (124,125). FGF2R and FGF2 contribute to angiogenesis in androgen-independent prostate cancer (123). HIF-1alpha, matrix metalloproteinase-2 and 9, cyclooxygenase-2, epidermal growth factor, angiopoietin-1 and 2, tissue factor, angiogenin, interleukin-8, hepatocyte growth factor, transforming growth factor-β, and platelet-derived endothelial cell growth factor (PDECGF) are pro-angiogenic factors in Pca. Matrix metalloproteinases (MMP-2 and 9) are involved in the degradation of basement membrane, an initial step in the angiogenesis process (123,126,127). Therefore, aberrant regulation of angiogenesis plays a key role in the progression of prostate cancer.
ANIMAL MODELS OF PROSTATE CANCER AND BONE METASTASIS

Improved understanding of prostate cancer carcinogenesis and progression and development of effective therapeutics are hampered, in part, by a lack of relevant preclinical models (128). Animal models currently in use include those derived from rats, mice, dogs, and men. Xenografts of human and dog prostate cancer cell lines in immunocompromised mice are commonly used to investigate the pathogenesis of prostate cancer and develop novel therapeutics. Genetically engineered mouse models (GEM) are of increasing popularity because of the autochthonous development of prostate cancer and the ability to study the mechanistic and temporal qualities of tumorigenesis in an immunocompetent host without the need for exogenous carcinogens.

**Genetically engineered models (GEM) of prostate cancer:** GEM models of prostate cancer involve either the overexpression of an oncogene controlled by a prostate-specific promoter or the targeted deletion of specific genes in the prostate epithelium (129,130). These models vary in the ease of colony generation and maintenance, consistency in lesion development and extent of progression, and time required for pathologic changes to occur. No mouse model recapitulates the entire continuum and molecular diversity of the human disease and each has its unique attributes that should be considered in light of the specific scientific question it is intended to address. Given the diversity of lesion development and progression across the various models, it is important
that a standardized nomenclature is used. A review of the definitions and classifications of murine prostate cancer models and their pathology is available from the Mouse Models of Human Cancer Consortium Prostate Pathology Committee (32). In addition to appropriate terminology, it is important to provide thorough descriptions of specific pathologic alterations when characterizing a new model or when communicating the effects of an interventional therapy on an existing model.

Some GEM mouse models of prostate cancer have used a prostate-specific promoter, such as probasin, for the expression of viral oncogenes in the luminal epithelium. The TRAMP and LADY models are well characterized first generation models. These models have provided information contributing to our understanding of prostate cancer progression and resistance to anti-androgen therapy, and can be crossed with other transgenic or knock-out models to increase their molecular diversity (129,130).

The TRAMP model was one of the first viral oncogene systems to be developed. It is currently widely used and is one of the best characterized preclinical models of prostate cancer (128). Tumorigenesis in TRAMP mice is caused by the large and small T antigen early SV40 oncogenes under an androgen-responsive, short probasin promoter expressed specifically in the tall columnar epithelial cells of the prostate (131). The large T antigen disrupts p53 and retinoblastoma (Rb) functions, while the small T antigen interferes with protein phosphatase 2A (PP2A) activity and represses the transcriptional activity
of AR (132). The multistage process of tumorigenesis in the TRAMP model shares common histopathological features with the human disease including progression through hyperplasia, PIN, and carcinoma with metastasis (46). We have shown that epithelial hyperplasia and mild PIN is well established in this model by 6 weeks of age, primarily in the dorsal and lateral prostate lobes, and is associated with increased weights of the urogenital tracts and prostate lobes compared to nontransgenic littermates (133). By 8 weeks of age 100% of TRAMP mice have PIN, which progresses to well, moderate, and poorly differentiated carcinoma by 16-32 weeks of age (Fig 6) (46) with variable metastasis to the regional lymph nodes, lung, liver, and infrequently to the kidney, adrenal glands and spine (134). Poorly differentiated tumors in TRAMP mice can be palpated when the tumors are approximately 8 mm in diameter and weigh 400 mg (135). Advanced tumors exhibit neuroendocrine features including synaptophysin and chromogranin A expression (46,133).

The age-dependent progression of disease in the TRAMP model can be exploited for the design of preclinical prevention, intervention, and regression trials with strategies aimed at countering cancer progression. Comparison between studies is often difficult, due to the variability in experimental design and endpoints to test agents as diverse as small-molecule compounds, immunomodulatory agents, and dietary factors. For chemoprevention studies, endpoints used in the TRAMP and other models include urogenital tract weight, individual prostate lobe weight, histopathologic scoring, incidence and distribution
of specific prostate lesions (hyperplasia, PIN, well, moderate, and poorly
differentiated carcinoma), and incidence and sites of metastasis. If applicable,
molecular targets in the prostate may also be accomplished to associate an
observed effect with drug action.

Several natural and pharmaceutical compounds have been used as
chemopreventives in the TRAMP model, including signaling kinase modulators,
anti-androgens, and epigenetic regulators of gene transcription (130).
Investigation of the histone deacetylase (HDAC) inhibitor OSU-HDAC42, for
example, showed that it prevented the development of poorly differentiated
carcinoma and suppressed the absolute and relative weights of the urogenital
tracts by 86% and 85%, respectively, in mice treated from 6 through 24 weeks of
age. Blockade of tumorgenesis was associated with the intraprostatic modulation
of HDAC and other targets regulating cancer cell survival and differentiation
(133). Body weight of drug-treated mice was not affected, which is important
since dietary restriction has been shown to ameliorate tumor growth in this model
(136,137).

Other models have been created using the probasin promoter to
increase SV40 oncogene expression specifically in the prostate. The LADY
model is similar to the TRAMP model with regards to the pathogenesis of
prostate cancer, but the rate of progression from PIN to carcinoma and
metastasis is slower due to use of a different form of the rat probasin promoter
and expression of only the large T antigen (138). The more recently created
transgenic rats with adenocarcinomas of the prostate (TRAP) also express SV40 large T antigen under probasin promoter control (139). The rats develop proliferative lesions of the acinar epithelial cells as early as 4 weeks of age and progress to a 100% incidence of adenocarcinoma within 15 weeks (140). The rats have been used in evaluating the effects of raloxifene, nimesulide, atrazine, dietary restriction, gamma-tocopherol, antiandrogens, and dietary restriction on prostate tumorigenesis (141~143) with the advantage of greater tissue and blood volume (compared to mice) for experimental analysis.

Second generation GEM mouse models of prostate cancer involve targeted manipulation of gene expression in the prostate to mimic specific genetic aberrations found in the human disease. Examples of single-gene overexpression models driven by prostate-specific promoters include Akt kinase, AR, Skp2, fibroblast growth factors (FGFs), FGF receptors, and c-Myc, whereas models with loss of single gene function include PTEN, Nkx3.1, and RXRα (129). These models have been used to create compound mutations such as the concomitant loss of PTEN and p27KIP1 or Nkx3.1 (144). However, technical difficulties associated with the dual targeting of genes in a prostate-specific manner can make use of these models challenging (129). One exception is the knockout of p53 and Rb specifically in the prostate epithelium (p53PE−/−; RbPE−/−), which suggested that prostate cancer associated with p53 and Rb deficiency arises from the stem/progenitor cell–enriched proximal region of prostatic ducts (145). Second generation models have also been crossed with first generation
models to study the effect of specific genetic manipulations on a well characterized course of autochthonous prostate cancer development (Table 1). Loss of PTEN heterozygosity enhances malignancy and shortens the lifespan of TRAMP mice (146). Prostate tumorigenesis was impaired in TRAMP mice deficient in Egr-1 (147). Transgenic models of mouse prostate cancer commonly demonstrate metastasis to lymph node and lungs and infrequently to kidney, liver, and adrenal gland as summarized in table 1.

Despite advances in creating new GEMs that mimic human prostate cancer development, therapeutic intervention studies utilizing them often fail to predict results in human studies (128). Proposed reasons for this weak predictive power include inadequate or inappropriately used models that closely mimic human prostate cancer and a disparity in study design between the preclinical model and subsequent clinical trials (128). Since human prostate cancer is a complex and heterogeneous disease involving the epithelium and supporting microenvironment, future advances will depend on increasing the sophistication of GEM models, including targeting genetic events in the stroma and immune cells, to better integrate with the pathobiological evolution of human prostate cancer.

Rat models of prostate cancer and metastases: A few strains of rats develop spontaneous prostate cancer, and exhibit metastases to the lungs, peritoneal cavity and lymph nodes, but they do not develop spontaneous bone metastases
(Table 2). The Dunning tumor model represents spontaneous prostate cancer that originates in a Copenhagen rat. The MatLyLu cell line was developed from the Dunning model and the cell line develops osteolytic metastases in lymph nodes, lungs, vertebrae and hind limbs after intracardiac injections (148,149). The MatLyLu cells develop subcutaneous xenografts in syngeneic mice (150). The Lobund-Wistar strain of rats develops spontaneous cancer of the prostate in the dorsal and anterior lobes and seminal vesicles in about 25% of the rats at 26 months of age with metastases to the lungs, lymph nodes and the peritoneal cavity. The prostate adenocarcinoma cell line-III (PA-III) was developed from the Lobund-Wistar model and the cell line develops mixed osteoblastic and osteolytic metastases when transplanted over calvarium (151). PA-III cells develop subcutaneous xenografts in syngeneic mice and nude mice (Table 3) (152,153). Prostate cancer has been reported in Noble rats (0.5% incidence), but metastases do not occur (154).

**Xenograft mouse models of prostate cancer:** Prostate cancer xenograft models are established by orthotopic and heterotopic injection of tumor cells or tissue from men or animals, such as the dog, into immunodeficient mice (12,16). The most commonly used immunodeficient mouse strains used for xenotransplantation are athymic nude mice and non-obese diabetic severe combined immunodeficient mice (NOD/SCID), and SCID beige mice (155). Several prostate cancer cell/tumor lines from different species were established
and shown to be tumorigenic in the subcutis of immunocompromised mice and are summarized in table 3.

Orthotopic xenograft mouse models are useful to reconstruct the primary tumor microenvironment (11). Heterotopic xenograft models using injections into the subcutis, left ventricle of the heart, tail vein, and tibia are extensively used to model tumor growth and metastasis (11,156). Orthotopic xenograft models involve injection of cancer cells into the prostate dorsal, anterior, lateral and ventral lobes to study the differences in biological behavior compared to the heterotopic xenograft models (157~160). Glinkskii et al showed that orthotopic PC-3 xenografts exhibited a more aggressive metastatic phenotype characterized by increased local invasiveness, metastasis to lymph nodes, and viable cancer cells in circulation compared to subcutaneous xenografts (161). The highly aggressive tumorigenic and metastatic variants of LNCaP (LNCaP-Pro2, Pro3, Pro5, LN2, LN3, LN4) and PC-3 (PC-3M-Pro4 and PC-3M-LN4), were developed from orthotopic parental tumors and lymph node metastases, though not all the sublines exhibited discernible differences compared to parental cells (162).

Despite the aggressive behavior of cancer and increased metastatic ability to lymph nodes, bone metastases did not occur in the orthotopic models. To address this issue, Corey et al. developed a novel method of surgical transplantation of human prostate tumor lines in the SCID mouse prostate to generate an improved orthotopic model of metastasis using human prostate cell
Although gross bone metastases were not observed in the mice, micrometastases were detected by PCR for PSA in bone marrow eight months after surgical resection of tumor from the prostate glands. Mice transplanted with LuCaP 28.3 tumors and surgical resection had a higher number of lung metastases but there was no difference in mice with LuCaP 35 tumors (163).

Since there are limitations to orthotopic models to develop bone metastasis, experimental bone metastasis models were developed by direct growth of prostate cancer cells in the bone microenvironment by injection of cancer cells into tibias or femurs or left ventricle of the heart for the dissemination through the systemic circulation. Although these methods do not recapitulate the entire cascade of the events involved in the process of metastasis, they provide better model systems to investigate specific aspects of bone metastasis.

**Mouse models of prostate cancer bone metastasis:** Bone metastases occur in 65%-75% of men in advanced stages of prostate cancer and 24% of dogs with prostate cancer (5,71). Prostate cancer in dogs is most commonly diagnosed during advanced stages and has a low survival period (9). The incidence of prostate cancer is slightly higher in castrated dogs compared to the non-castrated dogs (71). The median age of dogs with prostate cancer is 10 years, and dogs diagnosed with prostate cancer at a younger age have a higher incidence of skeletal metastasis than older dogs (164). Prostate cancer
metastases to bone in men primarily results in the establishment of osteoblastic metastases and predominantly osteolytic metastases occur infrequently (7). Recent investigations have demonstrated that most osteoblastic metastases also have an osteolytic component, which was evident by radiolucent areas on radiographs, loss of preexisting bone, increased eroded bone surface area, and increased serum osteoclast activity marker TRAP5b and bone resorption crosslink markers (7,165). Similar to men, prostate cancer bone metastases in dogs can be osteoblastic, osteolytic or mixed (Fig 7 and 9) (9). Several dog prostate cancer cell lines were established to model the human prostate cancer (Table 4).

Although bone is the most common site of metastasis, prostate cancer sporadically metastasizes to other organs including the brain, lung, liver, eye, testis, thyroid and adrenal glands (166~169). Prostate cancer patients with high PSA concentrations and poorly differentiated cancer had a higher incidence of skeletal metastases (170). Mouse models of prostate cancer bone metastasis are usually developed by direct injection of cancer cells into long bones, such as the tibia or femur or injection into the left ventricle of the heart (171~173).

Intracardiac (IC) injection of cancer cells into the left ventricle results in direct entry into systemic circulation and cancer cells extravasate from blood vessels in bone to develop bone metastases if the tumor and host microenvironment interactions are favorable as suggested by Paget (4). However, this method does not recapitulate the earlier events of metastasis such
as soft tissue and vascular invasion (156). This method can be useful to study the incidence of bone metastases, time to first skeletal-related event, the mechanisms of tumor, bone and marrow interactions and novel therapeutics. A second model of bone metastasis is direct transplantation of cancer cells into the bone marrow of the tibia (IT) or femur. The host bone marrow microenvironment must also be favorable to permit cancer cells to proliferate, but this model avoids the need of cancer cells to survive in the circulation and extravasate through bone marrow blood vessels. Normalization of tumor growth is challenging in direct bone injections due to the technical difficulty of injection of cancer cells in small volumes (20ul). Both intracardiac and intrabone injection models permit investigations on cancer cell interaction with the bone and bone marrow microenvironment.

The human prostate cancer cell line, PC3, develops osteolytic bone metastases at long bones, mandible, ribs, and vertebrae, and is the most commonly used model to study the osteolytic phenotype of prostate cancer in experimental settings. Inoculation of PC3 cells through intracardiac, tail vein and orthotopic routes results in the development of bone metastases in immunodeficient mice. Yang et al demonstrated that orthotopically injected PC3 cells developed not only skeletal metastases but also soft tissue metastases to the brain, spinal cord, liver, kidney, lung, pleura and adrenal gland. Soft tissue metastases were not detected with other routes such as intracardiac and tail vein injection of PC3 cells, but bone metastases did develop (174).
Human fetal or adult bone fragments transplanted to the subcutis of mice have been used to investigate the role of species specificity on prostate cancer bone metastasis (175). Tail vein injection of PC3 cells in SCID-hu bone mice resulted in metastasis to human bone implants and mouse lungs but not to other mouse organs and human lung and intestinal implants. Human DU145 and LNCaP cells did not develop any metastases. Direct injection of all three cell lines into human bone implants resulted in tumor growth (33). In contrast, Yonou et al. showed that tail vein injection of LNCaP cells resulted in metastasis to human bone explants and mouse lungs but not to mouse bones (176). These findings demonstrate a significant role of species specificity on prostate cancer metastasis to bone.

Prostate cancer cells can induce osteoclast activity by secreting higher concentrations of osteoclastogenic factors such as receptor activator of NF-κB ligand (RANKL), nuclear factor-κB (NF-κB) (177) and by increasing the receptor activator of NF-kappa B ligand (RANKL)/osteoprotegerin (OPG) ratio of osteoblasts (178). RANKL binds to the RANK receptor on the surface of osteoclast precursors to trigger a cascade of signaling pathways, resulting in differentiation and maturation into active osteoclasts (175). OPG functions as a soluble decoy receptor for RANKL and inhibits osteoclastogenesis (175).

Expression of both RANKL and OPG is increased in several prostate cancer cell lines and primary carcinomas compared to normal tissues (179). RANKL and OPG detection was higher in skeletal metastases compared to
primary carcinomas (179). Although both RANKL and OPG are increased in prostate bone metastases, the RANKL/OPG ratio is low resulting in decreased osteoclast activity (180). It has been shown that MMP-7, either secreted by prostate cancer cells or osteoclasts in the bone microenvironment, contributes significantly in the prostate cancer-induced osteolysis by cleaving the RANKL from osteoblasts and tumor cells to promote RANKL-mediated osteoclast activation (181). Bonfil and colleagues showed that prostate cancer secreted membrane type-1-matrix metalloproteinase and increased bone resorption by degrading the bone matrix (182).

Roato et al demonstrated that TWIST transcriptional factor is expressed in osteolytic prostate cancer cell lines, PC3 and 22RV1, and TWIST contributed to the osteolytic phenotype by upregulating the osteoclastogenic factor, dickkopf-1, and downregulating the osteogenic factors, endothelin-1, bone morphogenic protein 6, fibroblast growth factor and RUNX2. Alternatively, the same group demonstrated that TWIST expression was upregulated in the 22RV1 prostate cancer cell line, which promoted bone induction by paracrine factors. In contrast to PC3 cells, TWIST in 22RV1 cells increased RUNX2 expression and transcriptional activity resulting in increased osteogenic potential. The divergent functional role of TWIST in its contribution to the phenotype of bone metastases is attributed to cell type-dependent activity (183).

Based on the process of bone remodeling, where osteoclastic bone resorption precedes bone formation, it has been hypothesized that prostate
cancer-induced osteoblastic metastases are dependent on osteoclast activity (175). While some studies supported this hypothesis, other investigations demonstrated that osteoblastic metastases were independent of osteoclast activity, suggesting that prostate cancer cells can have a direct effect on new bone formation (Fig 8) (184,185). Histopathological assessment of osteoblastic metastases revealed that new woven bone occurs at the tumor trabecular interface in the metaphyses, and along the endosteal surface in the diaphyses and in intramedullary regions. Osteolytic models can cause secondary periosteal new bone formation due to cortical instability, which should not be confused with an osteoblastic reaction (171).

Despite osteoblastic metastasis being common in patients with advanced prostate cancer, the mechanisms contributing to the osteoblast activity are not clearly understood. Most prostate cancer cells lines develop primarily osteolytic metastases (6). Several prostate cancer cell/tumor lines that develop bone metastases after IT/IC injection were summarized in table 5. We have developed a dog prostate cancer cell line (Ace-1) that metastasizes to bone with 100% incidence after intracardiac injection in nude mice and develops mixed predominantly osteoblastic metastases. (16). Using Ace-1 model, we showed that prostate cancer bone metastases and the osteoblastic phenotype of metastases were not dependent on bone resorption (171). Results obtained from this investigation were consistent with prostate cancer clinical studies in men.
Therefore, this is a valuable model for further investigations of pathogenesis of prostate cancer bone metastases.

Recent studies showed that prostate carcinoma express several Wnt family proteins that contribute to the osteoblastic phenotype by directly enhancing osteoblast activity (172). Wnts play a significant role in the process of osteoblastogenesis that involves osteoprogenitor commitment, differentiation and maturation into active osteoblasts. Wnts increase the proliferation and survival of osteoblasts and contribute to the prolonged function of osteoblasts (187). Wnts act through both canonical and non-canonical pathways in osteoblasts to promote bone formation (188). Wnts act on prostate cancer cells in an autocrine manner to induce expression of osteogenic bone morphogenic proteins and indirectly contribute to the prostate cancer-induced osteoblastogenesis (189). Wnts not only increase osteoblastic activity but also downregulate the expression of RANKL in osteoblasts and indirectly inhibits osteoclastogenesis contributing to the increase in bone density (190).

Prostate cancer cells produce several BMPs, which have osteogenic stimulatory properties. Human prostate cancer expressed BMP-6, which was shown to promote osteoblast differentiation and mineralization (189). BMPs promote prostate cancer induced osteoblastic activity by increasing vascular endothelial growth factor (VEGF) expression in prostate cancer cells in an autocrine fashion (189). Production of (VEGF) by prostate cancer cells was shown to contribute to increased osteoblast activity. Despite the fact that prostate
cancer-secreted VEGF promotes osteoblast activity, recombinant VEGF alone had no effect on the osteoblasts. This suggests that prostate cancer-secreted VEGF induces osteoblast activity only in the presence of other prostate cancer-derived factors (191). Mice bearing C4-2B prostate cancer cells develop mixed bone metastases. These cells became osteoblast-like (osteoblast mimickry) and expressed bone markers, such as RUNX2, osteonectin, bone sialoprotein, osteocalcin and alkaline phosphatase. C4-2B cells mineralized in vitro under standard mineralization conditions used for preosteoblasts (192). Leroy et al showed that canine prostate homogenates stimulated calvarial phosphatase activity through endothelin-1. Endothelin receptor A and B (ET_A and ET_B) antagonists decreased the endothelin-1-mediated osteoblast activity in calvaria (193).

Several other factors, such as insulin growth factor-1 (IGF-1), transforming growth factors-β (TGF-β), fibroblast growth factor-2 (FGF-2), urokinase plasminogen activator (uPA) and Wnt family genes were shown to possess osteogenic potential (Table 6). Therefore, multiple osteogenic factors may be important in the pathogenesis of prostate cancer-induced osteoblastic metastases.
ADVANCES AND USES OF IMAGING IN ANIMAL MODELS

Imaging technology has become invaluable in advancing prostate cancer research. Imaging techniques such as micro-computed tomography (uCT), positron emission tomography (PET) and bioluminescence imaging (BLI) facilitates noninvasive spatial and temporal monitoring of the effects of genetic manipulations and novel therapeutic agents in preclinical studies. The high sensitivity and noninvasive features of these techniques extends the ability to detect and visualize the functional and structural modifications at the molecular level including signal transduction pathways, protein-protein interactions and nuclear receptor activities.

Bioluminescent imaging (BLI) is used widely in rodent studies, because it is very sensitive and has high throughput. The principle of BLI imaging is based on the optical detection of light emitted as a result of chemical reaction between a luciferase reporter gene and its substrate, luciferin, in the presence of oxygen and ATP. Only viable cells produce light (194). The luciferase gene expressed by the North American firefly (Photinus pyralis) is commonly used to label tumor cells. BLI has low spatial resolution, which is offset by its sensitivity and speed. BLI is used to monitor tumor growth spatially and temporally and BLI is very effective at identifying individual metastases in bones and their progression in longitudinal studies (Fig 9) (171).
All cell lines will not have the same transfection efficiency and light production varies by cell line or clonal cell line. Furthermore, following stable transfection of the luciferase gene, spontaneous loss of expression may occur. Mouse cell lines tend to lose expression more readily than other species. Longitudinal studies can only be performed with stable cell lines that have consistent light production.

BLI should be used to confirm that left ventricular injection of luciferase-labeled tumor cells were deposited completely in the circulation. A diffuse light signal throughout the whole body of an animal immediately after the left ventricular injection is desired, whereas an intense signal in the thoracic region indicates injection of cells into the pleural or pericardial space (171). Measurement of tumor growth by BLI should always be confirmed by physical measurements, such as histomorphometry. Quenching of signal will occur in larger tumors.

Bioluminescent prostate cancer xenograft models have been established that grow in the subcutis, bone, lungs and prostate glands of mice (195). BLI has been efficiently used to measure the effects of drug therapy on tumor growth and incidence of metastases (196). Zhang et al demonstrated that BLI can be used to detect the regulation of transcriptional activity of RANKL gene promoter in prostate cancer bone metastasis (197). BLI also has been used to detect prostate tumor growth in prostate-specific luciferase transgenic mice. The site of the BLI signal correlated with the tumor location and signal intensity correlated
with tumor size (195). Interestingly, it has been reported that a HDAC inhibitor decreased tumor size as detected by caliper measurements, but BLI signal increased. This was attributed to the action of HDAC inhibitors on increasing CMV promoter activity that was used in the vector to regulate the luc gene (personal communication with Drs. Ching-Shih Chen and Aaron Seargent).

Microcomputed tomography (μCT) has been utilized in *in vivo* and *ex vivo* to quantitatively and qualitatively image the three dimensional microarchitecture of anatomical structures non-invasively (198). μCT has become an important modality to measure the histomorphometric parameters of bone because of the higher density contrast of mineralized tissue compared to soft tissues (199). Despite difficulties in imaging soft tissue due to a lack of variation in tissue density, contrast-enhancing agents have facilitated computed tomography imaging of soft tissues (200). μCt is a valuable tool to image and assess prostate cancer bone metastasis-induced alterations of bone microarchitecture such as trabecular density, cortical and trabecular thickness, and volume (201).

μCT is used to evaluate the phenotype of bone metastases, such as the osteoblastic phenotype characterized by increased new trabecular and endosteal woven bone. It can also quantify cancer-induced loss of cortical and trabecular bone volume. Finite element analysis of bone permits evaluation of bone strength and quality (202). Additionally, soft tissue imaging, using contrast-enhancement of prostate cancers and neoangiogenesis has been used with μCT in the TRAMP mouse model (199).
PET imaging captures the spatial and temporal images of a targeted biological process. Cancer cells, due to increased glycolysis, have increased demand for glucose uptake and metabolism. In PET, radioisotopes with a short decay time such as flourine-18 ($^{18}$F), carbon-11 ($^{11}$C), oxygen-15 ($^{15}$O) and nitrogen-13 ($^{13}$N) are used as positron emission tracers linked to the glucose analog, fluoro-2-deoxy-D-glucose (FDG), and injected into the circulation (203). PET imaging can be used as a potential screening method to identify susceptibility of a tumor to gene therapy (204). PET imaging is a useful tool in monitoring efficacy and predicting the response to therapeutics. Leyton et al. used PET imaging to demonstrate the specificity of targeting of a radioiodinated antibody (I124-2B3) for prostate stem cell antigen (PSCA) in prostate cancer xenografts (205). PET imaging detects the quantitative alterations in tumor metabolism in response to therapeutic agents where monitoring of anatomical response may be confounded by areas of necrosis or inflammation (206). In conclusion, multiple imaging modalities have enhanced the understanding of the pathogenesis of prostate cancer using in vivo mouse models.

CONCLUSION

Prostate cancer is a devastating malignancy with a high incidence of osteoblastic bone metastasis. Animal models are important to model different stages of the
pathogenesis of prostate cancer and bone metastases. Although no animal model can entirely recreate the human disease, the dog serves as a valuable model of human prostate cancer because of a high degree of genomic homology and similarities in clinical presentation of the disease. Prostate cancer in dogs provides a unique opportunity to study spontaneous prostate cancer and bone metastases and evaluate novel therapies. In addition, xenograft and genetically modified mouse models are indispensable to study the mechanisms of carcinogenesis and metastasis. Although animal models have contributed significantly to our understanding of prostate cancer, there are several questions which remained unanswered, namely the molecular pathogenesis of osteoblastic bone metastasis. Improvement of existing animal models and/or establishment of new animal models that more closely mimic the human disease are instrumental to improving our understanding of prostate cancer pathogenesis. Efficient utilization of animal models combined with advancement in biomedical research technologies will improve insights into understanding this disease. The process of developing novel treatment strategies for metastatic prostate cancer will benefit from efficient integration of animal models with genetic, proteomic, and \textit{in vivo} imaging approaches.
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FIGURE LEGENDS

**Figure 1:** Comparison of dog, human, rodent and nonhuman primate prostate glands. A coronal section of dog prostate gland shows bilobed structure with sparse stroma. Human coronal section of prostate gland shows three different glandular prostate zones; transitional, peripheral and central zones and a fibromuscular zone. Rodent prostate coronal section depicts dorsal lobes, anterior prostate, lateral prostate lobes and ventral lobes. Nonhuman primate prostate sagittal section depicts anterior and posterior prostate lobes covering the dorsal urethra.

**Figure 2:** Haemotoxylin and eosin (H&E)-stained sections of normal human and canine prostate glands. Note the few glands (arrow) surrounded by a band of fibromuscular stroma (S) in the human prostate gland. The clear cytoplasm in cells lining the gland is characteristic of the mucinous nature of the human prostate. Bar, 100 microns. H&E-stained section of normal canine prostate gland. There is a marked increase in glandular density (arrow) of the canine prostate gland compared to the human prostate gland. The densely eosinophilic
cytoplasm of glandular epithelial cells is characteristic of the canine prostate. Bar, 100 microns.

**Figure 3:** H&E-stained sections of human and canine prostatic carcinoma. The normal structure of the human prostate is released by prostate carcinoma characterized by multiple atypical acini with loss of cellular orientation. Bar, 100 microns. H&E-stained section of canine prostatic carcinoma with an intra-alveolar histologic pattern and anaplastic cells filling the acini. Bar, 100 microns.

**Figure 4:** Macroscopic photograph of the prostate from a 5-month-old rat. Notice the dorsal (DL) and ventral lobes (VL) of the prostate, anterior prostate (AP) or coagulatory gland (CG), lateral lobes (LL). Penis (P), seminal vesicles (SV) and urinary bladder (UB) are indicated. (mm scale).

**Figure 5:** H&E-stained section of human prostate gland with prostate intraepithelial neoplasia (PIN) characterized by irregularly crowded, proliferative and atypical acinar epithelial cells (arrow), and loss of continuity of basal cells (arrow head).
Figure 6: Dorsal prostate gland tissue in the TRAMP mouse model at 6, 10, and 24 weeks-of-age. (A) Normal prostate from a 10-week-old non-transgenic littermate of TRAMP mouse (C57BL/6xFVB). (B) Epithelial hyperplasia (small arrow) and early PIN lesions (thick arrow) in a 6-week-old TRAMP mouse. Note the multifocal pattern of lesion development in the dorsal prostate. (C) 10-week-old TRAMP mouse with more developed PIN lesions featuring a higher degree of nuclear atypia, cellular stratification, and the presence of cribriform structures extending into the acinar lumen. (D) Poorly differentiated carcinoma invading and surrounding residual prostate acini in a 24-week-old TRAMP mouse. H&E, 400x.

Figure 7: Prostate cancer induced osteoblastic metastases in human and dog. (A) Faxitron macroscopic photograph of dog prostate carcinoma osteoblastic metastases to humerus, characterized by increased radio-opacity in metaphysis and diaphysis (arrows). (B). Photomicrograph of H&E-stained human prostate carcinoma metastasis to vertebral bone demonstrating induction of new woven bone (arrow).
Figure 8: Schematic illustration of proposed model for pathogenesis of bone formation in osteoblastic metastases of prostate cancer. Prostate cancer cells either directly secrete osteogenic factors or induce stromal cells in bone microenvironment to secrete osteogenic factors such as bone morphogenic proteins (BMPs), urokinase plasminogen activator (uPA), C-terminal parathyroid hormone related protein (C-PTHrP), platelet derived growth factor-BB (PDGF-BB), insulin like growth factor (IGF), Wnts, endothelin-1 (ET-1) and fibroblast growth factor (FGF). The osteogenic factors induce preosteoblast proliferation and maturation into active osteoblasts which deposit new woven bone. ET-1 inhibits the anti-osteogenic activity of dickkopf-1(DKK-1) gene by down regulating its expression. In addition to directly promoting osteoblastic activity, prostate cancer cells also secrete osteoclastogenic factors such as N-PTHrP, receptor activator of nuclear factor kappa B ligand (RANKL), and matrix metalloproteases, which increase osteoclast activity and osteoclast-mediated bone resorption. Bone resorption releases bone matrix growth factors such as IGF, FGF, transforming growth factor-β (TGF-β) and BMP which are important for survival of prostate cancer cells and osteogenic activity. Activated osteoclasts also secrete PDGF-BB which promotes the survival of osteoblasts. Prostate cancer cells
increase bone formation metastasis directly, by secreting factors which stimulate osteoblasts, or indirectly, through modulating the bone microenvironment.

**Figure 9:** Dog prostate carcinoma (Ace-1) bone metastasis in nude mice. (A) Representative bioluminescent image of nude mice on day 28 after intracardiac injection of Ace-1\textsuperscript{YFP-Luc} cells. Bioluminescence is represented by red and blue signal overlying sites of boney metastasis in both hind and fore limbs. (B). Histopathologic evaluation of BLI-detected bone metastasis showing Ace-1 tumors in long bones. Bone marrow is replaced by tumor cells (T) in the medullary cavity. Bone metastasis is characterized by development of new woven bone (WB) adjacent to the tumor along the endosteal cortex (CT), in addition to lysis of cortical bone (arrow) in the diaphysis, and illustrates the mixed bone metastatic phenotype characteristic of the model.
Table 1: Transgenic mouse models of prostate cancer and their metastatic ability

<table>
<thead>
<tr>
<th>Transgenic mouse model</th>
<th>Site of mets</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>LADY (12T-10)</td>
<td>Regional lymph nodes, liver, lungs</td>
<td>(138)</td>
</tr>
<tr>
<td>PSP94/SV40 T,t</td>
<td>Lymph nodes and kidneys</td>
<td>(207)</td>
</tr>
<tr>
<td>PTEN conditional KO</td>
<td>Regional lymph nodes, lungs</td>
<td>(208)</td>
</tr>
<tr>
<td>PTEN +/- FGF8b</td>
<td>Lymph node, lungS</td>
<td>(209,210)</td>
</tr>
<tr>
<td>Pten +/- × Nkx3.1 −/−</td>
<td>Regional lymph nodes</td>
<td>(211)</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Lymph nodes and lungs. Occasionally to kidney and adrenal gland</td>
<td>(134)</td>
</tr>
</tbody>
</table>

PSP, prostate secretory protein; PTEN, phosphatase and tensin homolog C3(1), rat prostatic steroid binding protein; FGF, fibroblast growth factor; mets, metastases, refs, references
### Table 2: Rat prostate cancer models with metastatic ability

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Site of mets</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fisher F344</td>
<td>Abdominal cavity</td>
<td>(212)</td>
</tr>
<tr>
<td>Lobund-Wistar</td>
<td></td>
<td>(213)</td>
</tr>
<tr>
<td>Copenhagen (Dunning</td>
<td>Lungs, peritoneal cavity and lymph nodes</td>
<td></td>
</tr>
<tr>
<td>model)</td>
<td>Lungs</td>
<td>(214)</td>
</tr>
</tbody>
</table>

mets, metastases; refs, references
Table 3: Subcutaneous xenograft models of prostate cancer in immunocompromised mice

<table>
<thead>
<tr>
<th>Type of Xenograft</th>
<th>Species</th>
<th>Tumor line (TL)/ Cell line (CL)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>LuCaP 23.1, 35, 41 47, 49, 58, 69, 70, and 73</td>
<td>Human</td>
<td>TL</td>
<td>(215)</td>
</tr>
<tr>
<td>PC-295, 310, 329, 346, 324, 339, and 374</td>
<td>Human</td>
<td>TL</td>
<td>(216)</td>
</tr>
<tr>
<td>CWR21, 22, 32 and 91</td>
<td>Human</td>
<td>TL</td>
<td>(217)</td>
</tr>
<tr>
<td>LAPC-3, 4 and 9 22RV1</td>
<td>Human</td>
<td>TL</td>
<td>(77)</td>
</tr>
<tr>
<td>PC3</td>
<td>Human</td>
<td>CL</td>
<td>(218)</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Human</td>
<td>CL</td>
<td>(219)</td>
</tr>
<tr>
<td>DU145</td>
<td>Human</td>
<td>CL</td>
<td>(220)</td>
</tr>
<tr>
<td>VCaP</td>
<td>Human</td>
<td>CL</td>
<td>(221)</td>
</tr>
<tr>
<td>DUCaP</td>
<td>Human</td>
<td>CL</td>
<td>(222)</td>
</tr>
<tr>
<td>PacMetUT1</td>
<td>Human</td>
<td>CL</td>
<td>(223)</td>
</tr>
<tr>
<td>C4-2B</td>
<td>Human</td>
<td>CL</td>
<td>(224)</td>
</tr>
<tr>
<td>C4-2</td>
<td>Human</td>
<td>CL</td>
<td>(225)</td>
</tr>
<tr>
<td>MDA PCa 2a and 2b</td>
<td>Human</td>
<td>CL</td>
<td>(226)</td>
</tr>
<tr>
<td>Ace-1</td>
<td>Dog</td>
<td>CL</td>
<td>(227)</td>
</tr>
<tr>
<td>Leo</td>
<td>Dog</td>
<td>CL</td>
<td>(NP)</td>
</tr>
<tr>
<td>DPC-1</td>
<td>Dog</td>
<td>CL</td>
<td>(14)</td>
</tr>
<tr>
<td>CPA-1</td>
<td>Dog</td>
<td>CL</td>
<td>(228)</td>
</tr>
<tr>
<td>CT1258</td>
<td>Dog</td>
<td>CL</td>
<td>(15)</td>
</tr>
<tr>
<td>PAlIII</td>
<td>Rat</td>
<td>CL</td>
<td>(153)</td>
</tr>
<tr>
<td>TRAMPC1 and C2</td>
<td>Mouse</td>
<td>CL</td>
<td>(229)</td>
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Table 4: Dog prostate cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CK expression</th>
<th>IT growth</th>
<th>Mets after IC injection</th>
<th>Site of mets</th>
<th>Type of Bone mets</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace-1 Leo</td>
<td>8, 18</td>
<td>+</td>
<td>+</td>
<td>Long bones, vertebral; brain, spinal cord and adrenal gland</td>
<td>Mixed</td>
<td>(171)</td>
</tr>
<tr>
<td>DPC-1 CPA-1 CT1258</td>
<td>14, 19</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>(14)</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>(228)</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>(15)</td>
</tr>
</tbody>
</table>

CK, cytokeratin; IT, intratibial; IC, intracardiac; OL, osteolytic; Mixed, osteoblastic/osteolytic with a prominent osteoblastic reaction; ND, not determined mets, metastases, refs, references
Table 5: Prostate cancer cell or tumor lines used to model bone metastasis

<table>
<thead>
<tr>
<th>Cell/ Tumor line</th>
<th>IT growth</th>
<th>Mets after IC injection</th>
<th>Site of mets after IC injection</th>
<th>Type of bone mets</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC3</td>
<td>+</td>
<td>+</td>
<td>Vertebrae, long bones and mandible</td>
<td>OL</td>
<td>(163,230)</td>
</tr>
<tr>
<td>LNCaP</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>OL</td>
<td>(163)</td>
</tr>
<tr>
<td>C4-2</td>
<td>+</td>
<td>+</td>
<td>Vertebrae and lymph nodes</td>
<td>Mixed</td>
<td>(231)</td>
</tr>
<tr>
<td>C4-2B</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Mixed</td>
<td>(172)</td>
</tr>
<tr>
<td>DU-145</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>OL</td>
<td>(163)</td>
</tr>
<tr>
<td>DuCap</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>OB</td>
<td>(232)</td>
</tr>
<tr>
<td>VCaP</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>OB</td>
<td>(232)</td>
</tr>
<tr>
<td>LAPC-9</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td>OB</td>
<td>(233)</td>
</tr>
<tr>
<td>MDA</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td>OB</td>
<td>(234)</td>
</tr>
<tr>
<td>PCa 2a</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td>OB</td>
<td>(234)</td>
</tr>
<tr>
<td>MDA</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td>OB</td>
<td>(234)</td>
</tr>
<tr>
<td>PCa 2b</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td>OB</td>
<td>(235)</td>
</tr>
<tr>
<td>CWR22</td>
<td>+</td>
<td>+</td>
<td>Mandibles, tibias, liver and adrenal gland</td>
<td>Mixed</td>
<td>(236,237)</td>
</tr>
<tr>
<td>22RV1</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5 continued next page

IT, intratibial; IC, intracardiac; OL, osteolytic; Mixed, osteoblastic/osteolytic with a prominent osteoblastic reaction; ND, not determined; mets, metastases, refs, references; Osteoblastic, predominantly osteoblastic or mixed osteoblastic/osteolytic with a prominent osteoblastic reaction
## Table 5: Prostate cancer cell or tumor lines used to model bone metastasis

<table>
<thead>
<tr>
<th>Cell/ Tumor line</th>
<th>IT growth</th>
<th>Mets after IC injection</th>
<th>Site of mets after IC injection</th>
<th>Type of bone mets</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog Ace-1</td>
<td>+</td>
<td>+</td>
<td>Long bones, vertebrae</td>
<td>Mixed</td>
<td>(171)</td>
</tr>
<tr>
<td>Leo</td>
<td>-</td>
<td>+</td>
<td>Predominantly brain, infrequently to long bones, spinal cord and adrenal gland</td>
<td>OL</td>
<td>(NP)</td>
</tr>
<tr>
<td>Rat MatLyLu</td>
<td>ND</td>
<td>+</td>
<td>Lymph nodes, lungs, vertebrae and femur</td>
<td>OL</td>
<td>(148)</td>
</tr>
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</table>
Table 6: Potential osteoblastic factors that can induce bone formation in prostate cancer

<table>
<thead>
<tr>
<th>Osteoblastic Factor</th>
<th>Effect on Osteoblast</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>↑ Differentiation</td>
<td>(193,238)</td>
</tr>
<tr>
<td>BMP2</td>
<td>↑ Differentiation and mineralization</td>
<td>(239)</td>
</tr>
<tr>
<td>BMP3</td>
<td>↑ Differentiation</td>
<td>(240)</td>
</tr>
<tr>
<td>BMP6</td>
<td>↑ Proliferation and mineralization</td>
<td>(189)</td>
</tr>
<tr>
<td>VEGF</td>
<td>↑ Differentiation and mineralization</td>
<td>(191)</td>
</tr>
<tr>
<td>PTHrP</td>
<td>↑ Proliferation and differentiation</td>
<td>(241)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>↑ Proliferation and differentiation</td>
<td>(242)</td>
</tr>
<tr>
<td>uPA</td>
<td>↑ Differentiation</td>
<td>(243)</td>
</tr>
<tr>
<td>FGF-2</td>
<td>↑ Proliferation and differentiation</td>
<td>(238,244)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>↑ Proliferation and ↓ differentiation</td>
<td>(245)</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>↑ Proliferation and survival</td>
<td>(246,247)</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>↑ Proliferation and differentiation</td>
<td>(248)</td>
</tr>
<tr>
<td>Wnt4</td>
<td>↑ Differentiation</td>
<td>(249)</td>
</tr>
<tr>
<td>Wnt5a</td>
<td>↑ Survival</td>
<td>(250)</td>
</tr>
<tr>
<td>Wnt7b</td>
<td>↑ Proliferation and differentiation</td>
<td>(251)</td>
</tr>
<tr>
<td>Wnt11</td>
<td>↑ Differentiation and mineralization</td>
<td>(252)</td>
</tr>
<tr>
<td>Dkk2</td>
<td>↑ Terminal differentiation</td>
<td>(253)</td>
</tr>
</tbody>
</table>

↑, increases; ↓, decreases; ET-1, endothelin-1; BMP, bone morphogenic protein; VEGF, vascular endothelial growth factor; PTHrP, parathyroid hormone-related protein; TGF-β, transforming growth factor–β; uPA, urokinase plasminogen activator; FGF-2, fibroblast growth factor; IGF-1, insulin like growth factor-1; PDGF-BB, platelet derived growth factor-BB; Wnt, winglessint, Dkk-2, dickkopf-2
Figure 1: Comparison of dog, human, rodent and nonhuman primate prostate glands.
Figure 2: Haemotoxylin and eosin (H&E)-stained sections of normal human and canine prostate glands.
Figure 3: H&E-stained sections of human and canine prostate carcinoma.
Figure 4: Macroscopic photograph of the prostate from a 5-month-old rat.
Figure 5: H&E-stained section of human prostate gland with prostate intraepithelial neoplasia (PIN).
Figure 6: Dorsal prostate gland tissue in the TRAMP mouse model at 6, 10, and 24 weeks-of-age
Figure 7: Prostate cancer osteoblastic metastases in dog and man.
Figure 8: Schematic illustration of proposed model for pathogenesis of bone formation in osteoblastic metastases of prostate cancer.
Figure 9: Dog prostate carcinoma (Ace-1) bone metastasis in nude mice.
CHAPTER 2

ZOLEDRONIC ACID INHIBITS OSTEOLYSIS BUT NOT BONE METASTASIS IN A NUDE MOUSE MODEL OF CANINE PROSTATE CANCER WITH MIXED BONE LESIONS

ABSTRACT

Bone metastasis is the most common cause of morbidity and mortality in patients with advanced prostate cancer and is manifested primarily as mixed osteoblastic and osteolytic lesions. However, the mechanisms responsible for bone metastases in prostate cancer are not clearly understood due to the lack of relevant in vivo models that mimic the clinical presentation of the disease in humans. We previously established a nude mouse model with mixed bone metastases using intracardiac injection of canine prostate cancer cells (Ace-1). In this study, we hypothesized that tumor-induced osteolysis promoted the incidence of bone metastases and osteoblastic activity.
We studied the effect of inhibition of osteolysis with zoledronic acid (ZA) on the prevention and progression of Ace-1 bone metastases in nude mice using prophylactic and delayed treatment protocols. Bioluminescent imaging, radiography, and histopathological evaluation were performed to monitor the effect of ZA on the incidence, progression and nature of bone metastases.

Unexpectedly, there was no significant difference in tumor burden and the incidence of metastasis between control and treatment groups as detected by bioluminescent imaging and bone histomorphometry. However, radiographic and histopathological analysis showed a significant treatment-related decrease in osteolysis, but there was no effect on tumor-induced trabecular bone thickness in both treatment groups compared to controls.

Our results demonstrated that the incidence of prostate cancer bone metastases in vivo was independent of tumor-induced osteoclastic bone resorption, even though inhibition of bone resorption reduced bone loss associated with the mixed osteoblastic/osteolytic bone metastases in the Ace-1 model.

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and second leading cause of cancer-related deaths in men (1). In spite of the marked improvements
in early diagnosis and efficient local and systemic therapeutic approaches, 65-75% of the patients with advanced prostate cancer develop skeletal metastasis due to the resistance of tumor cells to conventional therapies (2-4). In prostate cancer, most patients die because of metastases to bone rather than the primary tumor (5).

Prostate cancer bone metastasis is frequently osteoblastic in nature with increased woven bone formation often preceded by osteoclastic activity (2, 6). Currently, in prostate cancer, the mechanisms responsible for osteoblastic bone metastases are not clear due to the lack of prostate cancer cell lines that consistently metastasize to bone and develop mixed osteoblastic and osteolytic lesions in animal models. Therefore, prostate cancer cell lines that reliably develop mixed osteoblastic and osteolytic lesions in vivo can be used to help understand the mechanisms underlying prostate cancer-induced mixed bone metastases as they occur in men. Recently, we have established a new canine prostate cancer cell line (Ace-1) from a prostate adenocarcinoma that consistently produces mixed osteoblastic and osteolytic bone metastases after intracardiac injection in nude mice (7, 8). Metastases of prostate cancer occur in the axial and appendicular bones in humans. Spontaneous prostate cancer in dogs has important similarities to human prostate cancer in the clinical presentation of disease including tumor growth over a long period of time, individual and intratumor heterogeneity, extensive genome homology, and metastasis to distant sites, such as bone. Metastasis of Ace-1 cells and
development of mixed osteoblastic/osteoblastic lesions in nude mice recapitulates the characteristics of human and canine prostate cancer metastases in a mouse model (9, 10). Therefore, the Ace-1 model is useful to study the pathogenesis of prostate cancer metastasis and investigate the complex interactions between tumor cells and the bone microenvironment.

Preferential metastasis and growth of prostate cancer cells in bone is associated with complex interactions between the cancer cells (seed), bone cells, and the bone marrow microenvironment (fertile soil) (3, 11). Metastatic prostate cancer cells in bone produce factors such as parathyroid hormone related protein (PTHrP) and receptor activator for nuclear factor κ B ligand (RANKL) that stimulate increased bone resorption (5). This results in the further release of growth factors and proteins from the bone matrix that promote the growth of cancer cells in bone and possibly increase the maturation and function of osteoblasts. Therefore, it has been hypothesized that bone resorption contributes significantly to the development of osteoblastic metastases. However, the role of osteolysis in prostate cancer bone metastasis, growth of metastases, and induction of osteoblasts is not well understood (5, 12-15). Therefore, targeting osteoclast activity in mixed osteoblastic and osteolytic bone metastases will improve our understanding of the mechanisms underlying prostate cancer bone metastasis. Insights into the pathogenesis of prostate cancer bone metastasis will help identify specific targets for effective therapeutic approaches to help treat this devastating malignancy.
Previous studies have shown that zoledronic acid (ZA) is a potent inhibitor of osteoclastogenesis and osteoclast-mediated bone resorption in animal models of bone metastasis induced by prostate cancer, breast cancer and myeloma (16-20). In the present study, we used ZA to inhibit osteoclast activity and investigate the role of osteoclastic activity in prostate cancer mixed bone metastasis.

MATERIALS AND METHODS

Cell culture: Ace-1 is a spontaneously immortalized canine prostate cancer cell line derived from a prostate adenocarcinoma that was previously established in our laboratory (7). Ace-1 cells were maintained at 37°C in Dulbecco’s Modified Eagle’s Medium/Ham’s Nutrient Mixture F12 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum, 250 U/ml penicillin, 250 µg/ml streptomycin, and 2 mM L-glutamine (Invitrogen Corp.) in a 5% CO₂-humidified chamber.

Establishment of Ace-1 cells stably expressing the YFP-Luc reporter gene: Ace-1 cells were transfected with 6 µg of pcDNA3.1(+)/YFP-LUC, a dual reporter gene construct under control of the CMV promoter (a generous gift from Dr. Christopher Contag, Stanford University, Stanford, CA), and 10 µl of
Lipofectamine 2000 (Invitrogen Corp.). Stably integrated cells were selected using 400 μg/ml of G418 (Sigma-Aldrich Co., St. Louis, MO) for 17 days. Flow cytometry (BD FACS Vantage SE; BD Biosciences, San Jose, CA) was used to sort and clone YFP-positive cells.

**Intracardiac inoculation of Ace-1 cells into nude mice:** Male nu/nu mice, 4-6-weeks-old (Charles River Laboratories, Wilmington, MA) were housed in microisolator cages, and were provided food pellets and water ad libitum. Animal care procedures were approved by the Ohio State University Institutional Lab Animal Care and Use Committee using criteria based on both the Animal Welfare Act and the Public Health Services “Guide for the Care and Use of Laboratory Animals”. Mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) administered intraperitoneally (IP) and positioned on dorsal recumbency. 1x10^5 Ace-1 cells were suspended in 100 μl of sterile Dulbecco’s PBS (Invitrogen Co) and were injected into the left ventricle using a 27 gauge needle after confirmation of location of the tip of the needle in the left ventricle indicated by pulsatile blood flow in the hub of the needle (21). Successful Ace-1 intracardiac injections were confirmed using bioluminescent imaging (BLI) at 10 minutes after injection and were characterized by a diffuse emission of light from the entire body. Mice were euthanized 28 days after Ace-1 inoculation.
**Treatment:** Nude mice were divided into three groups: (a) Control group (n = 11) received PBS from -1 to 4 wks. (b) Prophylaxis group (n = 9) received ZA from -1 to 4 wks. (c) Delayed treatment (n = 7) group received ZA from 2 to 4 wks. Mice were administered ZA at 100μg/kg B.W, (Novartis, Basel, Switzerland) or vehicle (PBS) twice a week subcutaneously (SQ) (Fig 10).

**Bioluminescent imaging (BLI):** Mice were injected intraperitoneally (IP) with 150 μl 40 mg/ml luciferin (Caliper Life Sciences, Hopkinton, MA) dissolved in PBS. Mice were anesthetized with 3% isoflurane-air mixture and transferred to the light-tight 37°C imaging chamber of an In Vivo Imaging System (IVIS; Caliper Life Sciences). BLI was performed 10 minutes after IP administration of luciferin. BLI was performed on mice with dorso-ventral positioning under anesthesia with 1.5% isoflurane-air mixture once per week for 4 weeks. The BLI signal intensity was analyzed using LivingImage software version 2.50 (Caliper Life Sciences) and was quantified serially by measurement of peak photon flux at the individual metastasis foci by selecting a region of interest (ROI) around the BLI signal.

**Faxitron radiography:** Radiographic images of mice were obtained using a Faxitron cabinet X-ray system (Hewlett-Packard, McMinnville, OR) at 45 kVp for 3.5 minutes at day 28.
**Histopathology:** Complete necropsies were performed on the mice. Bones were fixed in 10% neutral-buffered formalin at 4°C for 24 hours, decalcified in 10% EDTA (pH 7.4) for 2 weeks at 4°C, and embedded in paraffin. The specimens were sectioned (5 μm) and were stained with hematoxylin and eosin (H&E) to evaluate by histopathology or stained for tartrate-resistant acid phosphatase (TRAP) to identify active osteoclasts. TRAP staining was performed on non-stained sections that were deparaffinized by three 1 min washes with xylene (Hemo-De, Fisher Scientific, and Bay Shore, NY) and rehydrated in decreasing concentrations of ethanol (100%, 95% and finally 70%). For the effective staining of TRAP, antigen retrieval on the sections was performed using heat treatment at 60°C for 10 min in pre-heated antigen retrieval solution (Dako-Cytomation, Carpinteria, California USA) and then stained for TRAP (Acid Phosphatase Kit 387-A; Sigma Diagnostics, St. Louis, Missouri, USA) as directed by the manufacturer.

**Bone histomorphometry:** Bone histomorphometry was performed using computer software designed for histomorphometric analyses (Image Pro plus version 5.0; Media Cybernetics, Silver Spring, MD.). The number of large active osteoclasts (TRAP-positive osteoclasts with three or more nuclei) per millimeter of trabecular bone was measured along the tumor-bone interface in five different fields at 200X magnification for each bone. Trabecular volume in the metaphyses of long bones was measured in five different fields at 200X magnification for each
bone. Total tumor area in the medullary cavity of each bone was measured at 400X magnification. The terminology used to describe bone histomorphometric parameters was recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (22).

**Serum dickkopf-1 enzyme-linked immunosorbent assay (ELISA):** Serum DKK1 levels were measured using the DuoSet Human DKK1 ELISA Kit, as recommended by the manufacturer (R&D Systems, Minneapolis, MN, USA). The lowest standard of the assay was 62.5 pg/ml.

**Serum osteocalcin assay:** Serum mouse osteocalcin levels were measured using an immunoradiometric assay according to the manufacturer’s protocol (American Laboratory Products Company, Salem, NH). The polyclonal goat antibody used in this assay detects the mid-region and C-terminal portion of osteocalcin. The sensitivity of the assay is 0.1 ng/ml.

**Statistics:** Results were displayed as means ± standard error of mean (SEM). Data were analyzed using student’s T-test and multiple group comparisons were made by one-way ANOVA and Kruskal-Wallis test followed by Dunn’s post hoc test. Data with P values less than 0.05 were considered statistically significant. In ANOVA models used for the outcome variables, serum Dkk1 levels (Fig 15C) and serum osteocalcin (Fig 15A), Dunnet’s method was used to adjust for
multiple comparisons between control or vehicle groups. One observation from the “delayed” group was removed because statistical diagnostics indicated that it was highly influential and resulted in bad fits. For the outcome variable serum osteocalcin, the Bonferroni correction method was used to adjust for multiplicity for the 6 pairwise comparisons of interest. The statistical software SAS v.9 (Institute Inc., Cary, NC, and USA.) was used for all the analyses.

**RESULTS**

**Serial in vivo bioluminescent imaging (BLI) of Ace-1 tumor growth and metastasis incidence in nude mice:** To visualize and measure the effect of osteolysis on real-time tumor growth and incidence of metastasis in bone, Ace-1 prostate cancer cells stably expressing YFP-Luc reporter gene were injected into the left cardiac ventricle of nude mice. BLI of mice 10 minutes after intracardiac injection of Ace-1 cells revealed a bioluminescent signal over their entire bodies, which confirmed successful intracardiac injection of cancer cells. It was apparent from the signal intensity that tumor cells initially accumulated in the lungs, kidney, and brain immediately after intracardiac injection on day 0. However, metastases did not develop in these organs (Fig 11A). At day 7, the diffuse BLI signal over the entire body was gone. By day 14, bone metastases in control, prophylactic and delayed treatment groups were identifiable in the vertebrae, humeri, tibias
and femurs. There was no change in the number of metastatic foci during the course of the experiment, but there was increased intensity of bioluminescence signals at day 21 and 28 demonstrating progressive tumor growth in the bones. All mice in control, prophylactic and delayed treatment groups developed bone metastases over a period of 4 weeks after intracardiac injection of Ace-1 cells. Zoledronic acid (ZA) treatment had no significant effect on the BLI of tumor growth and incidence rate of metastases in prophylactic and delayed treatment groups compared to control mice (Fig 11B and C). The BLI signals correlated well with the metastases in bones as demonstrated by radiography and histopathology (see below).

**Faxitron radiography:** To characterize the Ace-1 bone metastases and confirm the metastatic sites observed by BLI, radiographs of all mice were taken on the day of sacrifice (day 28 after intracardiac inoculation of tumor cells) and representative radiographs are illustrated in Figure 12. In the tumor-bearing vehicle treatment group, the bone metastases had evidence of osteolysis characterized by loss of cortical and medullary bone in the metaphyses of long bones compared to intact cortical bone and radio-opaque medullary bone in the nontumor-bearing vehicle treatment group. In the delayed treatment group, intact cortical bone with mild osteolysis in the medullary region of the metaphysis was observed demonstrating inhibition of osteolysis by ZA. In the prophylactic treatment group, intact cortical bone with increased radio-opacity in the
metaphyseal regions of the long bones demonstrated inhibition of osteolysis and the osteoblastic nature of the Ace-1 tumors following inhibition of osteolysis by ZA.

**Histopathology:** Based on BLI and radiography, we selected 13, 12 and 7 long bones from the control, prophylactic and delayed treatment groups, respectively, and stained sections from the bones with H&E. In the control group, the prostate carcinoma cells induced woven bone formation in the metaphyses adjacent to the neoplastic cells demonstrating their osteoblastic phenotype (Fig 13A). There were multiple areas of cortical bone resorption caused by tumor-induced increased osteoclast activity along the cortical endosteum. In some of the bones there was reactive new bone formation in the periosteum adjacent to the tumor. In ZA-treated mice, Ace-1 metastases extended from the growth plate to the diaphysis and filled the marrow cavities in between metaphyseal trabeculae (13A and 13B). To determine the effect of ZA treatment on tumor-induced bone formation, we measured metaphyseal trabecular thickness adjacent to intramedullary metastases. Trabecular thickness adjacent to metastases was significantly (P<0.001) increased in all tumor-bearing mice compared to contralateral bones in non-tumor bearing mice. ZA had no effect on tumor-induced trabecular thickness in treatment groups compared to the control group (13C). Histomorphometry demonstrated that ZA had no significant effect on the tumor varea (Fig 13D).
TRAP staining was performed on the bone sections to identify the active osteoclasts. Numerous TRAP-positive osteoclasts (with three or more nuclei) were observed along the tumor-bone interface in vehicle-treated mice compared to few active osteoclasts in the mice treated with ZA (Fig 14A). Bone histomorphometry was performed on the TRAP-stained bone sections to quantify the effect of ZA on the osteoclast activity at the tumor-bone interface. ZA decreased TRAP-positive osteoclasts by 5-6-fold in tumor-bearing mice, as compared to vehicle-treated tumor-bearing mice (Fig 14B).

**Bone remodeling indices:** Our results showed that ZA inhibited the prostate cancer-induced osteolysis. To determine and compare the effect of ZA on bone remodeling in healthy mice and in tumor-bearing mice, we measured serum osteocalcin following treatment of mice for 4 weeks. ZA treatment significantly (P< 0.0001) decreased serum osteocalcin concentrations in nontumor-bearing mice. In contrast, ZA treatment did not reduce serum osteocalcin in tumor-bearing mice (15A). Bone histomorphometric analysis showed that ZA treatment of healthy mice for 4 weeks increased the trabecular volume by 2-fold (15B) compared to the vehicle-treated mice. The decreased serum osteocalcin and increased trabecular bone volume in ZA-treated healthy mice demonstrated the effect of ZA on the inhibition of normal physiological bone remodeling.

It has been shown that Dkk1 promotes osteolysis and inhibits osteoblast differentiation by antagonizing the Wnt signaling pathway in prostate cancer bone
metastases (23, 24). To determine the role of Dkk1 in this study, we measured serum Dkk1 concentrations. Dkk1 concentrations in tumor-bearing mice (avg = 4.17 ng/ml) were significantly increased compared to nontumor-bearing mice (avg = 1.9 ng/ml). ZA significantly decreased Dkk1 concentrations in the prophylactic treatment group but had no effect in delayed treatment mice as compared to vehicle-treated mice (15C). This could be due to the long duration of ZA treatment in the prophylactic treatment compared to the delayed treatment. These data suggest that ZA-mediated inhibition of Dkk1 might contribute to the decreased osteolysis.

DISCUSSION

Prostate cancer metastases to bone are typically characterized by a predominance of new woven bone formation with a lesser amount of osteolysis (‘osteoblastic’ metastases) (23). The contribution of osteolysis in the development of osteoblastic lesions in prostate cancer bone metastases is not well understood partially due to a lack of preclinical models that consistently develop mixed osteoblastic and osteolytic lesions (24-26). This study showed that bone metastasis and osteoblastic lesions are independent of osteolysis in a nude mouse model of canine prostate cancer-induced mixed bone metastases.

Studies have shown that normal bone homeostasis is maintained by the balanced coupling of bone resorption and bone formation (27). However, the
importance of the bone remodeling sequence in the development of prostate cancer bone metastasis is unknown. Metastasis of prostate cancer cells to bone disrupts the balanced coupling of bone remodeling. Although bone formation and bone resorption are both increased, bone formation is favored and the remodeling process becomes unbalanced, resulting in a net gain of bone. Many studies have reported that both osteoclast and osteoblast activity are important for prostate cancer bone metastases (13). Previously, the lack of preclinical models that recapitulate the sequential course of mixed bone metastases has prevented investigations on the in vivo significance of osteoclast activity in the development of osteoblastic lesions (23, 28-31). We have developed a canine prostate cancer cell line (Ace-1) that develops mixed bone metastases in nude mice (7, 8). This model allows studies on the sequential biological events involved in the development of prostate cancer bone metastases.

To investigate the role of osteoclastic bone resorption in the development of osteoblastic metastases, we inhibited osteoclastic-mediated bone resorption in Ace-1 tumor-bearing mice using zoledronic acid (ZA). As expected, radiographic and histopathological findings showed that ZA markedly inhibited osteolysis of cortical and trabecular bone when compared to the control group, regardless of the administration schedule. Decreased number of TRAP-stained osteoclasts and decreased serum Dkk1 levels in the ZA-treated groups additionally supported this observation. Dkk1 was shown to decrease osteoblast maturation and mineralization in vitro and has the potential to switch the phenotype of bone
metastasis from osteoblastic to osteolytic (32). In the bone microenvironment, bone marrow mesenchymal stem cells (BMMSC) are the source of DKK1 (33). The exact mechanism of ZA inhibition on DKK1 is not clear. Based on the findings in this study that ZA had no effect on the incidence of prostate cancer metastasis to bone or growth of bone metastases, but had decreased DKK1 levels, we speculate that prostate cancer metastasis to bone stimulated DKK1 expression in BMMSC. ZA inhibited the tumor-mediated stimulation of DKK1 expression by BMMSC. Decreased Dkk1 levels might be one of the potential factors contributing to the decrease in osteolysis and increased bone formation in this model. The ability of ZA to inhibit osteolysis in our study was consistent with previous reports (16-18). ZA was less effective in the delayed treatment group compared to the prophylactic group, suggesting that preventive therapy may be more effective to treat patients with osteolytic metastases in bone. On the other hand, the increased efficacy of prophylactic treatment in this study might be attributed to the longer duration of treatment (5 weeks) compared to shorter (2 weeks) administration in the delayed treatment group.

Recent studies have documented the ability of in vivo bioluminescent imaging (BLI) to measure tumor progression and response to therapy in animal models (34). The BLI data revealed that ZA treatment had no significant effect on the incidence of bone metastases or the rate of metastatic tumor growth. Furthermore, analysis of tumor area using histomorphometry revealed no significant difference between treatment and control groups despite the
significant inhibition of osteolysis. Our data is in agreement with the report from Saad et al. that showed ZA had no effect on tumor progression and survival rate of prostate cancer patients (35). Lee at al showed that ZA was effective in inhibiting bone resorption induced by the prostate cancer cell line (PC-3); however, there was no effect on osteoblastic lesions induced by the LAPC-9 prostate cancer cell line (16). In contrast, Corey et al demonstrated that ZA inhibited tumor progression and osteoblastic lesions in an experimental mouse model with LuCaP 23.1 prostate cancer cells (17). These findings suggest the ability of ZA to inhibit tumor progression and osteoblastic metastasis depends on the specific biology of the prostate cancer cell line evaluated. Variation in the nature of different cell lines can be attributed to the heterogeneity of the primary prostate cancer from which cell lines are derived. Our findings demonstrate that the osteoclastic component of Ace-1-induced bone metastases were not necessary for the survival and growth of tumor in bone or development of osteoblastic metastases.

Osteocalcin, which is secreted by osteoblasts, is a bone turnover marker because the osteoblast-secreted osteocalcin is deposited in bone matrix and released during bone resorption (36, 37). ZA significantly reduced serum osteocalcin in nude mice, indicating a reduction in bone turnover. This finding was consistent with previously published work showing that ZA reduced osteocalcin concentrations in patients with prostate cancer bone metastases (38, 39). There was no significant difference in serum osteocalcin levels in Ace-1-
bearing control mice compared to control nontumor-bearing mice. Koizumi et al previously showed that serum osteocalcin concentrations were similar in human patients with and without prostate cancer bone metastases (28, 40). This correlated with our findings and suggested that osteocalcin is not a useful marker to identify bone metastases. Serum bone specific alkaline phosphatase, secreted by osteoblasts, is a specific and reliable bone formation marker (41). We were unable to measure the bone specific alkaline phosphatase concentrations in the mice due to the lack of assays that detect this marker in mice.

Many studies have reported that osteoclast and osteoblast regulatory factors expressed by prostate cancer cells can alter bone homeostasis either directly or indirectly. Prostate (PC-3) and breast (MDA-231) cancer cell lines that induce osteolytic bone metastases express cytokines that include RANKL, interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), PTHrP and cathepsin K, which are associated with increased osteoclastogenesis. In contrast, C4-2B and LAPC-9 cells (prostate cancer) and ZR-75-1 and MCF-7 cells (breast cancer), which induce osteoblastic metastases, express abundant osteoprotegerin (OPG), bone morphogenic protein-2 (BMP-2), BMP-4, BMP-6, vascular endothelial growth factor (VEGF), endothelin-1 (ET-1), platelet derived growth factor-BB (PDGF-BB), insulin growth factor-1 (IGF-1) and fibroblast growth factor-2 (FGF-2), which are known to stimulate osteoblast activity (16, 24, 42-45). Yin et al showed that ET-1 production in ZR-75-1 cells stimulated osteoblast activity resulting in increased new bone formation (46). PDGF-BB, VEGF and urokinase
plasminogen activator (uPA) also contributed to the increased bone formation in prostate and breast cancer bone metastases (24, 47, and 48). PCR analysis revealed that Ace-1 cells express a wide variety of factors known to stimulate both osteoclasts (RANKL, IL-6, cathepsin K, PTHrP) and osteoblast activity (PDGF-BB, ET-1, VEGF, uPA, OPG, FGF-2 and IGF-1) (unpublished data). The cytokine expression profile of Ace-1 cells suggests that multiple factors contribute to the osteoblastic and osteolytic phenotypes at metastatic sites. However, further investigations will be required to understand the role various cytokines in the development of mixed bone metastases in this model. In this regard, the Ace-1 model will be a very useful translational model to study the pathogenesis and treatment of prostate cancer bone metastasis.
REFERENCES


FIGURE LEGENDS

**Figure 10:** Zoledronic acid (ZA) treatment protocol: Nude mice were divided into three groups: the control group received PBS from -1 to 4 wks (top line). The prophylaxis group received ZA from -1 to 4 wks (middle line). The delayed treatment group received ZA from 2 to 4 wks (bottom line). Mice were administered 100μg/kg ZA B.W., twice a week subcutaneously (SQ) to their respective groups. Ace-1^YFP-LUC^ cells were injected on week 0.

**Figure 11:** Effect of ZA on the Ace-1 tumor growth and metastasis incidence in nude mice was monitored using bioluminescence imaging (BLI): (A) Representative images of serial BLI of Ace-1 tumor progression from control, delayed treatment and prophylaxis groups taken at 0, 7, 14, 21 and 28 days, after intracardiac injection of cancer cells. On day 0, immediately after intracardiac injections, ACE-1 cells were present throughout the entire body and accumulated in the kidney, liver and brain. On day 7, BLI signals were gone. BLI signals were detected on day 14 at various sites of bone metastasis. At day 21 and 28, the intensity of BLI signals increased, which demonstrated progressive growth of the metastases. BLI on days 21 and 28 did not reveal any new metastatic sites compared to day 14. Panel (B) Graph represents the average...
intensity of the BLI signal measured at each metastatic region of interest (ROI) per group at the specific time points. Intensity of BLI was measured using LivingImage software Version 2.50. Panel (C) Graph shows the average number of metastases per group. Each region of interest was counted as one metastatic site.

**Figure 12:** Radiographic evaluation of ZA on Ace-1 metastases to long bones: Radiographs of mice were taken 28 days after injection of Ace-1 tumor cells into the left cardiac ventricle. Cortical and trabecular bone lysis (*thin arrows*) was observed in the metaphyseal regions of long bones of mice that received Ace-1 cancer cells and vehicle (C) compared to mice that received vehicle alone (A). ZA treated nontumor-bearing group (B) had mild increase in radio-opacity in the proximal metaphysis of long bones compared to vehicle treated nontumor-bearing group (A). Mild trabecular bone loss and intact cortices were present in the delayed treatment group (D), whereas in the prophylactic group (E), intact cortices and increased radio-opacity of bone (*thick arrow*) was present in the metaphyseal region.

**Figure 13:** Histopathological evaluation of ZA on Ace-1 bone metastases: Mice were sacrificed 28 days after injection of Ace-1 cells into left cardiac ventricle.
Panel A&B, H&E stained sections of long bones. In all tumor-bearing mice (a-f), Ace-1 cells (T) replaced bone marrow (BM) cells in the metaphyseal region of the long bones. In tumor-bearing mice that received vehicle (a,d), there was cortical and trabecular bone lysis (thin arrow) and new woven bone production (thick arrows) in the medullary cavity adjacent to the tumor. In prophylactic and delayed treatment groups (b,c,e,f), intact cortices and new woven bone formation (thick arrow) characterized by thickened trabeculae was present adjacent to Ace-1 cells in the metaphyseal region. Increased trabecular density (curved arrow) was present in tumor-bearing (b,c) and nontumor-bearing (h,j) mice that received ZA compared to the mice that received vehicle alone (g,i). Panel C, histomorphometric analysis showed that trabecular thickness in long bones adjacent to metastases was significantly greater than contralateral bones without metastases (D). * = p<0.001 (t-test) compared to their respective contralateral group and data represent the mean ± SEM. Panel D shows a vertical dot plot of the individual values of tumor area from each long bone that was quantified using Image Pro-Plus software.

**Figure 14:** Effect of ZA on tartrate-resistant acid phosphatase (TRAP) activity of osteoclasts in Ace-1 tumor-bearing mice: TRAP-stained sections of bones demonstrated numerous red TRAP-positive osteoclasts (thick head arrow) along
the tumor (T)-bone interface in Ace-1-bearing mice compared to few osteoclasts in ZA-treated mice (A, top panels). Higher magnification of TRAP-positive osteoclasts demonstrated multiple nuclei (thin head arrow) (A, lower panels). Histomorphometric analysis showed a significant decrease in TRAP-positive osteoclasts with three or more nuclei along the tumor-bone interface between control and ZA-treated groups (B). * = P< 0.01 (ANOVA and Dunn’s test for post hoc analysis). Data represent the mean ± standard error of mean (SEM)

**Figure 15:** Effect of ZA on trabecular bone, Dkk1 and osteocalcin: In nontumor-bearing mice, ZA significantly decreased serum osteocalcin compared to mice that received vehicle alone. In tumor-bearing mice, ZA had no significant effect on osteocalcin concentrations (A). * = p<0.0001 compared to vehicle alone group. Histomorphometric analysis revealed that ZA significantly increased the trabecular bone volume in nontumor-bearing mice (B). * = P<0.05, (t-test) and data represent the mean ± SEM. ZA decreased the serum Dkk1 concentrations in tumor-bearing mice in the prophylactic treatment group (C). * = P<0.05 (Dunnett’s simultaneous tests) compared to vehicle-treated tumor-bearing mice (control) and data represent mean ± SEM.
Figure 10: Zoledronic acid treatment protocol

Ace-1<sub>YFP-LUC</sub>

**Intracardiac injection**

- **Control**
  - (n=11) Vehicle (PBS), SQ, twice a week

- **Prophylaxis**
  - (n=9) ZA 100µg/kg body weight, SQ, twice a week

- **Delayed Treatment**
  - (n=7) ZA 100µg/kg SQ, twice a week
Figure 11: Effect of ZA on the Ace-1 tumor growth and metastasis incidence in nude mice was monitored using bioluminescence imaging (BLI)
Figure 12: Radiographic evaluation of ZA on Ace-1 metastases to long bones
Figure 13: Histopathological evaluation of ZA on Ace-1 bone metastases
Figure 14: Effect of ZA on tartrate-resistant acid phosphatase (TRAP) activity of osteoclasts in Ace-1 tumor-bearing mice
Figure 15: Effect of ZA on trabecular bone, Dkk1 and osteocalcin
CHAPTER 3

OVEREXPRESSION OF DICKKOPF-1 (DKK-1) STIMULATED PROSTATE CANCER GROWTH AND INHIBITED BONE FORMATION IN OSTEOSTATIC BONE METASTASES

ABSTRACT

Osteoblastic bone metastasis is the predominant phenotype observed in prostate cancer patients and associated with high patient mortality and morbidity. However, the mechanisms determining the development of this phenotype are not well understood. Prostate cancer cells secrete several osteogenic factors including Wnt proteins, which are not only osteoinductive but also oncogenic. Therefore, the purpose of the study was to investigate the contribution of the Wnt signaling pathway in prostate cancer growth, incidence of bone metastases and osteoblastic metastases by overexpressing Wnt antagonist, Dkk-1, in Ace-1 prostate cancer cells that develop mixed osteoblastic and osteoclastic bone metastases.

Stable Ace-1 cells expressing human Dkk-1 or empty vector were established and transduced with lentiviral yellow fluorescent protein (YFP)-
luciferase (Luc). The Ace-1/vector\textsuperscript{YFP-LUC} and Ace-1/Dkk-1\textsuperscript{YFP-LUC} cells were injected in subcutaneous, intracardiac and intratibial sites in the nude mice.

Unexpectedly, Dkk-1 increased Ace-1 subcutaneous tumor weights significantly and increased the incidence of bone metastases. Dkk-1 increased Ace-1 tumor growth by increasing phospho46 JNK via the noncanonical pathway. As expected, Dkk-1 decreased the Ace-1 osteoblastic phenotype of bone metastases, as confirmed by radiographical, histopathological, and microcomputer tomographical analysis. Dkk-1 decreased osteoblastic activity by the Wnt canonical pathway since it inhibited T-cell factor (TCF) activity in murine osteoblast precursor ST2 cells.

The present study showed that Dkk-1 is a potent inhibitor of bone growth in prostate cancer-induced osteoblastic metastases. However, its paradoxical effect on increased prostate cancer growth through Wnt noncanonical signaling warrants more studies to clearly delineate the role of Wnt signaling in prostate cancer.

**INTRODUCTION**

Prostate cancer patients frequently develop bone metastases in late stages of the disease (1). Most of the bone metastases are osteoblastic resulting in excessive, but poor quality, bone formation (2,3). Osteoblastic metastases result in spinal cord compression, pathological fractures and pain resulting in severe
morbidity of patients (4). Using the Ace-1 model we previously showed that prostate cancer directly induced new bone formation in nude mice (5). Mechanisms responsible for prostate cancer osteoblastic bone metastases are not clearly defined (6).

Prostate cancer produces Wnt proteins, which were shown to possess tumorigenic and osteogenic potential (7,8). Wnts are a large family of soluble proteins that contribute to normal development and embryogenesis (9). However, disregulated Wnt signaling has been implicated in the development of multiple cancer types, including prostate cancer (10). Additionally, several Wnts were reported to be upregulated in cancers resulting in increased growth (11-13). Wnts act through both a canonical and noncanonical pathway (14).

The canonical Wnt pathway acts by binding to low density lipoprotein receptor protein (LRP) and frizzled receptors on the target cells to induce Wnt signaling (15). Wnt signaling disintegrates the β-catenin destruction complex consisting of axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β), which phosphorylates β-catenin for further ubiquitination and proteosomal degradation in the cytoplasm (8). Therefore, in the presence of Wnts, β-catenin is stabilized and translocated into the nucleus where it binds to, and activates, T-cell factor (TCF). TCF acts on downstream target genes that are important for cell survival, proliferation and angiogenesis (16). Consequently, upregulation of the Wnt signaling pathway was shown to promote tumor survival and progression (17).
Wnts are important for the development and maintenance of bone by inducing the differentiation and maturation of precursor osteoblasts to active osteoblasts (18). Secretion of Wnts in prostate cancer was also shown to play an important role in the development of osteoblastic metastases (7). Dickkopf-1 (Dkk-1) antagonizes Wnt by binding to the LRP receptor on the target cells, thus resulting in internalization of the receptor and inhibition of Wnt-mediated canonical signaling (15). Dkk-1 has been reported to be downregulated in prostate cancer patients in advanced stages (19). Moreover, Dkk-1 was overexpressed in breast cancer patients who develop predominantly osteolytic bone metastases (15,20).

Therefore, the hypothesis for this study was that inhibition of Wnt signaling by Dkk-1 will decrease both prostate cancer growth in an autocrine manner and prostate cancer-induced osteoblastic metastases in a paracrine manner. To test this hypothesis, Ace-1 prostate cancer cells that develop mixed osteoblastic and osteolytic bone metastases were transfected to overexpress Dkk-1. In this study, the functional role of overexpression of Dkk-1 on prostate cancer growth, incidence of bone metastases and osteoblastic phenotype of bone metastases were investigated.
MATERIALS AND METHODS

**Cell culture:** Ace-1 cells were maintained at 37°C in Dulbecco’s Modified Eagle’s Medium/Ham’s Nutrient Mixture F12 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum, 250 U/mL penicillin, 250 µg/mL streptomycin, and 2 mM L-glutamine (Invitrogen Corp.) in a 5% CO₂-humidified chamber.

**Establishment of Ace-1 cells stably expressing the human Dkk-1 and yellow fluorescent protein-luciferase (YFP-Luc) reporter gene:** Ace-1 cells were transfected with 10 µg of pcDNA3.1(+)/Dkk-1 (Ace-1-Dkk-1) or empty vector (Ace-1-Vector) under control of the CMV promoter and 10 µL of Lipofectamine 2000 (Invitrogen Corp.). Stably integrated cells were selected using 2000 µg/mL of G418 (Sigma-Aldrich Co., St. Louis, MO) for 1 month. Ace-1-Dkk-1 and Ace-1-Vector stable cells were transduced with the retroviral YFP-Luc dual reporter gene and 8 µg/mL polybrene by spin inoculation at 2700 rpm for 1 hour at 32°C as described previously (19). The resulting cells were named Ace-1-Dkk-1<sup>YFP-Luc</sup> and Ace-1-Vector<sup>YFP-Luc</sup>, respectively.

**Dkk-1 ELISA:** Dkk-1 concentrations were measured using the DuoSet Human DKK1 ELISA Kit as described previously (5) (R&D Systems, Minneapolis, MN, USA). The lowest standard of the assay was 62.5 pg/mL.
Inoculation of Ace-1 cells into nude mice: Subcutaneous, intracardiac and intratibial injections of Ace-1-Vector<sup>YFP-Luc</sup> cells and Ace-1-Dkk-1<sup>YFP-Luc</sup> cells were performed in six-week-old male nu/nu mice (National Cancer Institute, Frederick, MD) under general anesthesia using a 3% isoflurane (Abbott Laboratories, North Chicago, IL)-oxygen mixture. A total of 5x10<sup>5</sup> Ace-1-Vector<sup>YFP-Luc</sup> cells or Ace-1-Dkk-1<sup>YFP-Luc</sup> suspended in 250 µL of sterile Dulbecco’s phosphate-buffered solution (PBS) (Invitrogen Corp) were injected subcutaneously (N=12 per group) over the dorsal scapular area of the mice. Left cardiac ventricular (N=12 per group) and intratibial (N=15 per group) injections were performed with 1X10<sup>5</sup> tumor cells suspended in 100 uL of PBS. Mice with subcutaneous, intracardiac and intratibial injections were sacrificed at 6 weeks, 4 weeks and 19 days, respectively.

Bioluminescent imaging: Bioluminescent imaging was completed with the IVIS 100 in vivo imaging system (Caliper Life Sciences, Hopkinton, MA) as previously described (21). The photon signal intensity was quantified using LivingImage software version 2.50 (Caliper Life Sciences)

Radiography: Radiographic images of mice were obtained immediately after sacrificing on day 19 using a Faxitron laboratory radiography system LX-60 (Faxitron X-ray Corp., Wheeling, IL) at 30 kVp for 10 sec.
**Histopathology:** Complete necropsies were performed on mice 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, sectioned (5 µm) and stained with hematoxylin and eosin (H&E) or evaluated for tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts (Sigma-Aldrich, St. Louis, MO) as previously described (5). Bone histomorphometry was performed using the Image-Pro Plus, ver 6.3 software (Media Cybernetics, Silver Spring, MD.). Tibial trabecular bone area, new woven bone area, endosteal eroded surface and endosteal surface covered with new woven bone were measured.

**Microcomputed tomography (µCT):** Nineteen days after inoculation of Ace-1 cells, the mice were sacrificed, tibial bones were carefully demuscled and fixed in 10% neutral-buffered formalin at 4°C for 24 hr. Formalin was replaced with 70% ethyl alcohol and transferred to 1.7 mL microcentrifuge tube for high resolution µCT scanning (Siemens Corporation., New York, NY). Tibial bones were scanned at binning 2, 80 kV energy, 500 mA current for a total of 400 projections. Tibial images were reconstructed and analyzed for morphometry using Inveon research workplace software (IRW) (Siemens). Trabecular bone volume in the metaphyseal region and new woven bone volume in the diaphyseal region of
tibial bones between the Ace-1-vector and Ace-1-Dkk-1 groups were quantified using the IRW software.

**Western blotting:** Total protein was extracted from subcutaneous tumors using M-per reagent (Pierce, place). Approximately 50 μg of protein lysates were separated on 4-12% tris-glycine SDS-PAGE gels (Invitrogen). Proteins were transferred to a nitrocellulose membrane and probed with primary antibodies for Dkk-1 (R&D Systems), β-catenin (Abcam, Cambridge, MA) and β-actin (sigma), total c-Jun amino-terminal kinase (JNK) and phospho-JNK (Cell Signaling Technology, Inc. Danvers, MA) followed by incubation with horseradish peroxidase-conjugated secondary antibody goat anti-rabbit (Promega, Madison, WI) or goat anti-mouse (Abcam). The signal was detected by chemiluminescent ECL Plus™ western blotting detection reagent (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

**RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR):** Total RNA was extracted from Ace-1 cells, using Trizol reagent (Invitrogen). Total RNA (1 μg) was reverse transcribed using the Superscript II First Strand cDNA synthesis kit (Invitrogen) and PCR was performed on the cDNA for the genes Dkk-1, Wnt4, Wnt5, Wnt7, LRP5, LRP6 and beta2microglobulin (β2M) using canine-specific primers as shown in table 7.
**Transfections:** To investigate the effect of Ace-1 cells on T-cell factor (TCF) reporter transcriptional activity in murine bone marrow osteoblast precursor cells (ST2), approximately 80-90% confluent ST2 cells in 6-well plates in triplicate were transfected with 1 μg of either wild-type pGL2/TCF/Luc (Top), mutant pGL2/TCFP2/Luc (FOP) constructs using 5 μL of Lipofectamine 2000 reagent (Invitrogen Corp.) for 6 hours. Then medium was removed, washed with PBS and replaced with fresh medium. Later, 1X10^4 cells of either Ace-1-Vector or Ace-1-Dkk-1 lines were added to the ST2 cells transfected with TOP or FOP vector and co-cultured for 48 hours. The plasmid pβgal-Vector (200 ng) was included in each transfection reaction to correct for transfection efficiency. Luciferase activity of ST2 cells was measured with the luciferase assay system (Promega) using 20 μL of lysate. β-galactosidase activity was measured with the luminescent β-galactosidase detection kit II (BD Biosciences, Palo Alto, CA) and β-galactosidase activity was used to normalize for transfection efficiency. Finally, wild-type TOP luciferase reporter transcriptional activity was normalized with the mutant (FOP) luciferase reporter transcriptional activity.

**Statistics:** All groups with normally distributed variables, unless stated otherwise, were compared by two-tailed student’s t-tests. For non-parametric data with two groups, Mann-Whitney rank sum t-tests were performed (Fig 2A). For comparison of multiple groups, one-way ANOVA was used followed by Tukey’s post hoc test to perform pairwise comparisons (Fig 8A) using SigmaPlot
software Ver 11.0 (Chicago, IL). Results were displayed as means ± standard deviations (SD). Data with P values < 0.05 were considered statistically significant.

RESULTS

Ace-1 prostate cancer cells expressed Wnt genes and its receptors:
Because Wnts play an important role in oncogenesis and osteogenesis, we evaluated the expression of Wnt genes in Ace-1 cells by RT-PCR using dog-specific primers. Ace-1 cells expressed mRNAs for Wnt 4, Wnt 5A, and Wnt 7 (Fig 16A). Ace-1 cells also expressed Dkk-1, LRP 5 and LRP 6 receptors (Fig 16A). Although Ace-1 cells expressed Dkk-1 mRNA, secreted Dkk-1 protein was below the level of detection using the human ELISA kit. Ace-1 cells were transfected with human Dkk-1 resulting in Ace-1-DKK-1 cells stably expressing human Dkk-1. The Ace-1-Dkk-1 cells produced approximately 10 ng/mL/10^5 cells of Dkk-1 at 72 hours as detected by ELISA (Fig 16B).

Effect of Dkk-1 on Ace-1 subcutaneous growth and incidence of bone metastases: To measure the effect of Dkk-1 on Ace-1 tumor growth subcutaneous injections were performed with Ace-1-VectorYFP-Luc or Ace-1-Dkk-1YFP-Luc cells in nude mice. After 6 weeks, mice were sacrificed and tumors were weighed. There was a 3.5-fold (p<0.002) increase in tumor weight in Ace-1-Dkk-
compared to the Ace-1-Vector<sup>YFP-Luc</sup> group (Fig 17A). To evaluate the effect of Dkk-1 on bone metastasis incidence, left ventricular intracardiac injections were performed with Ace-1-Vector<sup>YFP-Luc</sup> or Ace-1-Dkk-1<sup>YFP-Luc</sup> cells and incidence of bone metastases was measured by bioluminescent imaging on day 14, 21 and 28. The Ace-1-Dkk-1<sup>YFP-Luc</sup> cells had significantly (p<0.05) greater number of bone metastases compared to Ace-1-Vector<sup>YFP-Luc</sup> cells (Fig 17B).

**Role of Dkk-1 in Ace-1 autocrine canonical and noncanonical signaling:** To confirm the expression of Dkk-1 in Ace-1-Dkk-1<sup>YFP-Luc</sup> tumors, total protein lysates were extracted and immunoblotted with antibodies to DKK1. As shown in Fig. 18A, Ace-1-Dkk-1<sup>YFP-Luc</sup> cells had marked Dkk-1 protein expression and no human Dkk-1 was detected in the Ace-1-Vector<sup>YFP-Luc</sup> tumors. Dkk-1 is shown to act primarily by interacting with LRP5/6 receptors to inhibit Wnt signaling. In order to assess the functional significance of the Wnts in Ace-1-Dkk-1<sup>YFP-Luc</sup> tumor growth, we measured the expression of β-catenin, which plays an important role in Wnt canonical signaling. (Fig 18A). Interestingly, as shown in Fig. 18A and 18B, there was no significant difference in the levels of β-catenin in the Ace-1-Dkk-1<sup>YFP-Luc</sup> tumors compared to Ace-1-Vector<sup>YFP-Luc</sup> tumors. β-actin was used as a loading control for protein and β-catenin expression was normalized to β-actin in Fig 18B.

Because phosphorylation of JNK is one of the functional consequences of Wnt noncanonical signaling, we measured phosphorylated JNK in Ace-1-Dkk-
1YFP-Luc tumors compared to Ace-1-VectorYFP-Luc tumors. As shown in Figs 18C and E, there was a significantly (P<0.001) greater level of phospho46 (P46) JNK isoform in the Ace-1-Dkk-1YFP-Luc tumors compared to Ace-1-VectorYFP-Luc. Whereas the levels of phospho54 (P54) isoform were slightly higher in Ace-1-Dkk-1YFP-Luc tumors compared to Ace-1-VectorYFP-Luc tumors, the difference was not significant (Figs 18C and 3E). Densitometry ratios in Figs 18E and F were normalized to total JNK protein expression.

Radiographic evaluation of Dkk-1 on Ace-1 osteoblastic metastases: To analyze the effect of Dkk-1 on the Ace-1-induced osteoblastic metastases, radiographs of all tibial bones were made on day 19 after intratibial injection. In the Ace-1 Vector group, there was increased radio-opacity in the medullary region of the diaphysis compared to the contralateral, uninjected tibia (Fig 19). In contrast, Dkk-1 overexpressing intramedullary tumors had marked loss of cortical and trabecular bone in the metaphyses and diaphyses of the tibias (Fig 19) compared to the Ace-1-vector group. This demonstrated that Dkk-1 inhibited the Ace-1-induced new bone formation in the intratibial model of bone metastases.

Histopathologic evaluation of intratibial osteoblastic metastases: To investigate the effect of Dkk-1 on the osteoblastic phenotype of bone metastases, H&E-stained sections of the tibias that were injected with Ace-1 cells from both groups were examined 19 days after the injection. Intratibial injections
of Ace-1 cells resulted in growth of tumor cells from the growth plate to the diaphyseal regions replacing most of the bone marrow. Since Ace-1 cells typically result in mixed (osteoblastic/osteolytic) metastases, mild trabecular bone lysis was present in the metaphyses of the vector group. Additionally, the Ace-1 vector group demonstrated marked tumor-induced new woven bone formation along the endosteal surface of the tibia and in the medullary region of diaphysis resulting in an osteoblastic phenotype of bone metastasis (Fig 20A (a and b)). In contrast, in the Dkk-1 overexpressing group, marked lysis of trabecular bone and tumor-induced resorption of the cortical endosteum was present demonstrating that Ace-1 cells overexpressing Dkk-1 significantly inhibited the Ace-1-induced osteoblastic phenotype (Fig 20A (c and d)). Histomorphometric analysis showed that the trabecular bone volume in tibias with Dkk-1 overexpressing Ace-1 cells was significantly decreased (approximately 4-fold) compared to the vector group (Fig 20B). Also, the new woven bone surface area was approximately 3-fold reduced in the Ace-1-DKK1 group compared to Ace-1-Vector group (Fig 20C).

**Dkk-1 decreased tibial endosteal eroded surface and decreased new woven bone formation:** To investigate whether Dkk-1 altered Ace-1-induced new woven bone surface and resorption surface along the tumor-tibial endosteal interface, we measured the length of tibial endosteal surface covered by new woven bone and eroded surfaces. Ace-1 cells overexpressing Dkk-1 significantly increased the tibial endosteal eroded surface by 2-fold compared to Ace-1-Vector
group (Fig 21A). In addition, the Dkk-1 group had markedly decreased new woven bone along (3-fold) the endosteal surface compared to the vector group (Fig 21B).

**Microcomputed tomographic evaluation of Dkk-1 on Ace-1 tibial metastases:** Tibial bones were scanned by microcomputed tomography after fixing in neutra-buffered formalin for 24 hours (Fig 22A). The effect of Dkk-1 on Ace-1-induced trabecular and new woven bone volume was measured using Inveon research workplace software. Analysis of the µCT images showed that Dkk-1 decreased trabecular bone volume approximately 6-fold (Fig 22B) and new woven bone by 5-fold (Fig 22C) compared to Ace-1-vector group.

**Role of Dkk-1 on paracrine canonical pathway in mouse preosteoblast ST2 cells:** Since Dkk-1 overexpression markedly inhibited the Ace-1-induced osteoblastic phenotype of bone metastasis, the effect of Dkk-1 on canonical signaling in mouse bone marrow osteoblast precursor cells (ST2) was tested. In Wnt canonical pathway, β-catenin binds to T-cell factor (TCF) and activates it for further transcriptional targeting of the Wnt-mediated pathway. Since TCF activity plays an important role in the canonical Wnt pathway, we measured the TCF activity in ST2 cells as an indicator of canonical Wnt signaling. Co-culture of ST2 and Ace-1-Vector cells resulted in a significant increase in the canonical TCF reporter transcriptional activity in ST2 cells. However, co-culture of ST2 cells with
Ace-1-Dkk-1 cells inhibited the Ace-1-induced TCF activity in ST2 cells (Fig 23). This indicated that Ace-1 cells induced osteoblast activity through canonical Wnt signaling and this pathway was inhibited by Dkk-1 in osteoblasts.

**DISCUSSION**

In the present study we have shown that Ace-1 prostate cancer cells express Wnts and Wnt receptors LRP5 and LRP6. Overexpression of human Dkk-1 in Ace-1 cells resulted in increased subcutaneous tumor growth and incidence of bone metastasis, but significantly decreased the osteoblastic phenotype of bone metastases.

Dkk-1 has been shown to increase cell proliferation and tumor growth in myeloma and HeLa cells (2,3). As with published data, Dkk-1 overexpression resulted in increased growth of subcutaneous tumors in mice. Overexpression of Dkk-1 also increased the incidence of bone metastases following intracardiac injection of Ace-1 cells. Dkk-1 was significantly increased in breast cancer patients with bone metastases compared to patients with non-bone metastases and healthy women (20). Therefore, the results of this investigation indicate that Dkk-1 may contribute to the increased incidence of bone metastases as reported with breast cancer.
Wnts act through both canonical and noncanonical signaling pathways (14). In the canonical pathway, Wnts function by binding to low density lipoprotein receptors (LRP) and frizzled receptors on the target cells to mediate Wnt signaling through β-catenin (15). In our study there was no upregulation of β-catenin in the Ace-1-Dkk-1 cells compared to the Ace-1-Vector-1 cells indicating that the signaling did not occur using the canonical pathway. In human prostate cancer, dysregulation of factors involved in the canonical Wnt pathway such as APC gene mutations (22) downregulation or absence of GSK3β expression (23), axin gene mutations (24) and β-catenin mutations at phosphorylation sites important for ubiquitination and further degradation (25) were reported. In addition, mutated forms of LRP5 or LRP6 receptors were also shown to elicit constitutive canonical Wnt signaling (26,27). Dysregulation of any of the pathway constituents could be the cause for the absence of β-catenin induction in the Ace-1 cells (28). Since Dkk-1 overexpression did not have a significant effect on the canonical pathway in Ace-1 cells, constitutive activation of canonical Wnt signaling must be considered.

Although the Wnt noncanonical pathway is not well understood, it has been shown that JNK, Rho and Rock pathways are involved (15). Dkk-1 increased JNK by increased phosphorylation leading to survivability of lymphoma cells (29,30). Endo et al showed that Dkk-1 increased the phosphorylation of JNK in the noncanonical pathway through the frizzled receptor (31). We investigated noncanonical signaling in Ace-1-DKK-1 cells by examining the phosphorylation of
JNK. Western blots showed that the p46 isoform was markedly increased although the p54 isoform were only mildly increased. Yaccoby et al reported that inhibition of Dkk-1 in myeloma using an anti-human Dkk-1-neutralizing antibody significantly decreased myeloma growth and osteolysis (32). Therefore, Dkk-1 may be contributing to the increased Ace-1 tumor growth seen in this study via the noncanonical pathway by upregulating phosphorylation of JNK. Moreover, Dkk-1 increased the incidence of Ace-1 bone metastases following intracardiac inoculation of cells. It is possible that increased survivability of Ace-1 cells through elevated p46 JNK levels might be contributing to the establishment of a greater number of bone metastases.

Secretion of Wnt 7B by prostate cancer was reported to induce osteogenic activity through the canonical signaling pathway mediated by the LRP5 receptor in immunodeficient mice and Dkk-1 blocked the osteogenic activity (33). The data from the TCF reporter transcriptional assay showed that Ace-1 cells induced activation of Wnt/TCF canonical signaling in ST2 cells. This demonstrated that Ace-1 cells likely induced osteoblastic activity through canonical signaling. Importantly, Ace-1 cells overexpressing Dkk-1 inhibited Ace-1-induced canonical TCF activity in ST2 precursor osteoblasts showed that Dkk-1 inhibited Ace-1-induced osteoblast activity through a paracrine mechanism. This was supported by the in vivo data that Dkk-1 inhibited Ace-1-induced intratibial woven bone formation at site of tumor cell inoculation.
Radiographic and histopathologic evaluation of tibias with Ace-1 cells showed that Dkk-1 over-expressing cells significantly decreased new bone formation induced by Ace-1 cells as demonstrated by a marked decrease in trabecular and new woven bone area. µCT imaging also demonstrated a marked decrease in trabecular and new woven bone volume. Dkk-1 increased endosteal eroded surface in the tibias and decreased endosteal new woven bone surface. This demonstrated that Dkk-1 secreted by Ace-1 cells not only inhibited osteoblast activity but also indirectly led to increased osteoclast activity.

Although Dkk-1 has no direct effect on the osteoclastogenesis, it has been reported that Dkk-1 inhibited the secretion of osteoprotegerin (OPG) and induced RANKL secretion from osteoblasts resulting in an increased RANKL:OPG ratio, which has been shown to be important for enhanced osteoclastogenesis (34). In the current study, it is possible that Dkk-1 inhibited Ace-1-induced osteoblastic phenotype of bone metastases by directly inhibiting osteoblasts via the canonical pathway and by stimulating osteoclastogenesis indirectly through increased the RANKL:OPG ratio.

Although Dkk-1 significantly decreased Ace-1-induced new bone formation in osteoblastic metastases, its unexpected role in the increased prostate cancer growth and bone metastases incidence further necessitates investigations to clearly understand the role of Wnt canonical and noncanonical pathways in the pathogenesis of prostate cancer.
REFERENCES


FIGURE LEGENDS

Figure 16: Expression of Wnt family genes in Ace-1 prostate cancer cells. (A) Expression of Wnt genes, LRP receptors and Dkk-1 was evaluated by RT-PCR in Ace-1 cells using dog-specific primers. (B) Ace-1 cells were transfected with human Dkk-1 and secreted human Dkk-1 was measured by ELISA at 72 hours.

Figure 17: Effect of Dkk-1 on Ace-1 prostate cancer subcutaneous growth and bone metastasis incidence. (A) Subcutaneous tumor weights from the Ace-1-Vector and Ace-1-Dkk-1 groups were measured at 42 days post-injection. *, P=0.002 compared to Ace-1-Vector group. (B) Intracardiac injections were performed with Ace-1-Vector\textsuperscript{YFP-Luc} or Ace-1-Dkk-1\textsuperscript{YFP-Luc} cells. Incidence of metastatic sites was counted using bioluminescent imaging and number of metastases per animal per group was shown using vertical dot plot. *, p<0.05 compared to Ace-1-Vector\textsuperscript{YFP-Luc}.

Figure 18: Measurement of Dkk-1 autocrine canonical and noncanonical signaling in Ace-1 subcutaneous tumors. (A) Western blotting was performed on total protein lysates isolated from three different subcutaneous tumors from each group for Dkk-1, β-catenin and β-actin. (B) β-catenin protein expression was
analyzed by densitometry and normalized to the loading control, β-actin. (C) Total protein lysates from subcutaneous tumors were western blotted for phospho-JNK and total JNK. (D and E) Densitometric analysis of phospho46 (p46) and phospho54 (P54) JNK isoforms compared to total JNK concentrations. *, P<0.001.

**Figure 19:** Radiographic analysis of Dkk-1 on Ace-1 intratibial bone metastases. Radiographs were taken 19 days after the intratibial inoculation of Ace-1-Vector<sup>YFP-Luc</sup> or Ace-1-Dkk-1<sup>YFP-Luc</sup> cells. Tibias in the Ace-1-Vector<sup>YFP-Luc</sup> group had increased radio-opacity in the diaphyseal region (black arrow) compared to the control bone that did not receive any tumor cells. Tibias in the Ace-1-Dkk-1<sup>YFP-Luc</sup> group had cortical and trabecular bone lysis in the metaphyseal and diaphyseal regions (white arrows).

**Figure 20:** Histopathological evaluation of intratibial bone metastases 19 days after intratibial inoculation of Ace-1-Vector<sup>YFP-Luc</sup> or Ace-1-Dkk-1<sup>YFP-Luc</sup> cells. (A) H&E-stained sections of tibias demonstrated that Ace-1 tumors (T) extending from the growth plate to the diaphyseal region in both groups. In the Ace-1-Vector<sup>YFP-Luc</sup> group (a and b) new woven bone (WB) was present along the tumor and endosteal surfaces of the cortex (CT) in the tibial diaphyseal region. In the
Ace-1-Dkk-1YFP-Luc group (c and d) new woven bone was absent. Bar, a and c = 500 microns, b and d = 100 microns. Histomorphometric analysis showed that Dkk1 significantly decreased the trabecular bone (B) in the metaphyseal region and new woven bone (C) in the tibia compared to the vector group. *, P<0.001

**Figure 21:** Histomorphometric evaluation of endosteal eroded surface and tibial endosteal surface occupied by new woven bone. (A) Dkk-1 significantly increased the tibial endosteal eroded surface compared to vector group. *, p<0.001. (B) In vector group, greater tibial endosteal surface was occupied by new woven bone compared to the Dkk-1 group. *, P<0.001

**Figure 22:** Microcomputed tomographic (µCT) evaluation of Dkk-1 on Ace-1 tibial bone metastases trabecular bone and new woven bone volume. (A) Representative µCT images demonstrating trabecular bone (TB), woven bone (WB) and cortex (C) in both the Ace-1-vector and Ace-1-Dkk-1 groups. (B and C) Bar graphs represent trabecular and woven bone volume measured using IRW software. These data showed that Dkk-1 significantly inhibited trabecular and new woven bone volume. *, P<0.001.
Figure 23: Effect of Ace-1 cells on *in vitro* paracrine canonical TCF luciferase reporter transcriptional activity of murine bone marrow precursor osteoblast cells (ST2). (A) Co-culture of ST2 cells and Ace-1-Vector cells increased TCF reporter activity in ST2 cells compared to ST2 cells alone. *, P<0.003. Ace-1-Dkk-1 cells co-cultured with ST2 cells decreased the ST2 TCF reporter activity compared to vector group ST2 TCF activity. **, P< 0.003
Table 7: Dog-specific primers used for RT-PCR amplification of expressed genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’- 3’)</th>
<th>Reverse Primer (5’- 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dkk-1</td>
<td>ATCGAGGAGAGATCGAGGA GA</td>
<td>ATGATCGGAGACAGACCCGA G</td>
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<tr>
<td>LRP5</td>
<td>ACATGTACTGGACAGACTGG</td>
<td>AGTCTGTCGAGTACAGAGTG</td>
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<td>LRP6</td>
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<td>TTGAACCATCCATTCCAGCA</td>
</tr>
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<td>Wnt4</td>
<td>AACCTGGAGGTGATGGACT C</td>
<td>ACTTCTCCAGCTCCCCGCT</td>
</tr>
<tr>
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<td>AACTGTGCCACTTGTATCA GGA</td>
<td>TAGCCGTAGTGCAGTTGTC C</td>
</tr>
<tr>
<td>Wnt7</td>
<td>TACCAGTCCGCTTCCGACG CT</td>
<td>AGTTGCTCAGGTACCTTG G CT</td>
</tr>
<tr>
<td>β2M</td>
<td>CTTGCTCCTCATCCTCCTC</td>
<td>TGACACGCTAGCAGTTGAG</td>
</tr>
</tbody>
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Figure 16: Expression of Wnt family genes in Ace-1 prostate cancer cells and overexpression of Dkk-1 in Ace-1 cells
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Figure 21: Histomorphometric evaluation of endosteal eroded surface and tibial endosteal surface occupied by new woven bone
Figure 22: Microcomputed tomographic (μCT) evaluation of Dkk-1 on Ace-1 tibial bone metastases trabecular bone and new woven bone volume
Figure 23: Effect of Ace-1 cells on *in vitro* paracrine canonical TCF luciferase reporter transcriptional activity of murine bone marrow precursor osteoblast cells (ST2)
CHAPTER 4

ESTABLISHMENT AND CHARACTERIZATION OF A BRAIN METASTATIC CANINE PROSTATE CANCER CELL LINE, LEO.

ABSTRACT

Prostate cancer in men has a high mortality and morbidity due to metastatic disease. The pathobiology of prostate cancer is not well understood due to lack of cell lines and animal models that recapitulate the complex nature of the disease. Therefore, the goal of the study was to establish and characterize a new prostate cancer line derived from a dog with spontaneous prostate cancer.

A new cell line (Leo) was derived from a dog with spontaneous prostate cancer. Immunohistochemistry and PCR were used to characterize the primary prostate cancer and xenografts in nude mice. Leo cells were transduced with lentiviral yellow fluorescent protein-luciferase(YFP-Luc). Subcutaneous tumor growth and metastases in nude mice were evaluated by bioluminescent imaging, radiography and histopathology. In vitro chemosensitivity of Leo cells to therapeutic agents was measured using an MTT cell viability assay.
Leo cells expressed the secretory epithelial cytokeratins (CK) 8, 18 and ductal cell marker, CK7. The cell line grew in vitro (over 65 passages) and was tumorigenic in the subcutis of nude mice. Following intracardiac injection, Leo cells metastasized to the brain, spinal cord, bone, and adrenal gland. The incidence of metastases was greatest to the central nervous system (80%) with a lower incidence to bone (20%) and the adrenal glands (16%). The brain and spinal cord metastases were multifocal and some grew to a large size (up to 50% of the cranium). *In vitro* chemosensitivity assays demonstrated that Leo cells were sensitive to velcade and an HDAC-42 inhibitor with IC$_{50}$ concentrations of 1.9 nM and 0.95 µM respectively.

The new prostate cancer cell line (Leo) will be a valuable model to investigate the mechanisms of the brain and bone metastases.

**INTRODUCTION**

Prostate cancer is the most commonly diagnosed cancer in men accounting for 25% of all cancers in United States of America (1). Prostate cancer in advanced stages has a high incidence of bone metastases that causes severe skeletal complications resulting in morbidity and mortality. Dogs are the only nonhuman mammals that develop spontaneous prostate cancer, which shares many characteristics in clinical presentation and pathogenesis of the disease as in men (2). In addition, dogs also have extensive genomic homology with the humans,
and recent sequencing of the dog genome and development of dog gene arrays make the dog an invaluable animal model to study the pathogenesis of prostate cancer in spontaneous or experimental settings (3). Previously, our laboratory established the ACE-1 canine prostate cancer cell line that metastasizes only to bone and induced mixed osteoblastic and osteolytic lesions in nude mice similar to bone metastases in men with prostate cancer (4).

Although bone is the primary site of metastases in prostate cancer, metastases also occur in lymph nodes, brain, lung, adrenal gland and liver (5-7). Soft tissue metastases, alone or in combination with bone metastases, were detected in 33% of prostate cancer patients with metastases (8). Brain metastases occurs in a small percentage of patients (1%), but is often a terminal event. Median survival rate is 1 month in untreated patients with brain metastases, 3.5 months in patients who received radiotherapy and 9 months in patients who had stereotactic radiosurgery (9). Although brain metastases are uncommon in prostate cancer, annual incidence of more than 150,000 cases of brain metastases from other cancers was reported in the US (10). It is important to develop new therapies for patients with brain metastases due to their serious consequences. Therefore, understanding the molecular pathogenesis of prostate cancer brain metastases will facilitate the development of novel treatment strategies.

Although numerous prostate cancer cell lines have been developed, a few cell lines develop bone metastases and no cell lines have been reported to cause
brain metastases (11). Despite the fact that the DU145 cell line was developed from a brain metastasis from a human prostate cancer patient, these cells have not demonstrated the ability to metastasize to the brain in animal models (12). Therefore, lack of prostate cancer cell lines that develop brain metastases have impeded studies of prostate cancer brain metastases.

In this investigation we report the establishment of a novel dog prostate cancer cell line and its ability to metastasize to numerous sites in the nude mouse including brain, spinal cord, bone and adrenal gland.

**MATERIALS AND METHODS**

**Establishment of prostate carcinoma cell line (Leo):** Tissue from a spontaneously arising prostate carcinoma was collected immediately following euthanasia of a tumor-bearing 5-year-old castrated male mixed breed dog and washed three times in DMEM/F12 (Invitrogen Corp., Carlsbad, CA) containing 50 μg/ml primocin (Invivogen, San Diego, CA) and minced into approximately 1 mm³ pieces. The tissue pieces were digested in 500 units/ml of collagenase type 1 (Worthington Biochemical, Lakewood, NJ) in serum-free DMEM/F12 for 2 hours at 37°C on a rocker platform with gentle rocking. The digested tissue was washed three times with DMEM/F12 containing 50 μg/ml primocin and 10% fetal bovine serum (FBS) (Invitrogen Corp.) and plated in a T-75 flask (Falcon; Becton-Dickinson, Franklin Lakes, NJ). Medium was changed every 3 days.
Differential trypsinization was performed to eliminate the stromal cell contamination of epithelial cells once every two days by treating the cultures with 0.05% trypsin ethylenediamine-tetraacetic acid (EDTA) (Invitrogen Corp.). Detachment of the cells was monitored every two minutes to remove the stromal cells, which detached earlier than the epithelial cells. The first phase of detached cells (mostly stromal cells) was removed and the remaining epithelial cells were collected and replated.

**Lentiviral yellow fluorescent protein-luciferase (YFP-Luc) vector production and transduction of Leo cell line:** Lentiviral vectors were produced by transient co-transfection of 293T cells with 10 μg packaging plasmid pCMVDR8.2, 2 μg envelope plasmid pMD.G and 10 μg transfer plasmid pHIVSIN-YFP-Luc using calcium phosphate (Sigma-Aldrich Co., St. Louis, MO) as described previously (13). The virus supernatant was collected at 24 hours, filtered through a 0.2 μm filter, and stored at -80°C until use. Leo cells were transduced with the retroviral YFP-Luc dual reporter gene and 8 μg/ml polybrene by spin inoculation at 2700 rpm for 1 hour at 32°C and transferred to a cell culture incubator overnight. Later, cells were maintained in DMEM/F12 medium containing 10% FBS and 50μg/ml normocin (Invivogen).

**Subcutaneous and intracardiac left ventricular inoculation of Leo cells in nude mice:** Subcutaneous (N=10) and intracardiac (N=25) injections of Leo YFP-Luc cells were performed in six-week-old male nu/nu mice (National Cancer
Institute, Frederick, MD) under general anesthesia with a 3% isoflurane (Abbott Laboratories, North Chicago, IL)-oxygen mixture. All the animal experimental procedures were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Ten million Leo cells suspended in 250 µl of phosphate-buffered saline (PBS) (Invitrogen Corp.,) were injected subcutaneously over the dorsal interscapular area of mice. Subcutaneous tumor growth in mice was measured weekly using vernier calipers and bioluminescent imaging (BLI) and sacrificed after 6 weeks. Left ventricular cardiac injections were performed with $1 \times 10^5$ cells suspended in 100 µl of PBS using a 26 gauge needle. Successful left ventricular intracardiac injections and metastases of tumor cells were monitored using BLI (BLI, see below). Immediately after intracardiac injection of Leo$^{YFP-Luc}$ cells, a diffuse BLI signal originating from the tumor cells throughout the entire body was considered successful left ventricular injection. Mice were sacrificed after 6 weeks.

**Bioluminescent imaging (BLI):** Mouse BLI was performed using an *in vivo* imaging system (IVIS 100, Caliper Life Sciences, Hopkinton, MA) as described previously (11). Briefly, 4.5 mg luciferin (Caliper Life Sciences) dissolved in 150 ul PBS was injected intraperitoneally into mice and imaging was carried out in an imaging chamber under general anesthesia with a 1.5% isoflurane–oxygen mixture until peak photon signal was attained. The photon signal intensity was quantified using LivingImage software version 2.50 (Caliper Life Sciences)
**In vitro growth rate:** Five hundred thousand cells \((5 \times 10^5)\) from passage 70 were plated in 10 cm plates (Falcon) in quadruplicates in DMEM/F12 medium with 10% FBS and 50 \(\mu\)g/ml normocin (invivogen) and incubated at 37°C in 5% CO\(_2\). The cells were harvested using 0.25% trypsin (Fisher Scientific, Pittsburgh, PA) at 24, 48 and 72 hrs after plating and counted with an automated cell counter (Nexcelom Bioscience, Lawrence, MA) using trypan-blue dye exclusion of dead cells. 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) (14).

**Radiography of mice:** Radiographic images of mice were obtained using a Faxitron laboratory radiography system LX-60 (Faxitron X-ray Corp., Wheeling, IL) at 30KVp for 10 sec on day 28.

**Histopathology and immunohistochemistry:** Complete necropsies were performed on the mice 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 (5 \(\mu\)m) and were either stained with hematoxylin and eosin (H&E) or evaluated immunohistochemically using human antibodies for the presence of CK5/14, 8, 18, 7, vimentin, androgen receptor (AR) and prostate specific antigen (PSA) to characterize the prostate carcinoma cells (see Table 1 for a list of primary
antibodies). Sections were deparaffinized in xylene (Hemo-De, Fisher Scientific, Bay Shore, NY) by two 3 min washes and rehydrated in 100%, 95% and 70% ethanol sequentially for 3 min and rinsed in water. Endogenous peroxidase activity was quenched in 3% H₂O₂ (Dako Corp., Carpinteria, CA) for 5 min at room temperature (RT) and washed in PBS for 15 minutes. To block nonspecific binding of proteins, sections were incubated in protein block (Dako Corp.) for 15 minutes at RT and rinsed in PBS. Antigen retrieval was performed using target retrieval solution and heated for 30 min in an oven at 60°C. Primary antibodies (see Table 8) were added to sections and incubated at RT for 30 minutes and sections were washed three times for 5 minutes in PBS/0.05% Tween. After washing, sections were incubated either with universal biotinylated goat-α-mouse secondary or biotinylated goat anti-rabbit IgG secondary antibody (1:250 dilution in protein block reagent) for 30 min at RT and followed by three 5 minutes washes in PBS/0.05% Tween. Sections were incubated with avidin–biotin complex for 30 min according to the manufacturer’s instructions (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame). To visualize the peroxidase activity on the sections, sections were incubated with 3, 3′-diaminobenzidine (DAB) reagent (1:50 concentrate:reaction buffer) for 5 min at RT and rinsed in distilled water. The slides were counterstained with hematoxylin for 1 min, dried and coverslipped with xylene-based mounting media.
RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR): Total RNA was extracted from Leo cells, Ace-1 dog prostate cancer cells, dog transitional cell carcinoma (TCC) cells (15) and dog brain using Trizol reagent according to manufacturer's protocol (Invitrogen). Total RNA (2.5 μg) was reverse transcribed using the Superscript II First Strand cDNA synthesis kit (Invitrogen) and RT-PCR was performed for parathyroid hormone related protein (PTHrP), matrix metalloproteinase (MMP-9), vascular endothelial growth factor (VEGF), beta2-microglobulin (B2M), receptor activator of NF-kappaB ligand (RANKL), osteoprotegerin (OPG), nerve growth factor (NGF), low affinity nerve growth factor receptor (LNGFR), and neurotrophin tyrosine receptor kinase 1 (NTRK1) using canine-specific primers (Table 9).

PTHrP immunoradiometric assay: PTHrP concentrations were measured in the Leo cell conditioned medium at 0, 6, 12, 24 hrs after the addition of serum-containing medium. Negative control medium was not conditioned by any cells. PTHrP concentration was quantified using a two-site immunoradiometric assay (DSL, Webster, TX) specific for PTHrP 1-80 using antibodies to the N-terminal region (amino acids 1 to 40) and mid-region (amino acids 57 to 80) according to manufacturer's instructions.

Data analysis: The half maximum inhibitory concentrations (IC$_{50}$) of the drugs were calculated using CalcuSyn software (Biosoft, Cambridge, UK).
RESULTS

Histopathology of the prostate carcinoma: In the dog, the normal prostate was replaced by a highly invasive carcinoma consisting of multiple lobules of polygonal to columnar cells arranged in large sheets of cells and nests of cells separated by dense fibrous connective tissue. Neoplastic cells had abundant cytoplasm with occasional cells containing a single, round, clear cytoplasmic vacuole which displaced the nucleus (signet ring formation). Binucleate cells were often apparent and up to 3 nucleoli were present in the nuclei (Fig 24A). Neoplastic tissue had regions of necrosis with secondary inflammation. The carcinoma invaded into the trigone region of urinary bladder and metastasized to the iliac lymph node, liver, lung and right proximal tibia. The central nervous system was not examined since the dog had no neurologic clinical signs.

Development of Leo prostate cancer cell line: The prostate cancer cell line, Leo, was established from the primary prostate carcinoma tissue. The cell line has been passaged over 75 times in vitro over a 2-year period. The cells grew in an anchorage-dependent manner in monolayer sheets with polyhedral cells exhibiting a tightly packed cobblestone growth pattern (Fig 24B).

Subcutaneous injection of Leo prostate carcinoma cells: Leo cells injected into the subcutaneous region of nude mice formed tumors that average 0.8 cm$^3$
as measured by vernier calipers in 6 weeks (Fig 27C). The microscopic appearance of the Leo subcutaneous xenografts was similar to the primary carcinoma. (Fig 24C,)

**Immunohistochemistry of the primary prostate carcinoma:** The primary prostate carcinoma was moderately positive for cytokeratin (CK) 8 (Fig 25A) and strongly expressed CK18 (Fig 25B), which are markers of prostatic secretory cells. The cancer cells were negative for basal cell cytokeratin markers CK5 and 14 (data not shown). Previously it was reported that prostate cancer can originate from ductal cells in dogs and express the cytokeratin marker, CK7 (16,17). The primary cancer cells expressed CK7 staining in the cytoplasm (Fig 25C). Androgen receptor, prostate specific antigen (PSA), and vimentin were not expressed by the carcinoma cells (Data not shown). Dog prostate glands do not express human PSA, but have a related kallikrein, arginine esterase (18).

**RT-PCR of the Leo prostate cancer cell line:** Leo cells expressed mRNA for MMP9 and VEGF (Fig 26), which have been reported to play an important role in the development of brain metastases (19,20). RANKL and OPG were expressed by the Leo cells (Fig 26) and might play a role in the bone metastases developed by Leo cells after intracardiac injection. Expression of PTHrP, NGF, LNGFR, and NTRK1 were not detectable. PTHrP concentrations in Leo cell conditioned media at different time points were similar to control media without cells.
**In vitro growth and in vivo tumorigenicity:** Leo cells grew exponentially *in vitro* with a doubling time of approximately 38 hours under standard cell culture conditions (Fig 27A). Leo cells were transduced with YFP-Luc to monitor the *in vivo* growth. The Leo^{YFP-Luc} cells were injected into the subcutis of nude mice to test their tumorigenicity. Subcutaneous tumor growth was detected in all mice (n=10) and was progressive tumor over 6 weeks (Fig 27B and C).

**Metastases of Leo cells in nude mice:** Left ventricular intracardiac injections of Leo^{YFP-Luc} were performed in 25 male nude mice. Metastatic ability of tumor cells was monitored weekly using BLI. At day 7, BLI signal was undetectable indicating that most Leo cells died. On day 14, metastatic foci were detected by BLI at different sites including the head, hind limbs, vertebrae and scapular region. BLI intensity of the metastatic foci increased at days 21 and 28. Metastases were detected in 23/25 mice. Mice were sacrificed on day 28 and *ex vivo* imaging of individual organs were performed immediately to localize the metastases. Metastases detected by BLI were confirmed by histopathological evaluation.

Histopathological analysis showed the presence of prostate carcinoma in the brain (19/25 mice), spinal cord (2/25 mice), adrenal gland (4/25), long bones (5/25) and scapula (1/25). Leo cells most commonly metastasized to brain (Fig 28A and B) when compared to other organs. Tumors in the brain ranged from 1mm to 5mm in diameter and multiple metastatic foci were observed in each brain section. Metastatic tumor in the brain and spinal cord (Fig 28C and D) was
most frequently located within the white matter. These lesions were expansile with compression of surrounding neuropil consistent with tumor size. Central necrosis was a feature of larger tumors within the brain. Tumor replaced the medulla and cortex of the adrenal gland (Fig 28E and F). In long bone metastases, such as tibia and femur, tumor cells caused marked osteolysis characterized by loss of cortical and trabecular bone in the metaphyseal region (Fig 29A, B and C). The single metastatic lesion to the scapula was osteoblastic and consisted of tumor cells surrounding the scapular bone and containing large radiating bands (50 um wide) of new woven bone proliferation lined by tall cuboidal osteoblasts (Fig 29D, E and F).

**In vitro chemosensitivity:** The *in vitro* chemosensitivity of the Leo cells to different doses of calcitriol, piroxicam, (S)-HDAC-42 and velcade was measured using the MTT assay at 72 hrs. All the drugs inhibited cell growth in a dose-dependent manner. The half maximum inhibitory concentrations (IC$_{50}$) were calculated using CalcuSyn software and were 6.5 µM for calcitriol (Fig 30A), 1.9 nM for velcade (Fig 30B), 0.95 µM for (S)-HDAC-42 (Fig 30C) and 518 µM for piroxicam (Fig 30D). The in vitro IC$_{50}$ concentrations of calcitriol and piroxicam were higher than what is accepted as physiologically achievable in patients. Leo cells were most sensitive to (S)-HDAC-42 and velcade compared to calcitriol and piroxicam.
DISCUSSION

Understanding the pathogenesis of prostate cancer and development of novel therapeutic agents have been hindered due to the lack of cell lines and in vivo animal models that mimic the condition in men with prostate cancer (7). Therefore, development of new cell lines and animal models that closely recapitulate the clinical disease in patients are crucial to understand the molecular heterogeneity and pathogenesis of prostate cancer. Dogs not only develop spontaneous prostate cancer but also share similarities to the clinical presentation of prostate cancer in men (2,11,21). In the present study, we described the establishment of a novel dog prostate cancer cell line (Leo) and characterization of its tumorigenicity and metastatic ability to various organs in nude mice.

The Leo cell line was developed from a 5-year-old castrated male mixed breed dog. The Leo cells formed tumors in the subcutis of nude mice demonstrating its tumorigenicity in vivo. Following intracardiac inoculation, the Leo cells metastasized to multiple sites including the brain, spinal cord, bones, and adrenal gland. The Leo prostate cancer cells exhibited a remarkable tropism for developing metastases in the brain based on the 80% incidence of brain metastases. It has been reported that MMP9 is upregulated in most brain metastases (19). MMP9 belongs to matrix metalloprotease family of enzymes that degrades the extracellular matrix leading to invasion of tumor cells (22). High expression of MMP9 by Leo cells suggests that it might play a significant role in
the development of brain metastases. Increased VEGF expression by tumor cells helps in the angiogenesis and transendothelial migration of cancer cells by regulating the permeability of brain microvascular endothelial cells (23). The Leo cells express VEGF and may be important in the development of brain metastases by angiogenesis and extravasation into the brain through transendothelial migration. Although VEGF and MMP9 may facilitate brain metastasis, other factors must also be involved because the Ace-1 and TCC cell lines also produce VEGF and MMP9 and do not metastasize to the brain. The brain is a rich source of NGF, which is important in the development and maintenance of nervous tissue (24). NGF regulates its action by binding and activating to two types of neurotrophin receptors, a low affinity nerve growth factor receptor (LNGFR or P75) and neurotrophin receptor tyrosine kinase (NTRK) (25). However, expression of NGF, LNGFR and NTRK1 were not detectable in Leo cells.

Waters et al reported that most dog prostate cancers simultaneously show mixed histologic features of adenocarcinoma and urothelial differentiation (2). It has been suggested that prostate cancer in dogs may originate from prostate ductal cells and are positive for CK7 (16,17). Expression of CK7 in addition to secretory epithelial cytokeratins, 8 and 18, by the Leo cells suggests that they have differentiated towards both secretory and ductal cells. Ductal cells in dogs may also have urothelial differentiation with CK 7 expression (17).
The androgen receptor in dogs is expressed in prostate secretory and basal epithelial cells, ductal cells, stromal cells and the prostatic urethra (16). However, in neutered dogs the AR is not expressed. Lack of expression of AR in the Leo cell line, developed from a prostate carcinoma in a neutered dog is consistent with previous reports (16). The Leo cells were negative for vimentin, which is generally expressed by stromal cells although it has been reported that vimentin was expressed in some prostate cancer cells suggesting epithelial to mesenchymal transition (4). Prostate specific antigen, a biomarker of prostate cancer in human, is expressed in glandular epithelium and liquefies the semen by degrading semenogelin (26,27). PSA belongs to the family of kallikrein genes and it is encoded by kallikrein 3. In human 15 kallikreins were identified whereas in dogs only 14 kallikreins are present. The human kallikrein 3 gene encoding PSA in humans is absent in dogs (28). However, dog kallikrein 2, encoding arginine esterase (AE), shares 58% amino acid homology with human PSA and both genes are regulated by androgens (29,30). Polyclonal and monoclonal PSA antibodies crossreact with human kallikreins 1 and 2 since the degree of amino acid homology is 80% (31). A few reports have demonstrated staining for PSA (using antibodies to human PSA) in normal dog prostate and canine prostate cancers (32). Presumably this may be due to crossreactivity to canine arginine esterase. However, since dogs lack a homolog to human PSA, PSA assay and immunohistochemistry generally are not useful in dogs (32).
The bone metastases that developed in nude mice with the Leo cells were predominantly osteolytic similar to that observed in the dog with the tibial metastasis. Dog prostate cancer metastases to bones mostly effect the axial skeleton and proximal long bones and can be osteoblastic, osteolytic or mixed (2). We have also developed a nude mouse model of dog prostate cancer using the Ace-1 cells that develop a 100% incidence of mixed osteoblastic and osteolytic bone metastases (4). The Leo cells have a unique metastatic pattern in vivo with a relatively low incidence of metastases to bone (20%). The RANKL and OPG axis has been to play an important role in regulating osteolytic bone metastases (33). RANKL expressed by prostate cancer cells binds to RANK receptor expressed on the surface of osteoclast precursors and induces their differentiation and maturation into active osteoclasts. OPG expressed by cancer cells acts as decoy receptor for RANKL and prevents it from binding to RANK resulting in inhibition of osteoclastogenesis (34). Leo cells, similar to the Ace-1 prostate cancer cell line, express high levels of both RANKL and OPG. Therefore, the role of RANKL and OPG axis in the development of osteolytic lesions is not clear at present, but presumably the amount of OPG is not adequate to inhibit the osteoclastic bone resorption in the metastases.

Velcade, a proteosome inhibitor (PS341) was shown to inhibit prostate cancer growth and osteolytic bone metastases in preclinical models (35). Leo cells were very sensitive to velcade with an IC$_{50}$ of 1.9 nm. Serum concentrations of velcade in human patients of 40 to 90 ng/L were shown to
exhibit antitumor activity without serious adverse effects (36,37). Therefore, it will be important to test the efficacy of velcade on tumor growth and metastases in the Leo model of prostate cancer. A novel HDAC-42 inhibitor was very effective in inhibiting the growth of Leo cells \textit{in vitro} with an IC$_{50}$ of 0.95 µm at after 72 hours of treatment. This novel phenylbutyrate-derived HDAC inhibitor was shown to possess potent anti-tumor activity in prostate cancer by inducing apoptosis (38). Despite the fact that calcitriol and piroxicam had inhibited the Leo cell growth, the IC$_{50}$ concentrations were too great to achieve relevant serum levels in clinical patients.

One of the most important findings of the Leo xenografts was the consistent and frequent development of brain metastases in nude mice. To our knowledge, this is the first prostate cancer cell line that consistently develops brain metastases in vivo. This provides a unique translational model to investigate the pathogenesis of brain metastases and develop potent therapeutic strategies to treat or prevent brain metastases. Though the incidence of bone metastases was not high, it may be feasible to develop a subline of the Leo cells with a high rate of bone metastases, which would be a valuable model to study the skeletal complications of prostate cancer. Therefore, the new Leo prostate cancer cell line provides a valuable tool to help understand the molecular mechanisms responsible for the pathogenesis of prostate cancer and metastases to the brain and bone.
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**FIGURE LEGENDS**

**Figure 24:** Photomicrographs of primary prostate carcinoma, in vitro cell line (Leo) and subcutaneous xenograft. (A) Photomicrograph of hematoxylin and eosin (H&E)-stained primary prostate carcinoma. Note the solid carcinoma consisting of multiple lobules of polygonal to columnar cells (alveolar pattern) separated by fibrous connective tissue. *Bar*, 50 microns. (B) Phase contrast microscopy of Leo cells (passage 65). The polyhedral cells have a tightly packed cobblestone growth pattern. *Bar*, 50 microns. (C) Photomicrograph of H&E-stained subcutaneous Leo xenograft. Tumors were multilobular with large polygonal cells. *Bar*, 50 microns.

**Figure 25:** Immunohistochemistry of the primary prostate carcinoma. (A) Cytokeratin 8 staining in the cytoplasm is moderately positive. (B) Cytokeratin 18 staining in the cytoplasm is strongly positive. (C) Cytokeratin 7 staining in cytoplasm is moderately positive.

**Figure 26:** Gene expression profile of Leo cells. Expression of MMP9, VEGF, OPG, RANKL were detected using RT-PCR and the relative expression was
compared between dog prostate cancer cells lines; Ace-1 and Leo and a dog transitional cell carcinoma cell line (TCC). Expression of β2microglobulin was used as a control.

**Figure 27:** *In vitro* and *in vivo* growth patterns of Leo cells. (A) *In vitro* growth curve of Leo cells. Data presented as mean + standard deviation of 4 replicates. (B) Graph represents the average intensity of the BLI signal measured at the indicated time points. (C) Graph represents the average tumor volume. Data presented as mean + standard deviation of 10 mice.

**Figure 28:** *In vivo* BLI and histopathologic evaluation of nude mice 28 days following intracardiac injection of Leo<sup>YFP-Luc</sup> cells (A) was performed in ventral (a) and lateral (b, c) recumbency and demonstrated metastasis to the cranium. (B) H&E-stained coronal section of brain revealed a large metastatic carcinoma (T, right side) with multiple small metastases in the brain parenchyma. BLI evaluation of spinal cord metastasis 28 days after intracardiac injection of Leo<sup>YFP-Luc</sup> cells (C). Histopathologic evaluation (D) showed a metastatic carcinoma (T) in white matter of spinal cord (H&E). BLI evaluation 28 days after intracardiac injection of Leo<sup>YFP-Luc</sup> cells revealed a metastasis to the lumbar region (E) which
was microscopically confirmed as an adrenal metastasis (F). Tumor cells are indicated by (T), adrenal cortex by (CT) and adrenal medulla by (M). H&E

**Figure 29:** Evaluation of bone metastasis on day 28 following intracardiac injection of Leo\textsuperscript{YFP-Luc} in nude mice. (A) BLI showed metastasis to the left hind limb. (B) Radiographic evaluation showed osteolysis (arrow) in the metaphyseal region of the humerus. (C) Histopathologic evaluation showed that tumor (T) replaced bone marrow in the metaphysis and diaphysis of humerus. The metastasis caused extensive bone osteolysis (arrow) characterized by loss of cortical (CT) and trabecular bone. Metastasis to the scapula was detected by BLI (D). Radiographs demonstrated a radiodense lesion (E) which was histologically composed of carcinoma interspersed with radiating trabeculae of woven bone (F).

**Figure 30:** Dose-response of Leo cell viability to calcitriol (A), velcade (B), (S)-HDAC-42 (C) and piroxicam (D) treatment. An MTT assay was performed with 6 replicates for each dose with a duration of 72 hours. IC\textsubscript{50} values for calcitriol, piroxicam, (S)-HDAC-42 and velcade were 6.8 μM, 518 μM, 0.95 μM, and 1.9 nM respectively. Bars represent mean + standard deviation of 6 replicates.
Table 8: Antibodies used for immunohistochemistry of the primary prostate carcinoma

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Clone</th>
<th>Company, City, state</th>
<th>Dilution</th>
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<tr>
<td>MA903 (CK5/14)</td>
<td>mouse</td>
<td>34βE12</td>
<td>Enzo Life Sciences, Farmingdale, NY</td>
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<td>CK18</td>
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<td>Sigma Aldrich, St Louis, MO</td>
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<td>CK8</td>
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<td>Chemicon, Temecula, CA</td>
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<td>Clone V9</td>
<td>Cell Marque, Hot Springs, AR</td>
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<td>OV-TL 12/30</td>
<td>Dako Corporation, Carpinteria, CA</td>
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<td>PSA</td>
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<td>Polyclonal</td>
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Table 9: Dog-specific primers used for RT-PCR amplification

<table>
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<th>Reverse Primer (5’- 3’)</th>
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</thead>
<tbody>
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<td>PTHrP</td>
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<td>GGAAGAATCGTCGCGCGTAAAG</td>
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<td>AGAAAGTCTTTCTTGTCGACCCĞ</td>
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<tr>
<td>VEGF</td>
<td>ATCGAGTACATCTTTCAAGCATCC</td>
<td>CTATGCTGCAAGAAACTCATCC</td>
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<td>TCAGCTGAAGATACTCTCTGTGC</td>
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<td>CTGCAAAAGCTGCAATGTGCTCTGG</td>
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<td>GAACTTGCAGTAGGAGGACT</td>
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<tr>
<td>B2-M</td>
<td>CTTGCTCCTCATCCTCGATC</td>
<td>TGACACGTAGCAGTTCAG</td>
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</table>
Table 10: Incidence of Leo\textsuperscript{YFP-Luc} metastases, by anatomic location, at 28 days following intracardiac injection.

<table>
<thead>
<tr>
<th>Site of Metastasis</th>
<th>Incidence (Number of mice with metastases/Total mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mice with metastases</td>
<td>23/25</td>
</tr>
<tr>
<td>Brain and Spinal cord</td>
<td>21/25</td>
</tr>
<tr>
<td>Bone</td>
<td>5/25</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>4/25</td>
</tr>
</tbody>
</table>
Figure 24: Photomicrographs of primary prostate carcinoma, in vitro cell growth and subcutaneous xenograft.
Figure 25: Immunohistochemistry of the primary prostate carcinoma
Figure 26: Gene expression profile of Leo cells
Figure 27: *In vitro* and *in vivo* growth patterns of Leo cells.
Figure 28: *In vivo* BLI and histopathological evaluation of nude mice 28 days following intracardiac injection of Leo$^{\text{YFP-Luc}}$ cells
Figure 29: Evaluation of bone metastasis on day 28 following intracardiac injection of Leo$^{YFP-Luc}$ in nude mice
Figure 30: Dose-response of Leo cell viability to calcitriol (A), velcade (B), (S)-HDAC-42 (C) and piroxicam (D) treatment
CHAPTER 5

SYNOPSIS AND FUTURE DIRECTIONS

Bone metastasis is the most common complication of advanced prostate cancer patients in the USA, although soft tissue metastases can also occur (1). Osteoblastic bone metastases are characterized by formation of excessive poor quality woven bone and are the predominant phenotype in the bone metastases of prostate cancer patients (2). As osteoblastic metastases enlarge they cause pain, spinal cord compression and pathological fractures resulting in poor quality of life (2, 3).

Although osteoblastic metastases are a primary concern of prostate cancer patients, mechanisms causing development of the osteoblastic phenotype are not clearly understood (4). Experiments presented in the second chapter of the dissertation demonstrated that osteoblastic metastases in prostate cancer are independent of osteoclast activity. These data suggest that prostate cancer secretes osteogenic factors that directly induce osteoblastic bone formation.

Data in chapter three showed that prostate cancer cells produced Wnt proteins that were important for the induction of bone formation and osteoblastic metastases. The Wnt antagonist, Dkk-1, decreased Ace-1-induced new bone
formation. Although inhibition of Wnt signaling inhibited the osteoblastic phenotype of prostate cancer bone metastases, Dkk-1 surprisingly increased prostate cancer growth and bone metastasis incidence in a JNK-dependent manner since, Dkk-1 increased phosphorylation of the JNK 46 isoform.

Finally, data in the fourth chapter reported the establishment of a new dog prostate cancer cell line that developed a high frequency of brain metastasis in addition to bone and the adrenal gland. Since brain metastasis is a terminal event with median survival of less than 9 months from the time of diagnosis (5), this model will be useful in studies to investigate pathogenesis of prostate cancer metastasis to brain.

These data demonstrate that dogs, which are the only mammals that develop spontaneous prostate cancer similar to men, are a valuable source of prostate cancer to develop new animal models to investigate the molecular pathogenesis of prostate cancer bone metastases. Chapter two reported that prostate cancer cells either directly secrete factors that are osteogenic or stimulate the bone microenvironment to form woven bone at sites of metastasis in bone.

Using in vitro osteoblast differentiation and mineralization assays we were unable to recapitulate the osteoblastic phenotype of bone metastases of Ace-1 cells that was seen in vivo. Therefore, it is speculated that the bone microenvironment might change prostate cancer gene expression by shifting the balance of
osteoblastic and osteoclastic factors towards an osteogenic potential. Therefore it will be important to understand the bone microenvironment in prostate cancer. Data from the chapter three raised questions about the role of Wnts in the prostate cancer growth. Therefore, in future, it will be important to understand the role of individual Wnts and Wnt signaling using prostate cancer animal models. In addition, although Dkk-1 is well characterized as a Wnt signaling antagonist, further experiments should to be conducted to identify the Wnt-independent mechanisms such as interaction with other major molecular signaling pathways in cancer including survival and apoptotic pathways. The investigations showed that dogs will be useful to model and investigate the molecular pathogenesis of prostate cancer and bone metastases. Our studies demonstrated that prostate cancer directly induced the osteoblastic phenotype of bone metastases and investigating the factors responsible will be helpful to design novel therapeutics.
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