

The regulatory role of osteoblasts in castration-resistant growth of prostate cancer

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UNIVERSITY OF GOTHENBURG

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Cover illustration: Immunohistochemical staining of RUNX2 in an osteoblastic tumor of castration-resistant prostate cancer

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To my parents

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ABSTRACT

Bone metastasis of a predominantly osteoblastic (sclerotic) nature is the outcome for the vast majority of patients with castration-resistant prostate cancer (CRPC). Pathologically, osteoblastic tumors are characterized by excessive bone formation resulting in decreased quality of life due to severe pain, fractures, nerve compression, and a suppressed immune system. Despite the success of novel therapeutic approaches, castration-resistant tumors remain the primary unsolved obstacle for patient survival. Therefore, an improved understanding of the molecular mechanisms behind the osteoblastic growth of CRPC is important in the search for novel therapeutic strategies. The aim of this thesis was to investigate the specific role of osteoblasts in the growth of prostate cancer in bone. By establishing and characterizing a novel model of sclerotic CRPC, it was demonstrated that both osteoblasts and prostate cancer cells are potential mediators of bone formation. It was further demonstrated that osteoblasts promote the osteogenic and metastatic progression of CRPC cells and potentiate the cross talk between CRPC and bone cells. Moreover, it was shown that osteoblasts induce and alter steroidogenesis in the CRPC cells by increasing the expression of steroidogenic enzymes in a similar manner to what has previously been described in bone metastases from patients. Further studies revealed that Runt-related transcription factor 2 (RUNX2) – which is under the control of osteoblasts – is a putative regulator of *de novo* steroid synthesis in osteogenic CRPC cells, and this mimics a mechanism of steroid synthesis previously only described in osteoblasts. Finally, a preclinical study with tasquinimod showed that this drug efficiently impaired the establishment of bone metastases in mice by interfering with the osteoblastic pre-metastatic niche and osteoblastic activity, thus emphasizing the role of osteoblasts in the early phases of the metastatic process. In summary, the studies performed in this thesis have characterized the role of osteoblasts in castration-resistant growth of prostate cancer in bone and suggest that osteoblasts could be an attractive target for the development of novel therapeutic approaches. A better understanding of the osteoblast–tumor cell interaction might facilitate the design of treatment strategies targeting the osteoblasts as a way to inhibit the metastatic process and thus bypass the castration resistance of CRPC bone metastases.

Keywords: castration-resistant prostate cancer, bone metastases, osteoblasts

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Prostatacancer är idag den cancerform som både drabbar flest och står för den högsta cancerrelaterade dödligheten bland svenska män. Varje år diagnostiseras cirka 9200 män och ca 2300 av dessa kommer att dö av sjukdomen. Vid tidig upptäckt är prognosen god, då canceren är lokaliserad i prostatan, men vid en spridd (metastaserad) sjukdom finns ingen botande behandling. I dessa fall behandlas canceren med kastrationsbehandling, med syftet att blockera produktionen av manligt könshormon som cancertumören är beroende av för sin tillväxt. För majoriteten av patienter är behandlingseffekten initialt god och har en hämmande effekt på tumören men effekten är tyvärr inte bestående. Inom loppet av två år övergår ca 80 % av fallen till en mer aggressiv form med spridning till andra delar av kroppen utanför prostatan. Denna form kallas kastrationsresistent prostatacancer (CRPC) och innebär att cancertumören kan växa trots sänkta nivåer av manligt könshormon. Det finns flera föreslagna mekanismer till hur tumören anpassar sig till fortsatt tillväxa utan könshormonet. En av förklaringarna är att tumörcellerna själva börjar producera de könshormoner de behöver för sin tillväxt. CRPC uppkommer huvudsakligen som metastaser i skelettet och bildar, till skillnad från andra cancerformer som ofta bryter ner ben, en ökad benmassa. Mekanismerna bakom tumörtillväxten i skelettet och vilken betydelse cellerna i benet har i denna process är till stor del okänt. För patienter med tumörer i skelettet är överlevnaden kort och ofta förenad med svåra smärtor på grund av tumörens växtsätt. På grund av svårigheter att få tillgång till kliniskt material från denna patientgrupp och bristen av modeller som liknar den kliniska bilden är utvecklandet av nya experimentella modeller av största vikt för att kunna studera bakomliggande mekanismer för denna idag obotliga sjukdom. Syftet med denna avhandling var att studera samspelet mellan osteoblaster, de benbyggande cellerna i skelettet, och tumörceller i CRPC i ben. Målet var att öka förståelsen för hur osteoblastiska benmetastaser bildas och växer och därmed hitta nya sätt att behandla dessa tumörer.

I denna avhandling karaktäriserades en ny modell, LNCaP-19, för att möjliggöra studier av tumörtillväxten av CRPC i ben. I denna modell påvisar vi hur osteoblaster driver på aggressiva egenskaper i tumörcellerna samt stimulerar dessa att förvärva benlika egenskaper som gör att de bättre smälter in i benmiljön. Vidare visar vi att samspelet mellan osteoblaster och tumörcellernas förmåga att själva bilda de könshormoner som kastrationsbehandlingen blockerar. Osteoplasterna påverkar tumörcellernas produktion av könshormon på ett sätt som stämmer väl överrens med det man sett tidigare i benmetastaser från patienter. Avhandlingen visar även att ett

ökat uttryck av RUNX2, ett protein som är viktigt för osteoblasters funktion, ökar i tumörcellerna genom samspelet med osteoblaster. Denna ökning av RUNX2 visas vara en nyckel till tumörcellernas förmåga att bilda könshormoner. I det avslutande arbetet utvärderas effekten av tasquinimod, en läkemedelskandidat, på den osteoblastiska tumörtillväxten av CRPC i ben genom den etablerade modellen. Detta läkemedel visade sig effektivt hämma bildandet av tumörer i skelettet genom att förändra egenskaper i benmiljön samt genom att angripa osteoblaster i det området i skelettet där tumörcellerna helst etablerar sig för att bilda metastaser.

Sammantaget visar denna avhandling att osteoblaster har en nyckelroll i benmetastaser av prostatacancer genom att anpassa tumörcellerna till miljön i skelettet, samt bidra till en kastrations-resistent tumörtillväxt genom att öka den egna produktionen av könshormon i tumörcellerna. Behandling med en läkemedelskandidat, tasquinimod, blockerar etableringen av tumören i skelettet genom att bland annat angripa osteoblaster i benmiljön. En potentiell framtida behandlingsstrategi skulle kunna vara att kombinationsbehandla prostatacancer med kastrationsbehandling och läkemedel som angriper osteoblaster och därmed förhindra metastasering till skelettet. Sammanfattningsvis visar detta avhandlingsarbete att osteoblaster utgör en potentiell måltavla för behandling vid prostatacancer.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

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- I. **Hagberg Thulin, M.**, Jennbacken, K., Damber, JE., Welén, K. Osteoblasts stimulate the osteogenic and metastatic progression of castration-resistant prostate cancer in a novel model for in vitro and in vivo studies, *Clin. Exp. Metastasis* 31 (2014) 269–283.
- II. **Hagberg Thulin, M.**, Nilsson, ME., Thulin, P., Céraline, J., Ohlsson, C., Damber, JE., Welén, K. Osteoblasts promote castration-resistant prostate cancer by altering intratumoral steroidogenesis. Submitted manuscript
- III. **Hagberg Thulin, M.**, Damber, JE., Welén, K. Putative role of RUNX2 in regulation of de novo steroidogenesis in osteoblastic CRPC. In preparation
- IV. Magnusson, L., **Hagberg Thulin, M.**, Olsson, A., Damber, JE., Welén, K. Tasquinimod inhibits prostate cancer growth in bone through alterations in the bone

CONTENT

ABBREVIATIONS	10
INTRODUCTION	12
GENERAL BACKGROUND TO PROSTATE CANCER.....	12
Incidence, cause and implications	12
Diagnosis and prognosis.....	12
The prostate – anatomy and function.....	13
Morphology of the normal and the malignant prostate	13
Endocrine regulation and growth of the normal and malignant prostate.....	14
Treatment of prostate cancer	15
CASTRATION RESISTANT PROSTATE CANCER.....	16
Intratumoral androgen synthesis.....	17
Androgen receptor mutations	19
BONE METASTASIS IN PROSTATE CANCER	19
Bone – a mineralized tissue	20
Bone cells	20
Bone remodeling	21
The vicious cycle of prostate cancer bone metastases.....	22
Hormonal regulation of bone.....	23
Osteotropism.....	24
The way to the bone – the metastatic process.....	25
Epithelial mesenchymal transition.....	25
Detachment, migration, attachment and colonization	26
Osteomimicry	27
The bone metastatic niche and tumor cell dormancy	27
Osteoimmunology and bone metastases.....	29
Pathophysiology of prostate cancer bone metastases	31
Markers of bone metastases.....	31
Treatment of bone metastatic CRPC	32

AIMS OF THE THESIS	34
METHODOLOGICAL CONSIDERATIONS	35
<i>IN VITRO</i> EXPERIMENTS.....	35
<i>IN VIVO</i> EXPERIMENTS.....	38
RESULTS AND COMMENTS	41
PAPER I.....	41
PAPER II	44
PAPER III	47
PAPER IV	49
GENERAL DISCUSSION	51
Models of prostate cancer bone metastases	51
Osteolytic versus osteoblastic tumors.....	53
The role of RUNX2 in osteolytic versus osteoblastic prostate cancer... 54	
ADT and prostate cancer bone metastasis	55
Intratumoral steroidogenesis in osteoblastic CRPC.....	57
Therapeutic approaches for osteoblastic metastasis of CRPC.....	60
CONCLUSIONS	62
ACKNOWLEDGEMENTS	63
REFERENCES	66

ABBREVIATIONS

ADT	Androgen deprivation therapy
ALP	Alkaline phosphatase
AR	Androgen receptor
bALP	Bone ALP
BMD	Bone mineral density
BMDC	Bone marrow myeloid stem cell
CDH2	N-cadherin
cDNA	Complementary DNA
CRPC	Castration-resistant prostate cancer
CTC	Circulating tumor cell
DHT	Dihydrotestosterone
DTC	Disseminated tumor cell
ECM	Extracellular matrix
E2	Estradiol
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ER α	Estrogen receptor alpha
ER β	Estrogen receptor beta
ET-1	Endothelin-1
FCM	Fibroblast-conditioned media
FGF	Fibroblast growth factor
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cells
IHC	Immunohistochemistry
IL-	Interleukin-
MET	Mesenchymal epithelial transition
mRNA	Messenger RNA
MDSCs	Myeloid derived suppressor cells
MMP	Matrix metalloproteinase
MSC MSC-	Mesenchymal stem cell
F GC/MS-	Macrophage stimulating factor
MS OCM	Gas chromatograph/tandem mass spectrometry
OPG	Osteoblast-conditioned media
PAP	Osteoprotegerin
PC	Prostatic acidic phosphatase
PSA	Prostate cancer
PTHrP	Prostate specific antigen
pQCT	Parathyroid hormone-related peptide
	Peripheral quantitative computed tomography

RANK	Receptor activator of nuclear $\kappa\beta$
RANKL	Receptor activator of nuclear $\kappa\beta$ ligand
RT-PCR	Reverse transcriptase polymerase chain reaction
RUNX2	Runt- related transcription factor 2
siRNA	Small interfering RNA
SNOs	Spindle-shaped N-cadherin positive Osteoblasts
SRD5A	5 alpha-reductase
SRE	Skeletal related events
TAMs	Tumor-associated macrophages
TNF- α	Tumor necrosis factor alpha

INTRODUCTION

GENERAL BACKGROUND TO PROSTATE CANCER

Incidence, cause and implications

Prostate cancer is the most common malignancy in men in Western countries and represents the second leading cause of cancer-related deaths [1]. The incidence of prostate cancer has increased during the last decades probably due to longer life span, but also due to the introduction of the prostate specific antigen (PSA) test in the clinic [2]. Despite good prognosis and recent advance in the management of locally defined disease, prostate cancer accounts for the highest death rate of cancer in Sweden (Cancer incidence in Sweden 2014, Socialstyrelsen). The vast majority of prostate cancer deaths are related to castration resistant bone metastases.

Prostate cancer is a highly heterogeneous disease in aging men, and the majority of cases occur in men over 60 years of age [3]. The cause of prostate cancer is multifactorial and several risk factors have been implicated in development of the disease. Epidemiological studies show that prostate cancer incidence and mortality incidence is highest in the US and Northern Europe with Sweden at the top – and lowest in Asia. Diet and lifestyle seem to influence the risk of prostate cancer development [3], and this is supported by the fact that US immigrants of Asian origin will eventually develop the same risk of prostate cancer as Americans. In addition several genes associated with prostate cancer have been identified [4-6], suggesting that genetic background might also be a risk factor.

Diagnosis and prognosis

In most cases, primary prostate cancer does not present with symptoms, and the cancer is detected by routine blood tests where elevated PSA levels might be indicative of cancer. There is an ongoing debate on the benefit of PSA testing due to its limited diagnostic specificity and low predictive value [7-9]. Traditionally, a PSA serum value < 3 ng/mL is considered normal and a PSA value > 10 ng/mL indicates a substantial risk of prostate cancer. A PSA value > 100 ng/mL indicates metastatic disease[10]. After a positive PSA test, digital rectal examination is performed to find a potential tumor and ultrasonography-guided biopsies are taken for histological examination to verify the diagnosis.

The Gleason system is used to grade tumors histologically from tissue-derived biopsies, and this system classifies tumors from 2-5 where 5 is the most malignant grade [11]. An overall Gleason score is the sum of two Gleason grades, the first is the most common grade in all samples and the second is the highest grade of what is left. The most common clinical classification system is the TNM (tumor, lymph node, and metastasis) system. The TNM classification system takes into account tumor volume, number of lymph nodes involved, and whether there are distant metastatic lesions. According to the TNM system, T1 and T2 stage tumors are still confined to the prostate. In stage T3 and T4, the tumors are locally advanced, and might have spread to organs outside the prostate (Union for International Cancer Control). In case of suspected metastasis, further investigations to determine metastatic spread are performed with bone scintigraphy, magnetic resonance imaging (MRI), computed tomography (CT) and sometimes positron emission (PET)/CT.

The prostate – anatomy and function

The prostate gland is a walnut shaped exocrine organ located in front of the rectum, below the urinary bladder, and surrounding the urethra. The main function of the prostate is to produce and secrete an acidic fluid consisting of proteins important for sperm motility and viability. The most abundant proteins found in secretions of the prostate are PSA and prostatic acidic phosphatase (PAP), both of which are used as clinical markers for prostate cancer. The prostate can be divided into three distinct zones: the peripheral, central and transitional zones [12]. The majority of benign prostatic hyperplasia lesions occur in the transitional zone, while most cancer arises in the peripheral zone [13-15] (**Figure 1A**).

Morphology of the normal and the malignant prostate

The prostate gland is enclosed by a fibromuscular capsule surrounded by stromal tissue. The prostate consists of three different cell types of epithelial origin, the luminal cells, the basal cells and the neuroendocrine cells. The luminal cells constitute the majority of cells and are terminally differentiated, express androgen receptor (AR) and require androgens for survival [16, 17]. Basal cells express low or no levels of AR and are not dependent on androgens for survival and growth [16, 18, 19]. It is believed that the basal membrane harbors stem cells or progenitor cells that can proliferate and differentiate into luminal cells in the presence of androgens and can repopulate the luminal layer if needed [20-23]. The neuroendocrine cells represent a small population differentiated AR negative cells [24, 25].

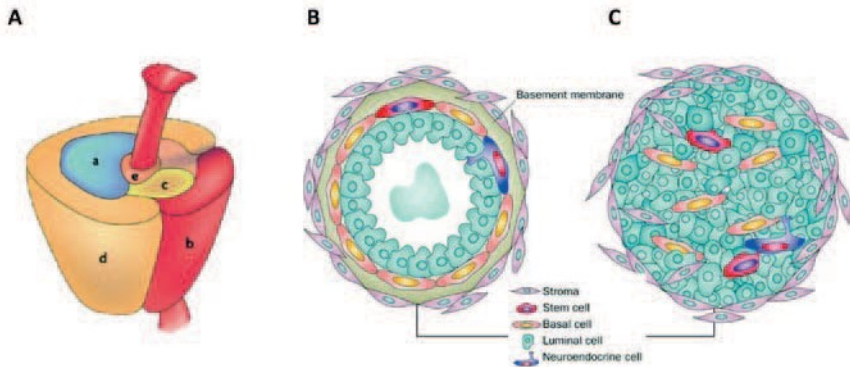


Figure 1. Anatomy and morphology of the normal and malignant prostate *A*) Illustration of the prostatic zones. Prostate cancer most often arises from the peripheral zone (*d*) whereas benign prostatic hyperplasia mainly develops in the transitional zone (*c*). *B*) Illustration of the cellular composition of the normal prostate and *C*) in primary prostate cancer. The distinct cell layers and cell compositions are rearranged in the primary tumor of prostate cancer. Adapted and modified from [26] and [27].

The surrounding stroma is biologically heterogeneous and composed of smooth muscle cells, endothelial cells, nerve cells, fibroblasts, dendritic cells and infiltrating immune cells along with growth factors, cytokines and numerous extracellular matrix (ECM) components. Fibroblastic stromal cells express AR and are androgen responsive [28-30]. The fibroblastic stromal cells produce growth factors in an androgen-dependent manner and the crosstalk between stroma and epithelial cells is an important regulator of prostate growth and differentiation [31].

In the malignant prostate, there exists a communication between prostate tumor cells and the stromal cells [18]. Interactions via paracrine signaling between tumor cells and stroma factors released from the tumor microenvironment are required for invasion, angiogenesis and metastasis of cancer cells to ectopic sites [19-21]. It is therefore generally believed that the stroma cells are important regulators of prostate cancer initiation and progression.

Endocrine regulation and growth of the normal and malignant prostate

The development, growth and function of the prostate are strictly dependent on androgens. Androgenic action in the prostate is primarily mediated by dihydrotestosterone (DHT), which is derived predominantly from the reduction of testosterone (T) or indirectly via adrenal dihydroepiandrosterone (DHEA). The cellular response to androgens is mediated via AR. Both T and DHT can bind to AR but DHT has stronger binding affinity and is thus a

more potent metabolite [32]. The catalysis of T to DHT occurs by locally produced 5 alpha- reductases (SRD5A) in the epithelial and stromal cells of the prostate and peripheral tissue [33, 34]. The testicular production of T accounts for 90–95% of the circulating androgens, and the remaining 5–10% is produced by the adrenal glands [35, 36]. Androgen levels are predominantly regulated through the hypothalamic-pituitary-adrenal /gonadal axis. The production of androgens is regulated by the hypothalamus through the secretion of gonadotropin-releasing hormone and the weak androgens – androstenedione and DHEA – which stimulate secretion of luteinizing hormone from the pituitary gland. Secreted luteinizing hormone stimulates the Leydig cells to produce and secrete T [36]. In addition, the corticotrophin-releasing hormone released by the hypothalamus induces secretion of adrenocorticotrophic hormone in the pituitary gland.

Treatment of prostate cancer

For localized prostate cancer, treatment methods such as surgery (radical prostatectomy) or radiation therapy can often cure the cancer. If the cancer is detected early and the life expectancy of the patient is long, active surveillance might be an initial option. For patients with locally advanced and metastatic prostate cancer, there is currently no cure and the therapy is given in the form of castration therapy. Androgen deprivation therapy (ADT) has been the mainstay of treatments for advanced prostate cancer since the recognition of the disease as being androgen-sensitive by Huggins and Hodges in 1941 [37]. The clinical use of ADT include medical therapies such as luteinizing hormone-releasing hormone agonist/antagonists or estrogens that target the hypothalamic-pituitary-gonadal axis [37], and this treatment leads to “chemical castration” with suppression of T from the testis and direct inhibition of AR action for patients with locally advanced disease or metastatic prostate cancer. The efficacy of ADT is based on achieving castration levels of serum T, defined as $< 20\text{ng/dL}$. This approach initially results in a beneficial suppression of tumor growth as evidenced by decreased tumor burden, decreased PSA levels and regression of symptoms in the majority of patients. However, regardless of the timing and nature of ADT, relapse of castration-resistant disease (CRPC) with bone metastases will occur. Despite initial good response to ADT, the majority of patients will experience disease progression/relapse within 24 months as evidenced by increasing PSA, radiological progression and/or progression of disease-related symptoms [38-40]. The mechanisms contributing to the development of castration resistance in metastases are not clear. However, it is known that acquired resistance to ADT often coincides with progression of metastasis to bone tissue [41]. Although prostate cancer also metastasizes to lymph nodes, these metastases are seldom resistant to therapy, suggesting that prostate

cancer has a unique relationship to the prostate and bone microenvironments [42, 43]. An adverse effect of castration/ADT is the negative effect on bone. In fact, studies have shown that men receiving ADT are four times more likely to develop significant bone deficiency [44].

The AR is composed of three domains. The COOH-terminal ligand binding domain binds androgens and anti-androgens, such as bicalutamide. The ligand binding domain of the AR contains a weak activation function-2 region and is separated from the highly conserved DNA binding domain by a hinge region that mediates nuclear localization [45]. The DNA binding domain is centrally located in the AR and binds to androgen response elements in upstream regulatory regions of androgen regulated genes, such as PSA. The NH₂-terminal domain [46, 47] is the most variable in terms of sequence homology between species and contains the activation function-1 region required for transactivation (reviewed in [48]). The inactive AR is predominantly located in the cytoplasm bound to heat shock proteins [49]. Upon ligand binding, cytosolic AR undergoes conformational changes including interactions between the C-terminal and N-terminal domains and dissociation from the heat shock protein, and this enables interactions with co-regulatory factors such as ARA70 [50]. The transformed AR undergoes dimerization, phosphorylation and translocation into the nucleus [51]. In the nucleus the AR dimer binds to androgen response elements located in the promoter or enhancer region of AR target genes [52], and it recruits various co-activators and RNA polymerase II to induce the transcription of AR-regulated genes needed for normal prostate function [53-55].

CASTRATION RESISTANT PROSTATE CANCER

It has also become evident that CRPC tumors are not androgen-independent because reactivation of the AR is frequently found in CRPC [56] and intratumoral androgen levels are maintained as levels sufficient to activate AR signaling pathways [34, 57-60]. Despite the fact that castration leads to low levels of circulating T (<50 ng/dL), castration does not eliminate androgens from the prostate tumor microenvironment. It has been shown that DHT and T in castrated men with locally recurrent CRPC are further elevated relative to serum levels, while tissue T levels in metastatic CRPC might actually be higher than in the prostate prior to castration [34, 60-62]. Several mechanisms by which prostate cancer cells can escape ADT, and restore AR activity have been described [48, 63]. The AR might become hypersensitive to DHT, it might become activated by other ligands than DHT, or it might become activated in the absence of a ligand. Furthermore, androgen signaling pathway might be completely by passed, or the tumor cells might begin to

express enzymes enabling the *de novo* synthesis of intratumoral androgens invoking an autocrine or paracrine mechanism for the development of CRPC [51, 64, 65].

Studies have shown that 20–30% of locally recurrent CRPC tumors harbor AR amplification [66-68]. Increased expression of AR enables AR-mediated signaling even at extremely low levels of DHT [51, 56, 69]. AR amplifications are rarely seen in hormone naïve prostate cancer, suggesting that amplification is selected for during emergence to CRPC. In addition, AR splice variants lacking the ligand binding domain are proposed to be constitutively activated [70]. AR regulates gene expression through recruitment of co-regulators complexes, and these co-regulators might act to enhance transcription or to suppress transcription of AR target genes [71, 72]. *In vitro* studies have shown that changes in components of the co-regulatory complex can modulate AR stability leading to an increase in overall AR activity and to broadened ligand specificity, in particular at low androgen levels [73]. Several co-activators such as members of the SRC family (SRC-1, SRC-2/TIF-2, SRC-3), TIP60 and ARA70 have been reported to be increased in CRPC [74-77]. The AR can also be activated in an androgen-independent manner by a number of factors, including interleukin-6 (IL-6), insulin growth factor-1 (IGF-1), epidermal growth factor (EGF) and cAMP [78-80]. Because the bone environment harbors many of these growth factors it has been suggested that bone-derived factors might facilitate the survival of prostate cancer and its progression to androgen independence by cross talk with the AR and alternative signal transduction pathways [81-83]. For example, soluble factors derived from osteoblasts have been shown to bind and transactivate AR, suggesting that AR might play a role in the progression of prostate cancer by a mechanism initiated by factors secreted from osteoblasts [84, 85].

Intratumoral androgen synthesis

There seems to be a gradual shift during prostate cancer progression from dependence on androgens from endocrine sources to dependence of androgens from paracrine, autocrine and intracrine sources [86]. Metastatic CRPC display a pattern of up-regulated steroidogenic enzymes, which could explain the elevated local levels of DHT and T found in bone metastases [60, 65, 87-89]. Intratumoral steroidogenesis might be initiated either via the uptake of weak adrenal gland precursors from DHEA [90] or via *de novo* steroidogenesis from cholesterol (**Figure 3**) [64, 91].

Several studies have identified increased expression of enzymes mediating the synthesis of T and DHT from weak adrenal gland precursors. Among

these the expression of *AKR1C3*, *SRD5A1* and *HSD3B2* have been reported in bone marrow biopsies of CRPC [60, 65, 87]. Besides the ability of prostate cancer cells to utilize weak adrenal androgens for adaptation to ADT, it has now been shown that cholesterol synthesis might be increased in CRPC [92, 93]. In a recent study investigating the metabolomics in CRPC bone metastases, increased cholesterol was demonstrated in bone metastatic tissue of CRPC [94]. Whether CRPC cells can synthesize physiologically significant amounts of androgen *de novo* from cholesterol is less clear. However, the enzymes required for *de novo* steroid synthesis including *CYP11A1*, *CYP17A1* and *HSD3B1/2* have been detected in metastatic CRPC bone marrow biopsies [65, 88, 95].

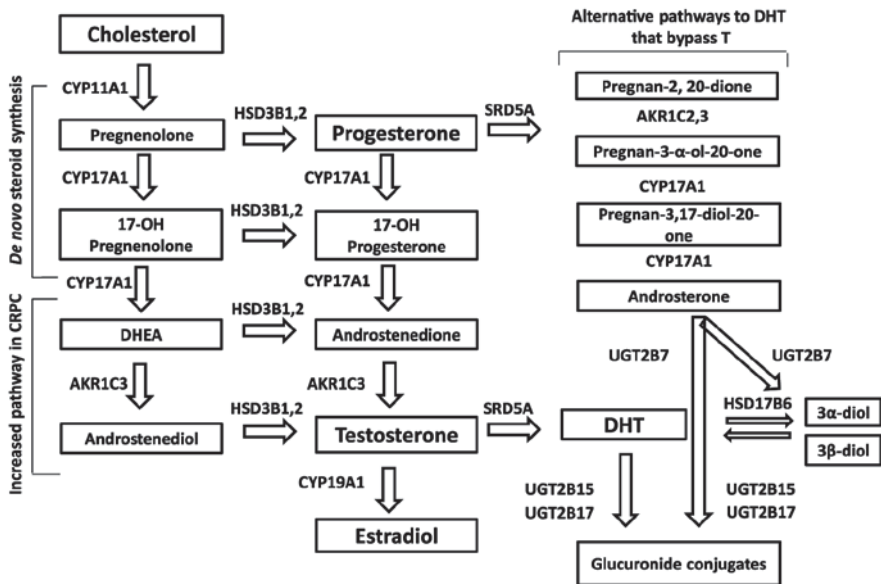


Figure 2. Androgen synthesis pathways in prostate cancer. Pathways that might contribute to androgen synthesis in CRPC are outlined. Redrawn and modified from [96]

Several reports have also shown increased expression of *CYP19A1*, which indicates increased synthesis of E2 from T [97, 98]. In addition, studies on CRPC bone metastases have shown increased expression of UDP glycosyltransferase 2, B15 (*UGT2B15*), which in conjunction with *UGT2B17* mediates glucuronidation of DHT metabolites [65]. Together these observations strongly suggest that the increased expression of androgen-metabolizing genes within metastatic castration-resistant tumors might contribute to the outgrowth of castration-adapted tumors (reviewed in [99]).

Androgen receptor mutations

AR mutations are rarely found in the early phase of prostate cancer [100, 101] but they are highly prevalent in advanced and metastatic CRPC, especially in those treated with ADT [102], suggesting that AR mutations play a role in tumor progression [102, 103]. In a recent summary of 27 studies, it was reported that AR mutations ranged from 10% to 40% in CRPC compared to 2% to 25% in androgen sensitive tumors [72]. Most of the mutations are found in the ligand binding domain, and these mutations result in broadened ligand specificity that allows binding of non-androgen ligands such as DHEA progesterone, estrogen and cortisol [104-108]. The most frequently reported mutation in prostate cancer is a substitution of threonine to alanine at amino acid 877 (T877A). This point mutation was initially described in the LNCaP cell line [109] and has also been found in clinical samples [110].

BONE METASTASIS IN PROSTATE CANCER

It has long been recognized that primary cancers spread to distant organs with characteristic preferences [111] and the skeleton is a major metastatic site of several carcinomas. In this regard, prostate and breast cancers are the most common malignancies that metastasize to bone, hence they are referred to as osteotropic cancers. Metastases to bone occur in about 70% of all patients with prostate and breast cancers. Bone metastases represent 98% of malignant bone tumors and are the most frequent occurring metastasis occurring in prostate cancer [112]. Around 90% of patients with metastatic prostate cancer will develop bone metastases. For these patients, the prognosis will be dramatically changed, and there will be increased morbidity and a drastic fall in survival expectancy. Once tumor cells have entered the bone prostate cancer cannot be cured [113]. As a result the majority of men with CRPC die from bone metastatic disease within 2 – 3 years [114]. In every second patient bone metastases lead to so-called skeletal-related events (SREs), which include pathological fractures, spinal cord compression and severe bone pain requiring palliative radiotherapy, and/or orthopedic surgery and subsequently an impaired health-related quality of life and reduced survival [115].

Bone metastases behave differently depending on their tumor origin. Typically, breast and lung cancer form osteolytic metastases due to enhanced activity of bone-resorbing cells, the osteoclasts, resulting in increased bone degradation [116-119]. Bone metastases from prostate cancer are predominantly characterized by increased bone mass due to the exaggerated activity of the bone-forming cells – the osteoblasts. These types of tumors are

referred to as osteosclerotic or osteoblastic [117, 118, 120-123]. This unique phenotype suggests that osteoblasts are of particular importance for the bone metastatic disease of prostate cancer.

Bone – a mineralized tissue

Bone provides structural and protective functions and stores calcium, and the bone marrow is the major hematopoietic organ, and a primary lymphoid tissue. Bone tissue consists of a fibrillous network made up of collagens and non-collagenous proteins. The main component is type 1 collagen (COL1A1) which accounts for 95% of the extracellular bone matrix and the remaining 5 % includes a variety of non-collagenous proteins such as bone morphogenetic proteins (BMPs), bone sialoprotein and osteocalcin (OCN). The mineralized matrix consist of hydroxyapatite $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ and crystal depositions.

Bones are divided into long bones (e.g. the tibia, femur, and humerus) and flat bones (e.g., the skull, ileum, and mandible). Both types are composed of cortical (compact) bone and trabecular (cancellous) bone. The trabecular bone is metabolically active and has, in contrast to the compact cortical bone, unorganized, porous matrix. The cortical bone is 80 - 90% calcified and constitutes the protective layer of bone whereas the trabecular bone is only 15 - 25% calcified and is located in the interior of the bone, near the ends of the bone marrow cavity. Long bones are anatomically divided into three sections; epiphysis, diaphysis and metaphysis. The metaphysis is located just below the growth plate near the ends of bone and is mainly composed of trabecular bone, surrounded by blood vessels, hematopoietic marrow and fatty marrow [124].

Bone cells

Osteoblasts are the bone-forming cells and account for 4-6% of the total resident cells in the bone. Osteoblasts are found lining the layer of the bone matrix that they are producing before it is calcified. This layer is referred to as osteoid, which will mature to form calcified matrix. Osteoblasts arise from local mesenchymal stem cells (MSCs), which are the precursors for many cell types in the bone that are involved in bone formation, including chondrocytes, fibroblasts, myoblasts, adipocyte and neural cells [125].

To become osteoblasts the MSCs must undergo a strictly regulated differentiation process with sequential steps of proliferation, be committed to pre-osteoblasts producing alkaline phosphatase (ALP), and subsequently mature osteoblasts producing osteocalcin and calcified matrix [126]. The transcription factors Runt- related transcription factor 2 (RUNX2) and the downstream factor osterix are crucial for the commitment of the osteoblast lineage and for driving differentiation process to become mature mineralizing

osteoblasts. After maturation, osteoblasts undergo apoptosis, remain as bone lining cells or become embedded in the bone matrix and differentiate into osteocytes. A small fraction remain on the bone surface, becoming flat lining cells or become osteocytes (up to 30%) [127]. The BMP, Hedgehog and Wnt signaling pathways are three major pathways known to regulate the commitment of MSCs to the osteoblast lineage. These pathways are activated by for example parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), fibroblast growth factors, transforming growth factor beta (TGF- β), sex steroids, and other hormones (reviewed [128]). Besides being crucial for the bone forming process, osteoblasts have been implicated as a key regulator in several physiological and malignant contexts. Osteoblasts participate in osteoclast formation by secreting osteoclast stimulatory factors, such as macrophage colony stimulating factor 1 (CSF-1) and the receptor activator of nuclear factor $\kappa\beta$ ligand (RANKL) on their surface [129]. There is also evidence that osteoblasts have an endocrine function (reviewed in [130]). Moreover, osteoblasts regulate hematopoietic stem cells (HSCs) in the bone marrow niche. Exaggerated osteoblast activity also suggests an important role in the bone metastatic process of prostate cancer.

Osteoclasts are specialized multinucleated macrophage-like cells with bone resorptive capacity that arise from the HSC monocyte/macrophage lineage. Macrophage colony stimulating factor (M-CSF) tumor necrosis factor alpha (TNF α) and RANKL are important growth factors that support osteoclastogenesis, and they are primarily produced by osteoblasts. The bone resorption process by osteoclasts occur by generating an isolated microenvironment between the cell's plasma membrane and the bone surface in which matrix mineral is mobilized in an acidic milieu, and the organic matrix is degraded by the lysosomal protease cathepsin K. Osteoclasts are important for the development of osteolytic metastases.

The **osteocyte** is the most abundant cell type in bone, representing 95% of all bone cells in mature bone tissue [131]. Osteocytes arise at the end of the mineralization phase from the osteoblast lineage after its entrapment in bone matrix [132, 133]. Osteocytes produce sclerostin [134] and are believed to play a primary role in directing bone remodeling via RANK/RANKL. However the impact of osteocytes in osteoblast regulation is controversial and not fully characterized (reviewed in [135]). The role of osteocytes in prostate cancer remains to be investigated.

Bone remodeling

Bone remodeling is a continuous process that is vital to maintain calcium stores and bone homeostasis [136]. Under physiological conditions, the

number and activity of osteoclasts and osteoblasts are balanced so that the bone resorption and formation is equivalent. The remodeling occurs in so called basic multicellular units, in which both osteoclasts and osteoblasts cooperate in a remodeling cycle [137-139]. This cycle starts with recruitment of monocytes to the bone surface. Osteoblast secreted RANKL binds to the RANK receptor on the surface of monocytes to form pre-osteoclasts. In the presence of CSF-1, RANKL further promotes the fusion of pre-osteoclasts to become mature multinucleated osteoclasts. The osteoclast starts resorption by digesting the mineralized matrix. At the end of the resorption phase pre-osteoblasts migrate to the resorption site where they mature and start forming new bone by producing matrix (osteoid), which is subsequently mineralized. A network is formed in the bone by the osteocytes, osteoblasts and bone lining cells, and this network responds to signals such as mechanical load and specific metabolic and hormonal requirements. These signals are integrated in the basic multicellular units leading to a controlled remodeling process.

Bone remodeling is regulated both systemically and locally. The major systemic regulators include PTH, calcitriol, glucocorticoids and estradiol (E2). It is now well known that this process can be corrupted by tumor cells and associated immune cell infiltrates to provide a favorable growth environment for bone metastases [116, 140]. The role of the OPG/RANKL system has been studied in patients with osteotropic tumors such as those from breast, lung and prostate in relation to their bone metastatic phenotype. Osteolytic tumors appear to exert their osteolytic actions through the up-regulation of the OPG /RANKL system, whereas prostate cancer seems to provoke profound elevations of OPG, thus promoting a shift toward increased osteoblastic activity [141].

The vicious cycle of prostate cancer bone metastases

The reciprocal communication between tumor cells, bone cells and the bone microenvironment fuels a vicious cycle of tumor growth and bone remodeling. The phenomenon referred to as “the vicious cycle” was first coined in the context of breast cancer metastases by G. Mundy [142] et al in 1997, and this term described the cross talk in osteolytic tumors. Once the tumor cells enter the bone, growth factors will be released from the bone matrix. The bone matrix is a store house of latent growth factors such as IGF-1, TGF- β , BMPs, and vascular endothelial growth factor (VEGF). The release of these factors during bone remodeling might promote homing of tumor cells to the bone, and stimulate the colonization and proliferation in the bone marrow. Depending on the tumor phenotype either osteolytic or osteoblastic factors will be secreted. The mechanisms through which prostate cancer cells promote osteoblastic growth and bone mineralization remain

poorly understood. However, a variety of bone-stimulating factors produced by CRPC cells, including PSA, endothelin-1 (ET-1), BMPs, IGF-1, and OPG have direct and indirect effects on bone [143-147] (**Figure 3**).

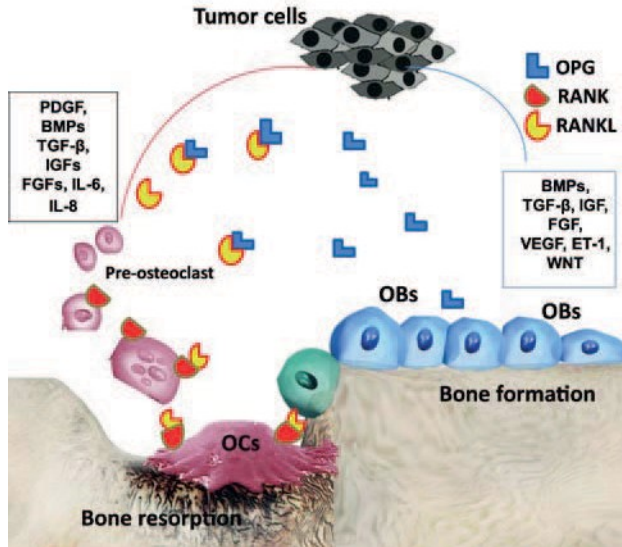


Figure 3. Illustration of the vicious cycle of prostate cancer bone metastases. Depending on which factors that are released from the tumor cells either osteoclasts or osteoblasts will be activated. OCs = osteoclasts, OBs = osteoblasts. Modified and redrawn from [148]

Hormonal regulation of bone

Steroid hormones are the key regulators of bone growth and homeostasis [149]. In the human male, both androgens and estrogens are involved in modeling and remodeling of the skeleton. Male skeletal androgen action can be mediated directly through activation of the AR, but also indirectly through aromatization into estrogens [150]. The direct role of androgens in bone is less clear, but AR signaling in osteoblasts has been reported to be important to maintain the trabecular bone mass [151, 152]. The direct role of E2 is to inhibit bone resorption by affecting osteoblast secretion, including increased $23^* \text{ DQG GHFUHDVHG } 5\$ 1./ \text{ DQG } 71) \text{ I}$ [153-157]. E2 has been shown to inhibit bone resorption by promoting apoptosis and differentiation of osteoclasts [152, 158]. In addition, it has also been proposed that E2 induce the commitment of precursor cells to the osteoblast lineage at the expense of the adipocyte lineage and to prevent osteoblast apoptosis [137, 159, 160].

Bone tissue and osteoblasts express steroid receptors and all enzymes necessary to convert the adrenal androgen precursors DHEA and androstenedione into active androgens and estrogens [161]. This provides significant evidence that bone is an endocrine organ with local paracrine and/or intracrine synthesis and a site of actions for steroids. Men receiving ADT have reduced bone mineral density (BMD) and increased risk of fracture because of significantly suppressed levels of serum T and E2 (reviewed in [162]). Due to the absence of androgens and estrogens the balance of bone turnover will shift towards bone degradation. In this process growth factors that have been embedded in the bone matrix are released, and these factors can function as chemotactic stimulants for prostate cancer cells, hence supporting their invasion, colonization and proliferation in the bone niche [116, 120, 163].

Osteotropism

The proclivity for prostate cancer cells to metastasize to bone has been explained by several mechanisms. The retrograde flow of tumor cells through Batson's venous plexus is the main anatomical explanation of the route for metastatic spread [164, 165]. In addition to anatomy, the British pathologist Stefan Paget published the seed and soil theory in the *Lancet* in 1889 [111]. After analyzing over 900 autopsies comparing primary breast cancer tumors with their metastases, Paget proposed that metastasis does not occur by chance, but depends on cross-talk between selected cancer cells (the 'seeds') and specific organ microenvironments (the 'soil'). In other words, certain tumor cells will selectively colonize to distant organs because of the presence of a favorable microenvironment for their localization and growth. Paget compared the seeding of cancer cells to the dispersal of the seeds of plants:

“When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil”.

Ever since, this theory has remained a basic principle in the field of cancer metastasis [166, 167] and is particularly relevant to bone metastasis because osteotropic cancer cells possess certain properties that enable them to grow in bone, and the bone microenvironment provides a fertile soil in which they grow [128]. The seed and soil theory was reinforced in recent studies showing that the acquisition of specific gene expression profiles [168] or the activation of specific signaling pathways [169, 170] dictates the specificity of cancer cells growing in bone.

Bone provides an especially attractive site for a variety of reasons. The continuous and dynamic turnover of the bone matrix and bone marrow

provides a fertile soil for tumor cells. The osteoblasts, as well as the marrow cells, provide an environment rich in growth factors, cytokines and chemotactic factors. As already mentioned certain areas of the bone are metabolically active – such as the metaphysis at the ends of the long bones – and these are well vascularized and allow various cells to easily enter and exit the bone. The vascular supply is sinusoidal in nature rather than a bed of capillaries. These factors, and the vascular structure of the trabecular bone, are crucial for metastatic cancer cell colonization and growth. Most likely, the suitability of bone as a site for metastasis is a mixture of factors involving anatomy, properties of the tumor cells, and the composition of the pre-metastatic niche in the metastatic site.

The way to the bone – the metastatic process

Metastasis is the process through which cancer spreads from the original site (primary site) to other parts of the body. Overall, metastasis is an inefficient process. Of the several millions of disseminated tumor cells (DTCs) that are introduced into the circulation, it is estimated that only a very small fraction (0.001– 0.02%) succeed in forming metastatic foci [171-173]. The metastatic process consists of a long series of sequential, interrelated steps, including degradation of the ECM, detachment of the tumor cells from the ECM and from each other, and migration toward and subsequent entry into the blood or the lymphatic system [174, 175]. Each of these steps can be rate limiting, and a failure or an insufficiency at any of the steps can stop the entire process [176, 177]. It is believed that the dissemination of tumor cells might occur early during progression with tumor cells preferentially homing to bone marrow.

Epithelial mesenchymal transition

For metastasis to occur, tumor cells must first detach and reach the vasculature. Several carcinomas including prostate cancer can develop invasive and mesenchymal features that facilitate detachment and migration. In order to acquire a mesenchymal migratory phenotype, tumor cells must shed many of their epithelial characteristics, detach from the epithelial layer and undergo a drastic alteration and this is referred to as epithelial mesenchymal transition (EMT) [178-180].

EMT is characterized by loss of cell-cell adhesion, loss of apical-basal polarity, and reorganization of the cytoskeleton in a process largely induced by tumor infiltrating immune and stromal cells [181]. Therefore tumors are often considered to be corrupted forms of normal developmental processes and EMT is often considered to be the most fatal consequence in tumorigenesis [178, 182-184].

In addition to the loss of epithelial characteristics, EMT frequently coincides with the acquisition of motility, invasiveness, changes in the cytoskeletal proteins (such as expression of vimentin and α -SMA) altered adhesion receptor expression (switching from E-cadherin to N-cadherin (CDH2) or Cadherin-11 (Osteoblast-cadherin)) and proteinase secretion (especially metalloproteinases (MMPs) [178, 179, 185-189]. In prostate cancer the “cadherin switch” has been observed in the more aggressive/castration resistant cell lines [190-192]. Supporting these data N-cadherin and Cadherin-11 both have been reported to increase after androgen deprivation [192, 193]. Cadherin-11 is known to facilitate the interaction with osteoblasts in the bone [193, 194].

The next step of invasion and metastasis requires disruption of the basal membrane and remodeling of the ECM which is coordinated by proteases such as MMPs and cathepsins. The acquisition of a mesenchymal phenotype by the cancer cells is crucial for invasion of the underlying stromal compartment. This step is considered to be the as the most critically important for malignant progression because this switch facilitates dissemination and metastasis.

Detachment, migration, attachment and colonization

After EMT, prostate tumor cells must go through a multistep process to metastasize to bone. These steps involve detachment from the primary site, survival in the circulation, attachment to resident cells in bone, and survival and proliferation in the bone marrow. Circulating prostate cancer cells (CTCs) preferentially adhere to bone marrow endothelial cells and then migrate through the endothelial layer [195] in a process involving several adhesion molecules such as selectins, integrins and cadherins that are present on the surfaces of endothelial cells and DTCs. The final stages of the metastatic cascade involve adhesion to the bone marrow endothelium of the sinusoids vessels, extravasation and colonization of bone marrow.

After successful colonization of bone marrow, it has been shown that the metastasized bone tumors are largely composed of cancer cells showing a mixed epithelial–mesenchymal phenotype and many morphological characteristics similar to the primary tumor. This suggests that the metastatic bone tumor resembles the primary phenotype in the bone microenvironment [188]. It appears that a number of DTCs in the bone marrow can reactivate certain properties through a mesenchymal–epithelial transition (MET). At present, the role of EMT and MET in bone metastasis is not fully understood, but it is known that these transitional stages are strongly affected by the bone microenvironment [196].

Osteomimicry

Besides undergoing EMT to acquire mesenchymal features, the prostate cancer cells must acquire bone cell-like properties in order to thrive in the bone microenvironment [19]. This adaptation is referred to as osteomimicry and has been substantiated in animal models and humans [19, 197, 198]. This process enables prostate cancer cells to produce bone matrix proteins such as osteopontin, osteonectin and bone sialoprotein. The osteoblast transcription factor RUNX2 has also been implicated in the osteomimicry that is attributed prostate skeletal metastasis (123–125). Osteomimicry facilitates the conditions for the tumor cells to metastasize, adhere, survive, and grow in bone. However, it is not fully known if the cancer cells already possess osteomimetic properties when they detach from the primary tumor site, or whether some of these phenotypical changes occurs when the cancer cells reach the bone marrow. Tumor cells in the metastatic prostate lesion might transdifferentiate to become mesenchymal cells that are capable of osteoblastic activity, cancer cells might induce resident cells in the marrow microenvironment to enter the osteoblast lineage, and prostate cancer cells might induce the proliferation and/or differentiation of osteoblast lineage cells. Osteoblasts are a vital component in certain aspects of tumor localization in bone [199].

The bone metastatic niche and tumor cell dormancy

The concept of a pre-metastatic niche has emerged as a means through which a primary tumor is able to prepare sites of metastasis [199]. Primary tumors might condition the bone marrow through the production of circulating factors that target cells in the bone microenvironment and thus render it capable of facilitating tumor localization and colonization.

Preclinical evidence suggests that DTCs can home in and localize in the HSC niche and that they survive in a dormant state. During dormancy the DTCs either stop proliferating or they proliferate at a reduced rate before showing clinical evidence of metastasis. The period of dormancy can sometimes exceed 10 years [200–204]. Patients with bone marrow DTCs at diagnosis are at a higher risk of both skeletal and extraskelatal metastasis. Evidence exists that DTCs can persist in the bone marrow for years in a quiescent state and that these cells are resistant to cancer therapies [205–207]. Of patients with prostate cancer who have had a radical prostatectomy, 72% have DTCs in the bone marrow [208]. However it is still a matter of debate whether the DTCs actually form metastasis or whether they prepare the metastatic niche for tumor establishment.

Bone marrow comprises various cell types, including cells of hematopoietic origin and cells involved in bone formation and remodeling. In the bone marrow osteoblasts, endothelial cells, adipocytes, nerve cells and mesenchymal stem cells serves as a niche for HSCs and maintain the activities of HSCs such as homing, self-renewal, quiescence and differentiation. Recent evidence indicates that a subset of osteoblasts, named Spindle-shaped N-cadherin positive osteoblasts, plays an important role the regulation of HSCs [209]. In particular, these specialized osteoblasts are located next to the endosteal surface of bone – the osteoblast niche – where they have the specific function of maintaining the HSCs in a quiescent state. This is supported by the finding that conditional ablation of osteoblasts in mice leads to depletion of HSCs [210], while stimulation with PTH increases the number of HSC and the number of osteoblasts [209]. Malignant cells disseminate to and develop in the bone marrow by hijacking the osteoblastic niche [211, 212]. In fact, both prostate and breast cancer home to the marrow by mimicking the homing mechanisms of HSCs (**Figure 4**) [213, 214].

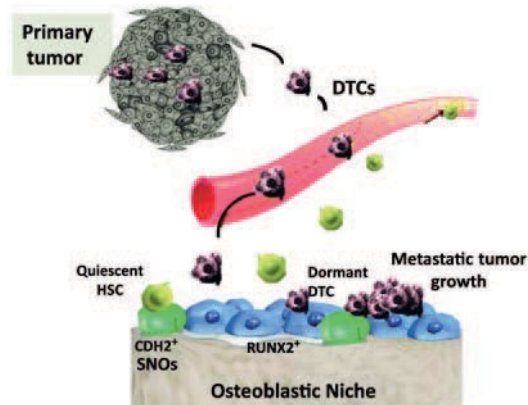


Figure 4. Illustration of the osteoblastic pre-metastatic niche in the bone marrow. Prostate cancer cells and HSC home to osteoblasts in the bone marrow (niche) using similar mechanism. HSC = hematopoietic stem cell, SNOs = Spindle shaped N-Cadherin (Cdh2) expressing osteoblasts, DTC = Disseminated tumor cell. Redrawn and modified from <http://www.sciencedaily.com/releases/2011/03/110323140237.htm>

As a result of this competition for the niche, disseminated prostate cancer cells displace HSCs from the marrow and induce the differentiation process of HSCs into hematopoietic progenitor cells (HPCs). Correspondingly, high levels of HPCs can be detected in the peripheral blood of prostate cancer patients with bone metastases. The mechanisms behind this phenomenon are

not clear, but several types of intercellular communication have been proposed, including exosomes containing mRNA, protein, or microRNA that are released by the tumor cells. The SDF-1/CXCR4 axis is considered to be important for tumor cell homing to bone [168, 214-216]. The homing into and the retention of HSCs, MSCs, as well as osteotropic cancer cells to the bone marrow niche is mediated by their expression of CXCR4, one of the receptors of the chemokine CXCL12 or stromal derived SDF-1 α expressed by osteoblasts [213, 217, 218]. The capability of tumor cells to mimic HSC cells, and evidence for factors that induce HSC dormancy could explain tumor cell dormancy in bone marrow niches. In addition, osteoblasts and endosteal cells express annexin II (anxa2) which activates the growth arrest-specific 6 (GAS6) receptors on prostate cancer cells. Differential levels GAS6 protein in the bone microenvironment induce dormancy in HSCs and has been found to reduce cell cycle progression in prostate cancer cells[219]. This evidentially shows that osteoblasts are facilitators for tumor dormancy in bone [220]. Similarly, homing was decreased when osteoblasts lacked the expression of annexin II (anxa2) in mice. Moreover, both HSC and prostate cancer cells express Anxa2 receptor (Anxa2r) and knockdown of this in prostate cancer cells decreased their homing to bone [221, 222]. Shiosawa et al further demonstrated that prostate cancer cells and HSCs both are co-localized in mouse bone marrow, next to cells expressing RUNX2 [221], supporting that prostate cancer cells are homing preferentially to the osteoblastic niche. In another study, metastasis to bone was decreased when osteoblasts were depleted in a transgenic mouse model, hence demonstrating an important role of osteoblasts in metastasis to bone.

Osteoimmunology and bone metastases

The immune system has long been known to play an important role in preventing tumor growth, but more recent evidence suggest the importance of the immune cell response to factors in the tumor microenvironment as main regulator of cancer progression and metastases. The fact that only a small fraction of DTCs succeed in colonizing the distant microenvironment suggests that the rate-limiting steps occur along the metastatic cascade after extravasation. These steps include angiogenesis, the establishment of a favorable growth promoting niche and evading immunosurveillance, in the bloodstream and in the bone [223, 224].

Bone marrow comprises marrow stem cells; the MSCs that give rise to osteoblasts and osteocytes, and the HSCs that develop into both myeloid derived suppressor cells (MDSCs) that differentiate into osteoclasts and lymphoid progenitors that give rise to immune cells. Moreover, recent studies have also indicated that certain cell of the immune system can help tumor

cells to become established within a niche. The bone marrow is a reservoir for immune cells such as macrophages, dendritic cells (DCs), MDSCs, B-cells and different T cell subsets that can directly impair the so called “tumor/bone vicious cycle” as reviewed in [225]. A hallmark of advanced bone metastasis is the prevalence of immunosuppressive cell populations such as MDSCs and regulatory T-cells. During tumorigenesis, the secretion of several factors such as IL-4, IL-13, VEGF, granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF) and TGF- β leads to the expansion, activation and recruitment of MDSCs, which might increase up to 25% in patients with bone metastases [226]. The MDSCs have multiple roles in immune suppression such as stimulating osteoclastogenesis [227, 228]. Moreover MDSCs supports the polarization of macrophages into a tumor-promoting phenotype [226] and the establishment of the pre-metastatic niche. Macrophages are like DCs and osteoclasts derived from circulating CD14+ monocytes, which originate from bone marrow myeloid stem cells [229]. Macrophages are frequently found in the marrow, where they help establish a favorable tumor microenvironment. These macrophages are referred to as tumor-associated macrophages (TAMs) and they have a significant amount of e plasticity that allows them to effectively respond to environmental changes [230-232]. Based on the activation route, TAMs can generally be divided into two major phenotypes: M1 tumor-inhibiting macrophages and M2 tumor-initiating macrophages. TAMs are suggested to play a pivotal role in the process of primary tumor growth, dissemination of cancer cells, and subsequent metastasis through the release of inflammatory cytokines and proteases such as cathepsin K [233, 234]. Moreover, it was shown that primary prostate tumor cells distantly instigate osteoblasts via PTHrP in the systemic circulation to increase the production of pro-metastatic VEGF (A), IL-6 and CCL-2 in the bone microenvironment which in turn stimulate MDSCs with increased angiogenic potential [230].

Although bone already represents an immune privileged site, tumor cells can further skew the balance of immune effector and suppressor cells towards an immunosuppressed niche to promote their outgrowth in bone (reviewed in [226]). It is now known that tumor cells can induce an immunosuppressed microenvironment in metastatic sites before their arrival via the secretion of immunosuppressive cytokines. For example, tumor cell and bone cell secretion of TGF- β has been associated with immunosuppression in the tumor microenvironment [235, 236]. Recent evidence supports a role of osteoblasts in osteoimmunologi by their release of cytokines and growth factors in the microenvironment [237].

Pathophysiology of prostate cancer bone metastases

Many patients with prostate cancer have bone metastases that appear on radiography as lesions with areas of increased bone density and on bone scan as hot spots of increased bone formation. Histopathological assessment of prostate cancer bone metastases show increased abnormal bone formation, with an elevated osteoid surface, i.e., unmineralized matrix. Osteoblastic lesions are composed of increased abnormal woven bone that is formed in marrow spaces from the tumor stroma and not from the bone surface [238]. Moreover, these metastases typically show an excessive number of osteoblasts adjacent to tumor cells. In contrast, osteoblasts are generally absent in bone metastases from other cancers (such as breast, lung and kidney), which instead are dominated by osteoclasts [43]. Although the overall tumor response of prostate cancer is osteoblastic, large heterogeneity of bone histomorphometry is seen in the same patients comprising lesions of both osteoblastic/osteolytic areas. The histological findings are consistent with clinical evidence that demonstrates increased systemic markers of both bone production and degradation in prostate cancer patients [239]

Markers of bone metastases

The tumor-associated activity can be visualized clinically, via radiographs, pathologically or by measurements of bone biochemical markers. Many markers of bone metabolism are elevated in bone metastases and are closely related with disease progression and have a prognostic value for diagnosis, determination of treatment and monitoring of treatment efficacy. Biochemical markers of bone turnover are generally categorized into bone resorption and bone formation markers. The bone formation markers include bone specific alkaline phosphatase (bALP), bone matrix proteins such as OCN and the procollagen extension peptides (PINP and PICP). bALP is an enzyme specifically produced by osteoblasts that is released into circulation during the mineralization process [240]. OCN is a noncollagenous protein synthesized by osteoblasts that binds to hydroxyapatite and is involved in calcium binding [241]. PINP and PICP are derived from the extracellular processing of the procollagen type I molecule, which contains amino-terminal and carboxy-terminal extensions that are enzymatically cleaved upon procollagen secretion [242].

Bone resorption usually occurs before bone formation, and thus an increased level of bone resorption markers might be indicative of bone tumor activity. The clinical markers bALP and N-telopeptide of type 1 collagen are associated with higher rates of death and skeletal-related events in prostate cancer bone metastases. Although bone-remodeling markers might be helpful in better defining the prognosis and the risk for bone complications in

patients with bone metastatic disease, the level evidence is not yet sufficient to recommend them as part of the guidelines for clinical practice [243].

Treatment of bone metastatic CRPC

Tumors are generally incurable once they have metastasized to bone. However there are strategies to prolong survival and to manage SREs. The first line-therapy for patients with metastatic CRPC after relapse from ADT is systemic (chemo) therapy in the form of the cytotoxic drug docetaxel [244, 245]. Systemic treatment with docetaxel has been used as the standard treatment demonstrating both improved quality of life and overall survival rates [244]. The second line therapies include chemo therapy with cabazitaxel which, like docetaxel, is a microtubule stabilizer [246]. Docetaxel and cabazitaxel are taxenes that exert their therapeutic effect by stabilizing the microtubules, and thus they block cell division, induce apoptosis and inhibit nuclear translocation of the AR [247]. Although chemotherapy has been shown to improve overall and progression free survival [246, 248], it is not well tolerated by all CRPC patients because the majority are elderly men with limited bone marrow and concurrent medical conditions [249]. In recent years, a wide variety of novel therapeutic options have become available for patients with metastatic CRPC. These strategies consist of cytotoxic, anti-androgen, immune and radiopharmaceutical therapies. The new anti-androgen therapies include abiraterone acetate (Zytiga®) [250] and enzalutamide (XTANDI®) [251]. Abiraterone is a highly efficient inhibitor of the CYP17A1 complex, thereby making it an androgen synthesis inhibitor in the adrenal glands, testes and prostate cancer cells and their microenvironment [86]. Treatment with abiraterone requires concomitant use of prednisone to decrease symptoms of mineralcorticoid excess. Enzalutamide is an AR-signaling antagonist that binds to the AR ligand site and thereby inhibits nuclear translocation of the AR, DNA binding, and co-activator recruitment. Both enzalutamide and abiraterone show beneficial effects on CRPC patients with bone metastatic disease [250, 252, 253].

Another approach to pain relief in patients with bone metastases has been the use of radiopharmaceuticals. Strontium, samarium, and radium have strong avidity for the calcified matrix of bone. These agents only exert a small antitumor effect through localized radiation, but they have substantial effects on bone pain [254]. The radioisotope radium-233 (Alpharadin®) is a new bone-seeking alpha-emitter radionucleotide that might be added to the therapeutic regimen of patients with CRPC who have either received or are considered unsuitable for docetaxel treatment. The radiation from the decay of radium-233 kills tumor cells by inducing double-strand breaks, and radium-233 was recently reported to extend survival in men with

symptomatic CRPC and bone metastases [255]. The bone marrow also serves as a reservoir for DTCs that can resist chemotherapy, and these tumor cells can emerge later as new metastases in bone or other organs [206, 256]. Drugs that directly interfere with bone remodeling, such as bisphosphonates (zoledronic acid) and RANKL antibodies, that target osteoclastogenesis have shown to significantly decrease the incidence of skeletal complications and are the current standard of care for patients with bone metastases regardless of symptoms. However, both bisphosphonates and RANKL therapies act to inhibit osteoclast-mediated bone resorption and can prevent bone loss in patients receiving ADT, i.e., they can indirectly increase BMD [257, 258]. There are emerging data that these anti-resorptive agents can also have direct anti-tumor effects. However, 30–50% of patients on such therapies still develop new bone metastases, skeletal complications and disease progression, emphasizing the need for new therapies.

AIMS OF THE THESIS

GENERAL AIM

Bone metastasis is the major cause of morbidity for men with advanced prostate cancer. Once the cancer has entered the bone, there is no cure. Prostate cancer forms osteoblastic metastasis – which is rare in other osteotropic cancers such as breast cancer – gives rise to predominantly osteolytic tumors in bone. The molecular mechanisms of osteoblastic tumors are poorly understood, and it is not known how prostate cancer in the bone microenvironment develops castration and drug resistance. Therefore, the overall aim of this thesis was to investigate the specific role of osteoblasts in bone metastatic prostate cancer.

SPECIFIC AIMS

- To develop and characterize a novel model suitable for in vitro and in vivo studies of the osteoblastic function of CRPC.
- To explore the role of osteoblasts in the development and growth of sclerotic CRPC tumors.
- To investigate the specific role of ectopically expressed RUNX2 in the growth of osteoblastic tumors.
- To evaluate the effect of a novel therapeutic (tasquinimod) on osteoblastic CRPC in bone using the in vitro and in vivo model.

METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are based on *in vitro* experiments to explore the role of soluble factors in the bi-directional cross talk between tumor cells and osteoblasts and *in vivo* experiments to verify the findings in a physiological context comprising the bone microenvironment.

What follows is an overview and considerations of methods used in this thesis. For detailed description of material and methods see the corresponding paper.

IN VITRO EXPERIMENTS

Cell lines and culture conditions

Although cell lines used *in vitro* do not recapitulate the heterogeneous condition in human prostate cancer, utilizing a representative *in vitro* model will spare cost and minimize ethical issues in decreasing the use of animal experiments. The cell lines used in this thesis were selected based on their specific phenotypic properties in bone. In the *in vitro* set-up a murine pre-osteoblastic cell line was chosen for studies of the bi-directional interplay with the human prostate cancer mimicking xenografts *in vivo*.

For all experiments Minimum Essential Medium (α MEM) was used supplemented with 1% antibiotics and 1 – 10 % Dextran treated Charcoal stripped fetal bovine serum (DCC- FBS) to minimize artifacts due to different cell culture conditions. Unless not mentioned, cell lines were bought from ATCC or ETCC and used in maximum 10 passages from the original passage in our laboratory. All cell lines used in this thesis were routinely tested for mycoplasma.

LNCaP FGC clone was originally derived from lymph node metastases and is androgen – dependent [259] and grows poorly in bone [260].

LNCaP-19 was established in our laboratory as an *in vitro* derived castration-resistant subclone of LNCaP [261]. LNCaP-19 has previously been shown to form osteoblastic tumors in the tibia of nude mice [262] and possess many of the characteristics of human CRPC. Compared to its parental cell line, LNCaP-19 grows without androgens, has an increased angiogenic and invasive potential [263, 264]. It also forms metastases to lymph nodes and lungs when orthotopically implanted in SCID mice [265, 266] and occasionally to bone (unpublished).

C4-2B₄ was originally derived from vertebral mouse metastases (kindly provided by Professor George Thalmann, Department of Urology, University of Bern, Switzerland). A second generation of LNCaP, C4 [267] was co-injected with a bone stromal cell line, MS in mice to generate C4-2. The C4-2 cells were then injected orthotopically or subcutaneously back into castrated mice and formed a bone metastatic subline, C4-2B₄. C4-2B₄ forms mixed osteoblastic/osteolytic tumors in mice [260].

The **PC-3** cell-line is castration resistant and forms highly osteolytic lesions in bone (ref). It was originally isolated from a vertebral metastatic prostate tumor [268].

MC3T3-E1, clone 4, is a murine pre-osteoblastic cell line established and characterized by Wang et al [269]. The MC3T3-E1 is the most commonly used cell line for studies of osteoblast differentiation to be able to induce the complete differentiation process of a pre-osteoblast to a mature mineralizing mature osteoblast.

NIH3T3 is a murine fibroblast cell line, and was used as a control to MC3T3-E1 in this thesis for valuation of osteoblast specific effects on the prostate cancer cells.

Conditioned medium (Paper I-IV)

The cross-talk between cells is mediated by either direct physical contact or released factors. Both forms of communication have shown to link several gene expression-directed signaling pathways. For the effect of osteoblasts on prostate cancer cells, communication via released factors appears to play a decisive role [84, 270]. Conditioned media was used in order to investigate bi-directional interplay between osteoblasts and tumor cells and the effect of secreted factors from either cell type. Abbreviations used in this thesis: prostate cancer cell-condition media (CM), fibroblast-conditioned media (FCM) and osteoblast-conditioned media (OCM).

Differentiation assays

The mineralization process in osteoblasts is well documented and can be studied by several methods. By using media without ascorbic acid it is possible to maintain the cells in an immature stage, and by adding promineralisation agents (β -glycerophosphate and ascorbic acid) differentiation can be induced. In this thesis an ALP activity assay and the Von Kossa method were used for visualization of steps in osteoblast differentiation and finally mineralization (**Paper I, IV**) and OsteoImage™ Mineralization Assay (LONZA) was used for quantification of formed hydroxyapatite in osteoblasts (**Paper IV**).

Proliferation assay (Paper I-III)

To study effects of treatment on proliferation of LNCaP-19 cells and MC3T3 cells, we used the established method of BrdU-incorporation. BrdU is incorporated into the DNA of proliferating cells. It is an indicative method of proliferation.

RNA and Protein preparations (Paper I-IV)

RNA from cells were extracted by RNEASY mini plus kit (Qiagen) and RNA from femoral bone with ALLprep kit (Qiagen) according to manufacturer's instructions. RNA concentrations were measured using a spectrophotometer (NanoDrop). Protein concentration was determined by the BCA Protein assay (Pierce Chemical)

Real time - Quantitative Polymerase Chain Reaction (RT-qPCR) (Paper I-IV)

cDNA was prepared by reverse transcription of total RNA with VILO Superscript III (Invitrogen) according to the manufacturer's instructions. RTqPCR was performed with an ABI Prism 7500 Fast Sequence detector. Evaluation of mRNA expression was investigated using individual TaqMan MGB probes purchased as TaqMan Gene expression assays (Applied Biosystems). For characterization and comparative analysis of basal properties and effects of osteoblast stimulation (OCM), human gene signature arrays of osteogenesis and androgen pathway were used (Applied Biosystems; TaqMan® Array Gene Signature plates plates #4418741 and # 4418728 respectively) with 96 selected genes including 4 endogenous control genes were used. Genes were considered to be non-detected in cases where Ct values were above 36.

Western blot (paper III)

Samples were separated on a 4-12% Bis Tris gradient gel under reducing conditions and subsequently transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 2% blocking buffer followed by anti - RUNX2 and anti - CYP11A1. Beta actin was used as loading control. Protein expression was visualized using secondary anti-rabbit antibody and an ECL Advanced detection kit (GE Healthcare).

RNA interference (Paper III)

Transfection of LNCaP-19 cells was performed with Amaxa™ transfection cell line Nucleofector kit™ (VCA-1001; Lonza) according to the manufacturer's protocol. All siRNAs were purchased from Ambion, Silencer Select. Transfection efficacy was evaluated using 2 different siRNA and 2 non targeting control siRNA and was confirmed by RT-qPCR and Western blot after 12, 48 and 72 h post transfection.

***IN VIVO* EXPERIMENTS**

Animal models

Mouse models of prostate cancer are critical for understanding the biology of PC initiation, progression and therapeutic implications. As of yet, no model exists that can successfully reproduce the whole metastatic process and all of the environmental interactions that occur in the human disease. However, available models have been utilized in the study of different aspects of human prostate cancer bone metastasis and hence provide useful data. The injection of tumor cells into a metastatic site (long bone such as femur, tibiae, humerus) does not exclude the possibility to evaluate the factors associated with tumor cell migration, invasion and preferential homing to a particular metastatic site [271].

Limitations of this xenograft model is the lack of T-cells, and thus it does not mirror a full immune response. Nude Balb/c mice have B-cells and NK cells but the role of these in T-cell deficient mouse is not fully known. This is of special importance to consider for the results in paper IV. Like in humans, castration leads to decreased levels of steroids in mice and also results in bone loss. Also the main production of sex steroid in mice takes place in the testis and levels of sex steroids are drastically decreased after castration. However, there are also some major differences in sex steroid metabolism between humans and mice. In humans the adrenal gland is an important source of weak androgens that can be further converted to more potent androgens or estrogens. Rodents do not express adrenal CYP17A1, and thus the adrenal androgen production in mice is considered as insignificant, resulting in lack of circulating testosterone, DHEA and androstenedione synthesis [272]. Another difference is that mice lack SHBG, which is a high affinity carrier protein for sex steroids in serum in humans [273]. These points are of special importance to consider for the results in paper II.

Animals and tumor cell implantations

In this thesis male athymic BALB/c Nude mice were used for studies of the sclerotic growth of LNCaP-19 in bone. Intratibial implantations of LNCaP-19 cells were used to study the osteoblastic tumor growth locally in bone. Orthotopic and subcutaneous tumors of LNCaP-19 were used as controls for the bone specific effects. Castration was performed prior to implantation, via scrotal incision, to mimic the human condition of CRPC. Mice were 8-9 weeks of age when the experiments started, this age corresponds to when mice turn sexually mature, which is an adequate start point for studies on prostate cancer. The use of the basement membrane product, Matrigel™ was used for all types of tumor cell injections.

Intratibial implantation Paper (I, II, IV)

In the works of this thesis an intratibial injection model was used to study the interaction between tumor cells and bone microenvironment at the metastatic site. This method was established by Corey et al in 2004. In brief, tumor cells were inoculated directly in the bone marrow via the knee joint. Orthotopically and subcutaneous implantations were performed according to standardized methods (Paper II).

The use of animals was approved by the animal ethic committee in Gothenburg.

Steroid measurements (Paper II)

The most accurate method to measure sex hormones in mouse serum is the gas chromatography/tandem mass spectrometry (GS/MS-MS). This ultra-sensitive method [274] was used to measure steroids in tumor bearing mice in paper II. Serum concentration of progesterone, androstenedione, T, DHT, E2 and estrone were measured in serum in a single run by GC-MS/MS. The limits of quantification for estradiol, estrone, T, DHT, progesterone and androstenedione were 0.5, 0.5, 8, 2.5, 74 and 12 pg/ml respectively. A limitation of this method is the amount of serum needed per sample- 250 µl. In Balb/c nude mice the maximum serum volume that can be obtained is 200 – 250 µl per mouse, and therefore samples were pooled to be able to use serum for other analysis.

Enzyme-linked immunosorbent assay (ELISA) (Paper I, II & IV)

Markers of bone remodeling (OPG, RANKL) and osteoblast activity (OCN) were monitored in serum of mice to reflect the bone response of the tumor. Treatment effects of tasquinimod on pro- and anti-inflammatory mediators in serum were evaluated using a multiplex MCCYTOMAG- 70K assay (EMD, Millipore), based on magnetic bead technology to quantify 32 selected cytokines, chemokines and growth factors. The use of mouse-specific assays enables to study the tumor induced effect in bone.

Immunohistochemistry (Paper II-IV)

Immunohistochemistry was used in paper II for identification of steroidogenic enzymes and steroid receptors of intratibial, subcutaneous and orthotopic tumor tissue from LNCaP-19 xenografts and in paper III to verify RUNX2 and CYP11A1 localization and expression pattern in tumor cells and osteoblasts in intratibial sections. Antibodies for PSMA (prostate-specific membrane antigen) and P504s (alpha-methylacyl-CoA racemase) were used to distinguish the human tumor cells from mouse osteoblasts. In paper IV CD206, CD11b and Gr1 were used for detection of immune cells. To avoid false positives due to unspecific binding, a matched isotype control to the

antibodies was used in similar concentration. The choice of decalcification method is of importance for the performance of IHC. To minimize bias due to preparation of the tissue control staining was performed with both EDTA and formic acid treated bones.

Biopix (Paper IV)

In paper IV, the sclerotic effect of intratibially injected LNCaP-19 cells was abolished and hence there was no use to measure tumor response by BMD. Instead tumor mass was used as a parameter of tumor response in tibia of tasquinomod treated mice. For quantification of tumor area and number of Gr1 and CD206 positive cells BioPix iQ 2.3.3 computerized color selection was used.

Computed tomography (CT) (Paper I)

To measure BMD, the peripheral quantitative computed tomography (pQCT) or can be used. The pQCT creates a 3D image of the bone and the true volumetric BMD and also separate the trabecular bone from the cortical bone. For trabecular bone analysis in mice, the sites investigated most often are of the proximal tibia, distal femur and vertebral body. In paper I, pQCT was used to calculate the sclerotic response of LNCaP-19 tumors in tibiae. Total and trabecular BMD were measured, *ex vivo*, in the metaphysis with the growth plate as a reference point.

Statistics

Statistical differences between groups were established using two-sided Student's *t*-test (Paper I-IV). Mann-Whitney *U* test was used to analyze differences between groups in the *in vivo* experiments (Paper I, IV). Correlation was determined with Spearman's rank correlation test (Paper I). Statistical calculations were performed using the latest version of SPSSv20 software package (SPSS, Chicago, IL). A *P*-value of ≤ 0.05 was considered statistically significant.

RESULTS AND COMMENTS

PAPER I

Nearly all deaths from prostate cancer are the result of development of osteoblastic CRPC. There is a lack of knowledge regarding the role of osteoblasts in the sclerotic process because of the limited amount of material from patients and a lack of experimental models that resemble the clinical characteristics of CRPC. To be able to further characterize the osteoblastic function in CRPC, new models are required.

In this work, we characterized a new cell line model, referred to as LNCaP-19, that was previously established in our research group [261]. This model was used to study both basal mechanisms of sclerotic tumor growth of CRPC and the interplay between osteoblasts and tumor cells. In contrast to most bone metastasis models, the LNCaP-19 was derived *in vitro* and thus has not been in contact with stroma or bone, and this enables studies of the initial interactions and first contact with the bone microenvironment *in vivo* or with osteoblasts via soluble factors (such as OCM) *in vitro*.

In order to grow and thrive in the bone microenvironment, tumor cells must be osteomimetic, i.e., they must have bone-like properties. To characterize the osteogenic properties of LNCaP-19, we used gene expression arrays to measure the basal expression of bone-associated genes and to identify genes that were affected by OCM stimulation. Expression analysis was performed on androgen-dependent and castration-resistant cell lines that were either bone-naïve or had been derived from bone. In accordance with the original study [19], we could show that prostate cancer cells express an inherent set of bone phenotypic genes, such as *BMPs*, *VEGF*, *ALP*, and the transcription factor genes *MSX2* and *RUNX2*. During the transition to the castration-resistant stage, additional genes are expressed for a more pronounced phenotype. For instance, *COL1A1* and *DSPP*, genes crucial for the mineralization process, were turned on during castration. The relevance of this gene expression could be demonstrated by the fact that the castration-resistant LNCaP-19 is able to induce mineralized matrix *in vitro* whereas androgen-dependent LNCaP lacked this capability. Interestingly osteolytic castration-resistant PC-3 cells did not have the capacity to mineralize *in vitro*, which could be explained by their expression of additional osteoclast phenotypic genes.

Koenemann suggested decades ago that the bone–stroma interactions are needed to fulfill the osteogenic phenotype in PC cells [19]. Our data support this theory and show that the stromal interactions with osteoblasts are likely the most important because fibroblasts do not induce bone-associated genes in CRPC cells. In accordance with observations by others, we could show that osteoblast-secreted factors stimulated proliferation [82, 84] and promoted the metastatic potential in osteogenic CRPC [275].

In addition, we could show that the osteoblast interaction further promoted the osteogenic progression in LNCaP-19 cells by increasing the expression of *RUNX2* and *CDH11* (osteoblast cadherin), which are suggested to be important for the bone tropism of prostate cancer [276, 277]. The interaction between tumor cells and bone cells is referred to as a vicious cycle and has been well characterized in osteolytic metastases from breast and lung cancer [278]. However, the vicious cycle of osteoblasts and prostate cancer cells in the sclerotic situation is poorly understood. Recently, *MMP2* was shown to be of major importance in the vicious cycle of osteoblastic tumors [279]. The upregulated expression of *CDH11* and *MMP2* in the LNCaP-19 cells upon OCM stimulation suggests that OCM from osteoblasts potentiates the interaction between osteoblasts and prostate cancer cells by regulating these factors.

The *in vivo* characterization of the intratibial LNCaP-19 model demonstrated that our model had many of the main characteristics of human adenocarcinoma of the prostate in bone, i.e., osteodense bone without resorption and excessive osteoblast activity. Measurements by pQCT confirmed the osteoblastic response by a prominent increase in BMD in trabecular bone and in cortical bone. Serum markers of OPG, OCN, and RANKL (Paper II) were measured by ELISA and used as parameters for the osteoblastic tumor growth in this model. Osteoblasts control bone remodeling by secreting OPG or RANKL, and this axis has emerged as a key regulator of osteoclastogenesis in physiological and pathological states, including bone metastases [280]. The elevated OPG/RANKL in serum in tumor-bearing mice indicates that local suppression of osteoclast activity might contribute to the overall osteoblastic response in CRPC tumors.

Previous studies have reported that stimulation by CRPC cells leads to a significant increase in the population of immature osteoblasts [269, 281]. These findings are in agreement with clinical observations of CRPC metastases, which are composed of a large proportion of immature newly woven bone and non-mineralized matrix, that is also clinically reflected by increasing bALP levels in serum [197]. We demonstrated that osteogenic CRPC cells altered the differentiation of osteoblasts as evidenced by

sustained ALP activity and the induction of a different mineralization pattern. We also demonstrate that BMD in tumor-bearing mice is positively correlated with OCN levels in serum, which is a clinical marker for osteoblastic tumors in prostate cancer patients. To summarize, the LNCaP-19 model resembles clinical CRPC thus making this model useful for studies of bone metastatic CRPC.

In paper I, we present a novel model resembling bone metastatic CRPC. Furthermore we show that osteoblasts promote the metastatic and osteogenic progression of prostate cancer cells and the bi-directional interaction between tumor cells and osteoblasts. Our model demonstrates that both tumor cells and osteoblasts are mediators of the bone-forming process of CRPC, and this suggests that osteoblasts could be an important target for osteoblastic CRPC.

PAPER II

As previously described, there seems to be a gradual shift during prostate cancer progression from dependence on androgens from endocrine sources to dependence of androgens from paracrine, autocrine, and intracrine sources [86]. Several studies on bone metastases from patients and from CRPC xenografts have demonstrated increased gene expression of enzymes involved in the androgen synthesis pathway [60, 65, 87-89].

The potential of osteoblasts in regulating steroidogenesis was investigated in paper II. Using an androgen gene signature array, we show that osteoblasts alter the steroidogenic transcription program in CRPC cells, mimicking the gene expression pattern described in metastatic bone tissue from CRPC patients. Osteoblast-stimulated LNCaP-19 cells displayed an increased expression of genes encoding steroidogenic enzymes (*CYP11A1*, *HSD3B1*, and *AKR1C3*), estrogen signaling-related genes (*CYP19A1* and *ESR2*), and genes for DHT-inactivating enzymes (*UGT2B15*, and *UGT2B17*). The altered expression pattern of steroid enzymes was bone specific and verified by immunohistochemistry in tissue specimens from LNCaP-19 xenograft tumors. Measurements with a GC/MS-MS further supported both the steroidogenic gene expression analysis *in vitro* and the immunohistochemical staining *ex vivo*. The overall steroidogenic effect was reflected by corresponding levels of progesterone and a tendency for increased T and decreased DHT in serum from castrated mice with intratibial LNCaP-19 tumors.

Despite the sclerotic response of CRPC, there are currently no reports that link CRPC bone metastases and E2, but several studies show increased CYP19A1 [97, 98] – which is responsible for the conversion of T to E2 – and ER β is reported to be increased in CRPC bone metastases [282-284]. In addition, in the intratibial tumors of LNCaP-19, both progesterone receptor and ER α were expressed in osteoblasts and osteocytes. During androgen deprivation there was also an upregulation of ER β in osteoblasts *in vitro* which suggest that there is a role for ER β in the sclerotic process of CRPC. In our model E2 levels were as expected not detectable in serum from the xenografts but in an additional study we could show that E2 is produced locally in bone harboring these tumors (unpublished data). Our studies show that osteoblasts have the capability to induce an intratumoral steroidogenic shift in favor of increased E2 production, which could explain the gain for the bone of hosting a CRPC. During castration, bone degradation occurs due to decreased T and E2 levels, and because E2 is the main anabolic factor for bone, it is likely that increased synthesis of E2 in the CRPC tumor supports bone formation under castrating conditions. In contrast to our data,

administration of E2 has been shown to have a direct inhibitory effect on tumor growth and prolonged survival in CRPC xenograft models [285]. However, these studies were based on subcutaneous tumors and cannot be directly translated to metastases in bone.

It is likely that part of the heterogeneity regarding expression of steroidogenic enzymes reflects the AR status in the tumor cells. The mutated AR in the LNCaP-19 model might influence the observed steroidogenic pattern and thus might select for one subgroup. The T877A mutation has been found in up to 30% of clinical CRPC bone metastases [102, 286]. This point mutation, in accordance with nearby mutations in the ligand binding domain, leads to promiscuous binding to other androgen ligands such as E2 and progesterone. Due to the increased progesterone levels in the *in vivo* experiments, we performed an AR activity assay to investigate the responsiveness to progesterone in relation to DHT and T under castration conditions. We could show that progesterone activated mtT877-AR ten times more efficient than DHT, under castration conditions, thus suggesting that progesterone might have an important role in AR activation in these cells. Interestingly, the effect of osteoblasts was like in (Paper I) exclusively found in osteogenic CRPC cells and not seen in PC-3 cells, which are predominantly lytic in nature and do not express AR. Collectively, our study together with a previous study on clinical CRPC bone metastases, which showed that AR amplification correlated with decreased osteoclast activity [287], suggests that AR might be of importance for the osteoblastic phenotype of CRPC. In line with this, OCM increased the expression of transcripts for the glucuronoid enzymes UGT2B15 and UGT2B17. These enzymes have been found to be elevated after ADT, and are considered as the major androgen inactivation pathway in prostate cancer cells [288]. UGT2B15 and UGT2B17 are regulated by AR and are not expressed in PC-3 [289]. This might indicate that predominantly DHT inactivation (and possibly also T and E2) occurs in CRPC bone metastases. This can be interpreted in two different ways. DHT inactivation could be increased due to an overall increase of DHT within the tumor, or DHT inactivation could occur to limit the amounts of DHT. However, these alternatives were not the focus of this study. Together, the increased gene transcripts of these enzymes could indicate that they are important for the osteoblastic response of CRPC.

The unchanged levels of CYP17A1 in response to OCM might be related to the fact that CRPC cells expressing the T877A mutant AR are not dependent on CYP17A1 for their AR activation. Instead, AR activity in these cells is mediated by intratumoral pregnenolone/progesterone synthesis [95]. Another plausible explanation could be that CYP17A1 is activated by other stimuli than of osteoblasts. The latter is supported by the majority of studies on

human CRPC tumors where no increased *CYP17A1* expression was detected in bone metastases [65, 87-89, 93]. However, blocking of *CYP17A1* activity by abiraterone efficiently inhibits tumor progression in CRPC patients, resulting in a prolonged survival, and thus points out this enzyme to be important for the overall steroidogenesis in CRPC. In accordance with previous studies, on CRPC bone marrow biopsies [60, 95], we showed an increased expression of *CYP11A1* in OCM stimulated LNCaP-19 cells. In contrast, Jernberg et al did not detect *CYP11A1* in the majority of bone metastases but identified a strong correlation between *CYP11A1*, *CYP17A1*, *HSD3B2* and *SRD5A1* in a subset of tumors [88]. This suggests that there are multiple ways for CRPC tumors in to produce steroids at levels adequate for sustained AR activity and maintained tumor growth.

These findings provide further evidence that osteoblasts might be important therapeutic targets in osteoblastic PC because osteoblasts are important both for bone formation (Paper I) and steroidogenesis (Paper II) in osteoblastic prostate cancer.

PAPER III

The gain of ectopic RUNX2 is suggested to be important in osteotropic cancers. However, little is known of the specific role of RUNX2 in osteoblastic tumors. The purpose of this study was to further characterize the acquisition of osteomimetic properties by CRPC cells and the role of RUNX2 in the context of osteoblastic tumor growth.

In Paper I, we identified the osteoblast transcription factor RUNX2 as one of the most up-regulated genes in osteogenic LNCaP-19 cells in response to osteoblast secreted factors (OCM). In contrast to the majority of studies on RUNX2 in prostate cancer (reviewed in [290]), we investigated the function of RUNX2 in a cell-line with osteoblastic properties, and we investigated the role of RUNX2 in the interplay between tumor cells and osteoblasts.

Besides its pivotal role in regulating osteogenesis, RUNX2 was recently demonstrated to control *de novo* steroidogenesis in osteoblasts through the regulation of CYP11A1, the enzyme responsible for the conversion of cholesterol to pregnenolone [291]. In paper II, we found that CYP11A1 was upregulated by OCM, and we further hypothesized that the gain of RUNX2 could be a mechanism in the acquisition of osteoblast-like properties as an adaptation for growing in bone. The purpose of this study was to address to what extent osteoblast-induced RUNX2 mediates the CYP11A1 expression and other osteoblast-induced phenotypes in LNCaP-19 cells.

By silencing RUNX2 in LNCaP-19 cells, we could show that the osteoblast-induced expression of CYP11A1 was dependent on RUNX2, and thus we could show that osteogenic CRPC cells might utilize RUNX2 for *de novo* steroid synthesis in a similar manner as osteoblasts. In further verification of sclerotic tumors in LNCaP-19 xenografts, we could show that CYP11A1 and RUNX2 were co-localized at the borders lining the tumor island and interlaced within the newly formed bone. CYP11A1 was exclusively expressed in tumor cells, whereas RUNX2 was expressed in both osteoblasts and tumor cells, thus supporting the induction of RUNX2 in tumor cells by osteoblast-derived factors. In fact, RUNX2-expressing osteoblasts were difficult to distinguish from RUNX2-expressing tumor cells. Koenen proposed in 1999 that the acquisition of RUNX2 might be a key event for the bone metastatic process of solid tumors [19], a further support for his theory has been provided by us and others [290, 292]. In addition, our observations also show that RUNX2 plays a novel role in osteoblastic metastases.

Depending on its cellular expression level and context, RUNX2 appears to have dual roles both as a tumor suppressor and as an oncogene.

Overexpression of *RUNX2* has been shown to also drive osteolytic cancer aggressiveness and the osteolytic phenotype through the regulation of osteoclast stimulatory factors such as *MMP-9*, *MMP-13*, and *IL-8* [292, 293]. In contrast to these reports, we found that these genes were upregulated by inhibition of *RUNX2* by siRNA. In addition, OCM significantly abrogated the upregulation of *IL-8*. This would indicate that osteoblasts regulate part of the osteogenic phenotype of prostate cancer cells by controlling *RUNX2* expression. In support of our finding, it has been shown that inhibition of *RUNX2* blocks the TGF- β -mediated stimulation of PTHrP, which is the most studied mechanism for the vicious cycle of osteolytic tumors [294, 295]. However, these experiments were mainly performed with cell lines such as the osteolytic AR-negative PC-3 cells, which do not represent the clinical phenotype of CRPC in bone. Other studies were performed using C4-2B, which, like PC-3, is derived from bone before being established in culture. This might influence the role and expression of *RUNX2*. However, *RUNX2* is known to be under the regulation of AR and ER (reviewed in [162]), and this regulation was not taken into consideration in paper III but might be of importance when considering our findings in a physiological context.

In conclusion, paper III reveals a novel mechanism for *RUNX2* in CRPC cells, which, under the regulation of osteoblasts, mediates *de novo* steroidogenesis and thus might be important for the intratumoral growth of CRPC in bone.

PAPER IV

Tasquinimod (ABR-215050; a quinoline-3-carboxyamide) is small-molecule compound that has proven to have immune modulatory [296, 297], anti-angiogenic [298, 299], and anti-metastatic [262] properties in several experimental tumor models. Recently, a randomized placebo-controlled pivotal clinical study with tasquinimod showed reduced risk of radiographic cancer progression compared to placebo (rPFS, HR=0.69, 95% CI: 0.60-0.80) in patients with metastatic castration-resistant prostate cancer who had not received chemotherapy. However, tasquinimod did not extend overall survival (HR=1.097, 95% CI: 0.938-1.282) [300]. In this pre-clinical study, we used the well-characterized LNCaP-19 model [301] to investigate the mechanisms of tasquinimod on osteoblastic growth of CRPC in castrated mice.

In accordance with the previous pre-clinical studies [262], we found that tasquinimod inhibits tumor establishment and tumor growth in intratibial LNCaP-19 xenografts. In the treatment group, only a few mice developed tumors and these were very small and thus no pronounced osteoblastic response could be detected. These data support previous observations and show that tasquinimod interferes with the early phases in tumor establishment of osteoblastic bone metastases.

Tasquinimod has previously shown inhibitory effects on the metastasis, probably through its binding to the S100A9 protein, which has been shown to be an important factor in the creation of the pre-metastatic niche as well as in the recruitment and differentiation of MDSCs [302-304]. Within the niche, the bone is lined with osteoblasts expressing genes such as *CDH11* and *RUNX2* and spindle-shaped N-cadherin-positive osteoblasts, which are special immature N-cadherin expressing osteoblasts (reviewed in [212]). In our study, the effect of tasquinimod on the niche was investigated in the tumor-free femoral bone marrow adjacent to the tumor cell-injected tibia. We found decreased expression of *runx2*, *cdh2* (N-cadherin), and *cdh11*. This result implies that tasquinimod targets the osteoblastic component of the pre-metastatic niche, thus indicating new putative mechanisms for tasquinimod in CRPC in bone.

A plausible mechanism for how tasquinimod inhibits the establishment of osteogenic prostate cancer cells could be by abrogating the expression of *RUNX2*, as seen in the femoral bone of tasquinimod-treated mice. *RUNX2* has proven to be important for the establishment of prostate cancer cells in the pre-metastatic niche where they attach to osteoblasts expressing *RUNX2* [221]. Moreover, *RUNX2* is essential for bone development and osteoblast

maturation [305]. For example RUNX2-null mice die directly after birth due to the complete lack of differentiated osteoblasts and thus a lack of mineralized skeleton, which demonstrates the crucial role of RUNX2 in bone formation. The lack of sclerotic response might be directly coupled to the inhibition of RUNX2 because both OCN and *colla2* levels were decreased in the mouse femur, which are both under the regulation of RUNX2 [306, 307]. The inhibitory effect of tasquinimod on LNCaP-19-induced mineralization in osteoblasts *in vitro* might be subsequent to the phenotypic switch by decreased osteoblast markers, and this was supported by the elongated fibroblast-like phenotype shown in tasquinimod-treated osteoblasts *in vitro*. In addition, a plausible explanation for the mineralization effect could be the downregulation of *lox*, which is necessary for the final stages of the mineralization process. The inhibitory effect on tumor-induced mineralization in osteoblasts was also demonstrated *in vitro* by decreased hydroxyapatite formation.

To evade an immune response, tumor cells directly modulate the immune system and induce an immune suppressive microenvironment by recruiting and activating immune-suppressive MDSCs and shifting the phenotype of tumor-infiltrating macrophages. We could show by immunohistochemistry that tasquinimod targeted MDSCs that was indicated by the decreased number Gr1 positive and CD11b positive co-localized per tumor area in the treated intratibial tumors. In accordance with this decreased immunosuppression, we showed a phenotypic switch from M2 to M1 macrophages in the femur adjacent to the tumor-injected tibia. The major evidence was the decreased expression of CD206 and Arg1 and increased expression of Nos2 (iNOS), which are markers of M2 and M1 macrophages, respectively, in mice [234, 308, 309]. In addition, it has been shown that inhibition of RUNX2 blocks the TGF- β -mediated stimulation of PTHrP, which is the most studied mechanism for the vicious cycle of osteolytic tumors. Interestingly, TGF- β leads to the expansion, activation, and recruitment of MDSCs [226]. In line with the observation in paper III, it is tempting to suggest that suppressed TGF β and the subsequent abrogation of MDSCs might be a cause of repressed *Runx2*.

In conclusion, paper IV shows that tasquinimod efficiently impaired the establishment of bone metastases in mice by interfering with the osteoblastic pre-metastatic niche and osteoblastic activity, thus emphasizing the role of osteoblasts in the early phases of the metastatic process of CRPC.

GENERAL DISCUSSION

Models of prostate cancer bone metastases

Multiple animal models of prostate cancer bone metastasis have been developed, but only a few effectively resemble osteoblastic bone metastases as they occur in men. Prostate cancer only arises spontaneously in men, dogs and more rarely in rodents. Therefore the use of xenograft models, in which human tumor cells are administrated into mice provides the opportunity to study human prostate cancer in a physiological context and allows for assessment of the interaction between tumor cells and the bone microenvironment in the metastatic process.

A major problem with the existing xenograft models of prostate cancer is that they, for unknown reasons rarely form metastases. Intravascular injections via the tail vein is used as a model of extravasation and metastasis, and intracardiac injection of cancer cells into the left ventricle permits cancer cells to localize to any tissue of the body depending on the cancer cells' inherent metastatic phenotype. However, these injection models can be controversial as metastasis models, because they eliminate the early steps of metastasis and thus fail to fully recapitulate human disease.

Intratibial implantations were first used to investigate the growth properties of different prostate cancer cell lines in bone [310]. The intratibial injection of tumor cells into a metastatic site (a long bone such as the femur or tibia) does not preclude the possibility to evaluate factors associated with tumor cell migration, invasion and preferential homing to a particular metastatic site [271]. Because femurs are larger than tibiae they can be used as a semi metastatic model [311]. Even though intratibial injections do not resemble the natural course of metastasis, we were able to use such a model to study steps such as modulation of/and attachment to the osteoblastic niche and tumor establishment using both the tumor injected tibia and the femoral bone (Paper IV).

The available prostate cancer cell lines that predominantly form osteoblastic tumors in bone are patient-derived bone marrow metastases (VCaP and LAPC-9) or are established by being co-injected with stromal cells in bone of mice before being established as cell lines from xenografts (C4-2B4) [312-314]. In both cases the stromal cell contribution and the adaptation to bone can influence the overall tumor response. Several CRPC cell lines have been developed *in vitro* by culturing the cells in steroid-deficient media for several passages as in the case of LNCaP-19 [261]. However few of them form osteoblastic tumors *in vivo*. An advantage of using *in vitro* derived cell lines

is that these cell lines are bone-naïve and thus are suitable for studies of the initial osteoblast – tumor cell interaction and the basal mechanism contributing to the establishment of CRPC in bone. In addition, some osteoblastic models do not have the capacity to grow or to form mixed osteoblastic/osteolytic tumors in the absence of androgens, and thus these models do not resemble clinical CRPC. Another challenge regarding the available osteoblastic prostate cancer models is their inability to propagate *in vitro*, which makes them unsuitable for use in *in vitro* assays to investigate the molecular mechanisms of pathogenesis in parallel with *in vivo* studies. Our model mimics the situation of osteoblastic CRPC both *in vitro* and *in vivo*, and thus making it a valuable tool for studies, both at the molecular level and under physiological situations in mice.

The heterogeneity in prostate cancer bone metastases is a major problem with regard to the development of resistance, and this heterogeneity can result in poor treatment response in some metastatic phenotypes when directed therapy is used [122, 197, 315]. In the clinic, PSA is used to monitor treatment effects and the progression of bone metastasis. Our model expresses low PSA levels, and does not respond to osteoblast-derived factors (OCM) with increased PSA expression *in vitro*. In a similar study, OCM has been shown to induce PSA secretion in prostate cancer cells [84]. Even if a high PSA value (> 100 ng/mL) is indicative of metastatic disease, it is probably more correlated to a high tumor burden rather than high PSA expression in the metastatic cells *per se*. Studies on patients with low PSA levels (< 10 ng/dl) [316], still identified bone metastases in a significant portion of patients. Thus, PSA levels alone cannot be used as a marker of bone metastases. Interestingly, patients with both primary tumors and bone metastases expressing high levels of PSA have been convincingly shown to have a better prognosis compared to patients with tumors expressing low levels of PSA [122, 315, 317, 318]. PSA expression is heterogeneous and the absence or low expression of PSA cannot be explained solely by populations of AR-negative neuroendocrine cells or stem cells, which are rarely seen in bone metastases [122, 319, 320]. Furthermore, there is no correlation between PSA and AR expression in human CRPC bone metastases [315]. Altogether, this might indicate that the expression of PSA could reflect some degree of tumor cell differentiation, dedifferentiation or transdifferentiation that can occur under influence of the bone microenvironment.

Subgroups of CRPC bone metastases – including splice variants, mutations, or the loss of AR – can also be characterized by AR status together with different steroidogenic patterns. Models like LNCaP-19, which harbors a mutated AR, might be relevant to the 10–30% of patients with CRPC who harbor this mutation [286]. Plausible explanations for the different growth

properties in bone observed in papers I and II, could be that there were differences in the osteomimetic properties of the cell lines and the origin of cell type, as well as AR status and the steroidogenic capability to adapt to the bone microenvironment under castration condition. Therefore characterizing major tumor subgroups and elucidating the underlying mechanism of the growth pattern in bone might lead to the development of therapeutic approaches targeting tumor cells in some cases and bone cells in others.

Osteolytic versus osteoblastic tumors

Although it is clear that the involvement of different interactions between the tumor and the bone microenvironment, the “vicious cycle”, driving the metastatic progress in bone metastases is far more complex than originally proposed. In general, osteoblastic tumors are described as slow growing tumors while osteolytic tumors are characterized to be more aggressive and fast growing. What drives the different tumor response of CRPC in this aspect is not known. In our study we show that osteoblasts influence sclerotic tumor progression in many steps of the metastatic process of CRPC (Paper I-IV). The respective phenotypes of dysregulate bone destruction and bone formation represent two ends of a spectrum, and most patients will have evidence of both processes to some extent. However, during disease progression one phenotype will be dominating and for patients with prostate cancer the vast majority will end up with a sclerotic phenotype. Even though this pattern is characteristic for prostate cancer, disease progression alone cannot explain the osteoblastic dominance in end-stage CRPC and the osteolytic dominance in late-stage breast cancer patients because a proportion of CRPC patients will develop osteolytic disease while a similar proportion of breast cancer patients will develop osteoblastic disease. The mechanisms responsible for tumor growth in bone are complex and involve tumor stimulation of osteoclasts and osteoblasts as well as the response of the bone microenvironment. Osteoblasts are implicated in the regulation of bone homeostasis and bone remodelling through the secretion of factors that will either stimulate or suppress osteoclast activity, thus suggest that osteoblasts might provide a regulatory mechanism of cytokines in the bone microenvironment [129, 237]. However, among experimental models the osteolytic phenotype tends to predominate, as exemplified by the AR-negative PC-3 cell line, indicating that the mechanism behind CRPC acquisition might in part also influence bone-related properties of the metastases. Our studies have shown that osteoblasts do not stimulate osteolytic CRPC cells (Paper I and II), and we and others have shown that osteogenic and osteolytic cell lines display different profiles regarding cytokines and osteoclast stimulatory factors [321-323]. This suggests that these different phenotypes should be treated as different forms of the disease.

The role of RUNX2 in osteolytic versus osteoblastic prostate cancer

The work in this thesis suggests that RUNX2 is involved in CRPC cell adaptation to the bone microenvironment by inducing genes related to both osteomimicry and steroidogenesis (Paper I, II and III). As evidence for the role of RUNX2 in the phenotypic fate of prostate cancer bone metastases, RUNX2 appear to behave differently in AR positive versus AR negative tumors of CRPC. In different types of tumor cells, RUNX2 cooperates with its co-activators or co-inhibitors to mediate the response of cells to various signaling pathways that are hyperactive in tumors [290]. Relevant for prostate cancer, are the AR and ER α pathways. RUNX2 directly binds to the AR and this interaction is potentially important for androgen signaling in various contexts including bone metabolism and prostate cancer progression [324, 325]. This regulation has not been addressed in our studies but it is important for understanding the complexity and function of RUNX2 in CRPC.

RUNX2 has been extensively studied in the context of osteoblastogenesis and bone development where it directs mesenchymal stem cells to the osteoblast lineage. Ectopic expression of RUNX2 is increased in breast cancer and prostate cancer cells that metastasize to bone, indicating pro-tumorigenic and pro-metastatic roles [290]. In prostate cancer the expression of RUNX2 positively correlates with Gleason score [292, 326, 327]. RUNX2 is suggested to be a key regulator of events associated with the metastatic progression of prostate cancer to the bone – and possibly contributing to their metastatic potential, in part by stimulating the expression of matrix metalloproteases such as MMP9 and MMP13 [328, 329] and by promoting EMT, tumor cell survival and invasion into bone tissue [292, 295, 330, 331]. In paper I, RUNX2 expression was shown to be increased by osteoblast-derived factors in osteoblastic CRPC cells while levels of RUNX2 in osteolytic CRPC cells were unchanged. This could indicate that osteoblasts regulate RUNX2 in CRPC bone metastasis and are a part of the osteoblastic vicious cycle. In our study, expression of RUNX2 was found in both tumor cells and osteoblasts in intratibial tumors of LNCaP-19 (Paper III) supporting a bidirectional role of RUNX2 in the sclerotic CRPC. In support of our hypothesis, short hairpin-mediated inhibition of RUNX2 in pre-clinical models suppresses the metastatic growth in bone of osteolytic prostate cancer (PC-3) and breast cancer with high expression of RUNX2 [292]. This indicates the importance of RUNX2 for the growth of osteotropic tumors in bone. However, the role of RUNX2 in bone metastatic CRPC needs to be further investigated pre-clinically and its clinical importance needs to be confirmed in bone metastatic tissue from patients.

The different roles of RUNX2 in tumorigenesis and the divergent development of osteolytic and osteoblastic tumors might be explained by the different roles of osteoblasts in these situations. Based on clinical and pre-clinical data including the data presented in this thesis, it appears that a population of immature RUNX2 expressing osteoblasts and the stimulation of ectopic expression in the tumor cells are important for the osteoblastic growth of CRPC. We have shown that RUNX2 is induced in tumor cells close to osteoblasts in the intratibial tumor model in mice. In addition, both breast and prostate cancer cells preferentially home to RUNX2 expressing osteoblast in the niche [221]. In contrast, osteoblasts do not stimulate RUNX2 expression in osteolytic tumor cells (PC-3) that have high RUNX2 expression. Interestingly, silencing RUNX2 has been shown to promote an osteoblastic phenotype, thus indicating that expression levels of ectopic RUNX2 are important for the different phenotypes. Together this suggests that osteoblasts play an important role in the regulation of the tumor phenotype through the expression of RUNX2. In addition, our data suggest that RUNX2 is important for homing to a niche and in the communication between tumor cells and osteoblasts within the niche.

For patients with CRPC a plausible therapeutic option could be to modulate RUNX2 with combined treatment for bone remodeling to maintain bone homeostasis. As indicated by our data (Papers I-III) targeting RUNX2 could be an effective strategy for targeting both osteoblasts and ectopically RUNX2 expressing osteoblast-like tumor cells. From a therapeutic point of view, RUNX2 could be an ideal target because CRPC patients have an adult skeleton and do not have the acute need to form new bone. Thus combining a RUNX2 modulator with currently used drugs such as denosumab and zoledronic acid might be a feasible treatment alternative.

ADT and prostate cancer bone metastasis

The optimal time point for ADT treatment is still a matter of debate. ADT remains the single most effective treatment of metastatic prostate cancer, but it is uniformly marked by progression to CRPC. The vast majority of patients who receive ADT have already developed lesions in the bone. Although the initial response to ADT is a decreased tumor mass in the prostate tumor and in distant lesions as well as a decrease in the metastatic spread of the cancer cells, ADT commonly gives rise to osteoporosis that leads to the release of tumor cell-stimulating growth factors. An increased number of tumor cells both in the prostate and in the circulation (DTCs) might be available for adaption to the castration conditions, and events such as osteomimicry might be promoted. Therefore, removing the prostate before ADT could be a

strategy to prevent or prolong the time to development of castration resistance.

It is important to identify prostate cancer subtypes and the proper timing for ADT depending on the type of tumor that we aim to treat. In some cases castration might actually trigger the metastatic process in bone, while in other cases it might inhibit metastasis. In our *in vivo* model, we could not detect AR in any of the bone cells while AR expression in the tumor cells interlaced within the bone was prominent and had a nuclear localization, thus indicating that AR is important for the sclerotic tumor growth of CRPC. A report by Efsthathiou et al [252], showed that the anti-androgen enzalutamide had significant therapeutic effect in patients with CRPC bone metastases who had strong AR expression before treatment. The effect of enzalutamide in these tumors was shown by subcellular translocation of the AR out of the nucleus and subsequently inhibition of AR signaling. However, a subgroup of patients (37%) who had the AR-V7 AR splice variant exhibited enzalutamide resistance.

Administration of an aromatase inhibitor in older men with low T levels increases T but decreases E2 levels and BMD, and this suggests that the increase in T cannot compensate for the negative effect of E2 deficiency on the skeleton [332]. In contrast, selective estrogen receptor modulators have proven effective in treating bone loss with ADT in prostate cancer patients [333]. In recent trials, monotherapy with anti-androgens such as bicalutamide and enzalutamide that prevent AR activation instead of reducing the synthesis of androgens did not show a significant effect on BMD [334]. The fact that peripheral aromatase activity might modulate circulating E2 levels is of particular interest because most estrogens in men are derived from conversion of androgens in peripheral tissue [335, 336]. Moreover, estrogen deficiency have a profound effect on the immune system leading to increased production of proinflammatory cytokines like IL-1, TNF- α , INF- γ , IL-6 and IL-17, both systemically and in bone marrow, and this might contribute to increased bone resorption [337].

To evaluate the clinical relevance of our model pQCT measurements of BMD were assessed to determine the bone-formation capacity of LNCaP-19 cells (Paper I). Interestingly, tumor-induced trabecular BMD was significantly elevated in castrated mice compared to non-castrated mice. Accordingly, in a recent experiment from our group, intratibially implanted LNCaP cells had the capacity to increase trabecular BMD in castrated mice but to a lesser extent than LNCaP-19 (unpublished data). In contrast, the commonly used C4-2 forms mixed osteoblastic/osteolytic tumors and leads to an overall decrease in BMD under castration conditions [338]. These data suggest that

castration influences the osteoblastic tumor response of CRPC. In paper II we demonstrated a low expression of AR in osteoblasts in tumor-bearing bone tissue and this could be an indication that a prominent population of immature osteoblasts is present in these tumors (Paper I-IV). In fact, AR expression in osteoblasts is known to increase along with maturation with the highest expression in the mineralizing phase [298]. This could indicate that immature osteoblasts in the pre-metastatic niche, which are characterized with RUNX2 expression, might not be severely affected by castration, and this might explain how metastasis can proceed during ADT and might even lead to a survival advantage for the pre-metastatic niche. In accordance with the osteomimetic changes in tumor cells that occur during the transition into a castration-resistant form, this indicates that castration/ADT might trigger the bone metastatic process of CRPC. How ADT affects the overall osteoblastic tumor growth of CRPC needs to be investigated further.

Intratumoral steroidogenesis in osteoblastic CRPC

Enzymes involved in steroidogenesis have been shown to be upregulated in clinical CRPC tissue and in mice under castrate conditions [60, 65, 87-89], and the increased steroidogenic potential of tumor cells has been suggested to contribute to the development of castration-adapted tumors within the metastatic site. Due to the fact that both prostate cancer cells in general and bone are dependent on sex steroids for their growth and maintenance of bone mass, we suggest there is a mutual interplay in the tumor microenvironment that is regulated, at least in part, by osteoblasts. We propose that osteoblasts facilitate the intratumoral steroidogenesis needed for sclerotic growth. In paper II, we could show that osteoblasts induced most of the enzymes described in human bone metastatic CRPC tissue (**Figure 5**).

We also demonstrated for the first time serum levels reflecting the changes in intratumoral steroidogenic enzymes on the mRNA level in CRPC bone metastases *in vivo*. In line with our study, Knuttilla et al confirmed steroid levels in serum to corresponding increases in mRNA in an orthotopic VCaP model [339], thus strengthening the relevance of the mRNA data in the present study. However, in contrast to the intratibial model in our study, most changes in hormonal levels were reflected in response to castration and not to the tumor. These data suggest that intratumoral steroidogenesis occur in sclerotic tumors of CRPC, and this supports the many studies, both clinical and preclinical, that report of increased mRNA levels of steroidogenic enzymes. The clinical relevance of these enzymatic changes and subsequent intratumoral steroidogenesis in bone metastatic CRPC, is demonstrated by the response to abiraterone, a CYP17A1 inhibitor, in many CRPC patients. Despite improvements targeting the AR-axis (enzalutamide) and the

enzymatic steps in steroidogenesis (abiraterone), resistance inevitably develops and remains the major obstacle for long-term survival. This again emphasizes the need for new treatment targets, in which the osteoblastic stimulation of osteomimicry and intratumoral steroidogenesis could be one possibility target.

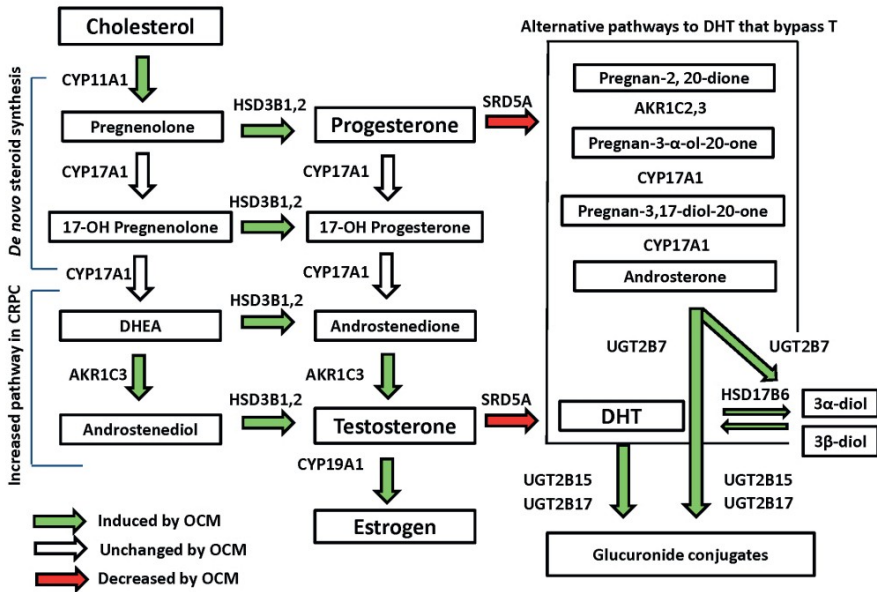


Figure 5. Osteoblast-induced regulation of steroidogenesis in a model of osteoblastic CRPC. The arrow indicates the enzymatic steps in the androgen synthesis pathway that exhibit increased versus decreased gene expression in LNCaP-19 cells in osteoblast-conditioned media (OCM). Green arrows = increased expression, white arrows = unchanged expression, and red arrows = decreased expression. Data are based on mRNA and levels of steroids in serum from *in vitro* culture and intratibial xenografts of osteoblastic CRPC.

It is well known that E2 is the main anabolic factor for bone and its formation. Also a marked up-regulation of *CYP19A1*, which mediates the aromatization of testosterone to E2, has been observed in human CRPC bone metastases compared to primary tissue [60, 97]. Despite this, E2 and progesterone are seldom mentioned in CRPC, and the intratumoral and serum ratio of steroid levels are uniformly reported as the T/DHT ratio. The sclerotic response CRPC resembles the effect of E2 in bone. In accordance we could show (paper II) that OCM significantly increased the expression of *CYP19A1* *in vitro* and in

intratibial tumors in LNCaP-19 xenografts. In an unpublished experiment by us, local steroid levels in tumor-bearing tibiae were measured in castrated mice, and showed detectable levels of E2, progesterone, DHT and T. This is the first study, to our knowledge, that actually has detected E2 levels in castrated male mice. In line with this, we could show corresponding expression of protein of ER α , ER β , and the E2 regulated- progesterone receptor in the intratibial tumors (Paper II). Although the role of ER β in human bone biology is not well understood, the fact that is expressed in the majority of bone metastases [282-284], strongly indicate that E2 signaling have a role in CRPC in bone. ER β expression was detected in tumor cells while both ER α and the progesterone receptor were expressed by osteoblasts (Paper II). This is in accordance with several studies that have shown increased expression of ER β in bone metastatic tissue whereas ER α has been undetected (reviewed in [340]). Interestingly, mRNA of ER α was expressed solely in the CRPC cell lines *in vitro* and not in androgen dependent LNCaP (Paper II). Hence suggest that ER α might be of importance for the transition into a castrated form while ER β might be important for the growth of CRPC in bone.

In our study (Paper II), the increased progesterone levels in the serum of tumor-bearing mice support the importance of progesterone for the osteoblastic response of CRPC in mice. Despite conflicting reports on the effect of progesterone on osteoblasts, with some studies suggesting an anabolic action and others showing no effect, the expression of progesterone receptor in osteoblasts plays a physical role [341]. The increased progesterone levels in response to LNCaP-19 cells in tibia (Paper II) could indicate that progesterone might be of importance for the total the sclerotic growth of CRPC, mediated by osteoblasts, while a sustained synthesis of androgens might be more crucial for the tumor cells. However, the steroidogenesis is complex machinery and the physiological importance of certain metabolites for the progression of CRPC need to be further investigated.

The heterogeneity in prostate cancer probably influences the responsiveness to treatment. A growing body of evidence shows that tumors in clinical CRPC and in pre-clinical material, tumors can be divided into subgroups with a distinguished expression pattern of steroidogenic enzymes. More recently it appears that specific features of AR correlate with these patterns. For example CYP11A1 and the *de novo* steroid synthesis pathway are mostly studied in the LNCaP sublines that harbor a mutated AR, and increased

expression of *AKR1C3* and *SRD5A1* is coincident with naturally occurring AR splice variants [88]. As we showed in paper II, osteoblasts induce steroidogenic changes in certain CRPC cells. It is thus plausible that only a modest increase in either key enzyme in the steroidogenic pathway together with the adrenal derived androgen precursors or cholesterol might be adequate to drive AR activity and tumor growth in CRPC.

Thus, it is reasonable that depending on the bone microenvironment and the origin of the tumor initiating cell, these changes results in tumor subgroups that have survival advantages in the local environment. ADT selects for certain subtypes of tumor cells that are capable to maintain steroid synthesis locally. Defining subgroups based on their steroid enzyme pattern and AR status might prove useful as a prognostic biomarker and also for guidance in the development of novel therapeutic strategies or patients with CRPC bone metastases.

Therapeutic approaches for osteoblastic metastasis of CRPC

Currently approved therapies against CRPC bone metastases are focused to target the tumor cells and do not fully consider the contribution of the host. One remaining question to address is why prostate cancer in a unique way induces osteoblastic metastases. Our studies have shown that osteoblasts are vital in several key mechanism of CRPC bone metastasis (Paper I-IV). In paper I, we showed that osteoblasts promote the osteogenic and metastatic progression of CRPC, which might explain part of the proclivity of prostate cancer cells to metastasize to bone. Despite their emerging role in the metastatic progression, few attempts have been made to specifically block osteoblast activity. One of the promising osteoblast targets investigated in preclinical and clinical trials has been ET-1. ET-1 is a vasoconstrictive agent that stimulates osteoblast proliferation and enhances osteoblast differentiation [145, 342]. Strong support for its ability to target prostate cancer skeletal metastases in animal models has led to investigation in human clinical trials. Atrasentan was the first ET-1 antagonist to be investigated in patients with prostate cancer, and currently, zibotentan, a promising more selective ET-1 inhibitor is in clinical trials [343]. It has also become clear that that interaction between tumor cells and the bone microenvironment is an early event in the metastatic process as the primary tumor prepares the niche metastatic site by releasing soluble factors such as VEGF, IL-6 and CCL2 [324], but when and how this event occurs is far from understood. In paper IV, we could show that by interfering with the osteoblastic pre-metastatic niche, the drug candidate tasquinimod inhibited tumor establishment and tumor growth possibly by interfering with the pre-metastatic niche, thus indicating the importance of osteoblasts for homing and the establishment of

metastasis of prostate cancer. However, the niche also serves as a reservoir for dormant tumor cells that can resist chemotherapeutic attack, and these tumor cells can emerge later as new metastases in bone or other organs [206, 256, 344]. It is suggested that dormant DTCs might start the process in the niche. In the bone marrow, cancer cells are often resistant to chemotherapy because they are held in the G0 phase of the cell cycle through contact with bone marrow stromal cells. Because these cells are considered to be resistant to radiotherapy and chemotherapy, a beneficial strategy could be to remove the prostate even in patients who are initially diagnosed with established bone metastases. In general the prostate is not currently surgically removed or locally treated in men with clinically evidenced bone metastases [3]. Taking into consideration of the paracrine signaling between the prostate and osteoblasts (niche) in bone, both before and after establishment in the bone, our study would suggest that by removing the prostate, it could be beneficial for patients with osteoblastic CRPC.

CONCLUSIONS

The main observations and conclusions of the present investigations can be summarized as follows.

- The established LNCaP-19 model resembles human sclerotic CRPC, sharing many pathological and molecular characteristics, and thus provides opportunities for *in vitro* and *in vivo* studies of the osteoblastic tumor progression of CRPC.
- The osteomimetic properties of CRPC cells might be inherent and some are increased during castration.
- Osteoblasts further promote the osteogenic and metastatic progression of osteogenic CRPC through the stimulation of genes associated with bone – such as RUNX2 – and they stimulate the expression of CDH11 and MMP2 in CRPC cells, which enables cross talk with osteoblasts.
- Osteoblasts induce and alter steroidogenesis in osteogenic CRPC towards increased progesterone, T, and E2. This bi-directional interplay allows for the growth of tumor cells and *de novo* bone formation, which might in turn explain part of the castration-resistant growth of CRPC in bone.
- Osteoblasts might regulate *de novo* steroid synthesis in CRPC through RUNX2-dependent CYP11A1 activation. This suggests that RUNX2 is a key factor in the sclerotic growth of CRPC and osteoblasts and might be a good target for inhibition of steroidogenesis.
- Targeting the osteoblastic niche has a profound inhibitory effect on both the establishment and growth of CRPC in bone, and this suggests that osteoblasts are a strong candidate for therapeutic intervention.

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