Prostate cancer and bone cell interactions: implications for metastatic growth and therapy

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To my family
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Abstract

The skeleton is the most common site of prostate cancer bone metastasis, and at present, there are no curable treatments for these patients. To further understand what stimulates tumor cell growth in the bone microenvironment and to find suitable therapies, reliable model systems are needed. For this purpose, we have developed an in vitro co-culture system that can be used to study interactions between tumor cells and murine calvarial bones. To validate the model, we measured the release of collagen fragments and monitored changes in expression levels of genes normally expressed during active bone remodeling.

One of the major reasons why prostate cancer cells colonize bone is the abundance of tumor-stimulating factors, such as insulin-like growth factors (IGFs), present in this milieu. We found that the IGF-1 receptor (IGF-1R) was one of the most highly activated receptor tyrosine kinases in tumor cell lines stimulated with bone conditioned media. Since IGF-1 is known to be a strong survival factor for tumor cells, we hypothesized, that concurrent inhibition of IGF-1R signaling can enhance the effects of apoptosis-inducing therapies, such as castration. We used our co-culture model to target human prostate cancer cell lines, PC-3 and 22Rv1, with simvastatin (an inhibitor of the mevalonate pathway and an inducer of apoptosis), in combination with anti-IGF-1R therapy. Tumor cell viability declined with either one of the therapies used alone, and the effect was even more pronounced with the combined treatment. The hypothesis was also tested in rats that had been inoculated with rat prostate cancer cells, Dunning R3327-G, into the tibial bone, and treated with either anti-IGF-1R therapy, castration, or a combination of both therapies. Immunohistochemistry was used to evaluate therapeutic effects on tumor cell proliferation and apoptosis, as well as tumor cell effects on bone remodeling. The tumor cells were found to induce an osteoblastic response, both in vivo in rats, and in vitro using the co-culture model. Interestingly, the therapeutic response differed depending on whether tumor cells were located within the bone marrow cavity or if they had leaked out into the knee joint cavity, highlighting the role of the microenvironment on metastatic growth and therapeutic response. Therapies targeting the IGF-1R have been tested in clinical trials, unfortunately with disappointing results. By immunohistochemical evaluation of bone metastases from patients with castration-resistant prostate cancer, we found a large variance in IGF-1R staining within this group of patients. Hence, we postulate that the effects of anti-IGF-1R therapies could be more beneficial in patients with high tumoral IGF-1R-activity than in IGF-1R negative cases. We also believe that side effects, such
as hyperglycemia, associated with anti-IGF-1R therapy, could be reduced if this treatment is administered only to selected patients and for shorter time periods.

In a separate study, using whole-genome expression data from bone metastases obtained from prostate cancer patients, we present evidence that a high activity of osteoblasts is coupled to a high activity of osteoclast. Moreover, we found that high bone remodeling activity is inversely related to tumor cell androgen receptor (AR) activity. The results from this study may be of importance when selecting therapy for patients with bone metastatic cancer, especially when bone-targeting therapies are considered, and could aid in the search for novel therapeutic targets.

In summary, we present an in vitro model for studies of the bidirectional interplay between prostate cancer cells and the bone microenvironment. We also demonstrate the importance of IGF-1 in prostate cancer bone metastases and suggest that inhibition of IGF-1R signaling can be used to treat prostate cancer as well as to enhance effects of other treatments such as androgen deprivation therapy. Furthermore, we emphasize the possibility of molecular tumor characterization when designing treatment plans for individual patients, thereby maximizing the therapeutic effects.
Populärvetenskaplig sammanfattning på svenska


För att vårt skelett ska behålla sin styrka sker hela tiden en nedbrytning av gammalt ben följt av uppbyggnad av nytt ben. Denna process kallas benombildning och utförs av två specifika celltyper; osteoklaster som bryter ner gammalt ben och osteoblaster som bygger upp nytt ben. När metastaser bildas i skelettet störs balansen mellan bencellerna. Då andra typer av cancrrar sprider sig till skelettet är det vanligast att det leder till bennedbrytning, men vid prostatacancer är det vanligast att det leder till en ökad bildning av ben. Det är sedan tidigare känt att tumörceller och benceller kan kommunicera med varandra och att detta kan stimulera tumörernas tillväxt i ben. Målet med denna avhandling var att öka förståelsen kring hur tumörceller och benceller kommunicerar med varandra.

blockerar IGF-1-signalering. Först undersökte vi detta i tumörceller samodlade med skallben från möss och fann att IGF-1-blockering kunde öka behandlingseffekten av simvastatin (som i sig själv också kunde inducera tumörcellsdöd). Därefter testade vi hypotesen i en råttmodell för prostatacancer som behandlades med kastrering. Resultaten från denna studie visade också att kombinationsbehandlingen gav en ökad effekt, däremot såg vi att tumörcellerna svarade olika mycket på behandling beroende på var i djuret de växte.

Det är ännu inte känt varför benmetastaser vid prostatacancer ger en ökad benbildning medan andra tumörformer oftast ökar nedbrytningen. För att förstå detta och öka kunskapen kring vilka faktorer som driver på benombildning i metastaser studerade vi vävnadsprover tagna från skelettmetastaser hos prostatacancerpatienter. Vi upptäckte att de metastaser som hade förhöjd osteoblastaktivitet oftast även hade en förhöjd osteoklastaktivitet. Det vill säga metastaserna hade antingen en hög eller låg total bencellsaktivitet.


Sammantaget visar denna avhandling att interaktionen mellan tumörceller och benceller är av yttersta vikt vid skelettmetastaser. Genom att blockera faktorer från benet som har förmågan att stimulera tillväxten hos tumörcellerna kan man direkt bromsa metastasers tillväxt samt öka effekten av andra behandlingar. Vidare stimuleras bencells aktivitet av tumörcellers närvaro och det är även centralt att identifiera de signaleringsvägar som huvudsakligen driver detta. Denna kunskap kan användas för att urskilja vilka patienter som har störst chans att svara på en viss behandling samt för att hitta nya möjliga behandlingsmetoder.
Abbreviations

ACP5  Tartrate-resistant acid phosphatase
Actb  Beta actin
ADT  Androgen deprivation therapy
ALP  Alkaline phosphatase
AR  Androgen receptor
BMD  Bone mineral density
BMP  Bone morphogenetic protein
BGLAP  Osteocalcin
BrdU  Bromodeoxyuridine
CATK  Cathepsin K
CTX-I  C-terminal cross-linking telopeptide of type I collagen
DKK-1  Dickkopf-1
ER  Estrogen receptor
ET-1  Endothelin-1
FGF  Fibroblast growth factor
IGF-1  Insulin-like growth factor-1
IGF-1R  Insulin-like growth factor-1 receptor
IGFBP  Insulin-like growth factor binding protein
INSR  Insulin receptor
M-CSF  Macrophage colony-stimulating
MMP9  Matrix metalloproteinase-9
Oc  Osteocalcin
Opg  Osteoprotegerin
OPLS  Orthogonal projections to latent structures
PCA  Principal component analysis
PDGF  Platelet-derived growth factor
PINP  Procollagen type I N-propeptide
PSA  Prostate-specific antigen
PTH  Parathyroid hormone
PTHrP  Parathyroid hormone-related protein
RANK  Receptor activator of nuclear factor-κB
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor-κB ligand</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SOST</td>
<td>Sclerostin</td>
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<tr>
<td>SPP1</td>
<td>Osteopontin</td>
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<tr>
<td>SRE</td>
<td>Skeletal-related event</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>1,25-dihydroxyvitamin D3</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-related integration site</td>
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**List of papers**

This thesis is based on the following papers, referred to in the text by their roman numerals in bold font:


Paper I was reprinted with permission from Springer. For paper II no permission was needed.

**Other papers by the author not appended in the thesis**

Introduction

Prostate cancer

General background
Prostate cancer is one of the most common forms of cancer among men worldwide [1]. In Sweden, approximately 10,000 men are diagnosed and about 2,500 men die from their disease each year (The National Board of Health and Welfare, Sweden). The risk of developing prostate cancer increases with age, though not all suffer from their disease. It is in fact not uncommon to, at autopsies, find previously undetected and asymptomatic tumors in the prostates of elderly men [2]. Although the death rate in prostate cancer has declined in most western countries, the incidence rate has increased over the last two decades [1, 3]. There is a large variation in incidence rate between different parts of the world, with more than a 25-fold difference between regions with the highest and lowest incidence [1]. Some of this variation might be explained by differences in screening, but there are also other factors involved, such as diet, environment and genetic background [1, 4].

One major reason why the reported prostate cancer incidence has increased over the last decades is the introduction of the prostate-specific antigen (PSA) test [3]. In healthy men, PSA is produced in the prostate and secreted in ejaculate to aid in liquefying the semen [5]. When tumors are formed in the prostate they can disrupt the normal architecture within the prostate, resulting in leakage of PSA out of the prostate gland [6]. Other conditions, such as benign prostatic hyperplasia and inflammation can, however, also increase PSA leakage. Nevertheless, serum levels of PSA is used to assess the risk of prostate cancer. The use of PSA testing means that prostate cancer can be detected earlier and in more patients. Unfortunately, it also results in overdiagnosis and even overtreatment of patients that would most likely not develop clinical symptoms [7]. It is even debated whether PSA testing provides any survival benefit [8, 9]. Patients with PSA levels above 10 ng/ml are considered to have a high risk of prostate cancer and are subjected to ultrasound guided needle biopsy of the prostate. If the histological examination show that tumor cells are growing in the prostate, the tumor is graded according to the Gleason system, which is used to predict prognosis [10]. After detection, digital rectal exam and bone scintigraphy are some of the methods used to
determine whether the disease is local, locally advanced or advanced. In local prostate cancer the tumor cells have not spread outside the prostate gland, whereas in locally advanced disease tumor cells have spread outside the fibrous capsule covering the prostate gland, though they have not formed distant metastases. If metastases are found at distant organs, it is classified as advanced prostate cancer.

The majority of patients with advanced prostate cancer will develop metastases to the bone [11, 12]. Prostate cancer cells generally metastasize to sites with a high content of red bone marrow, including the vertebral column, pelvic bones, ribs, skull and the femurs [13]. Patients often experience severe pain, and pathological fractures and compressions of the spinal cord are also common [14].

**The androgen receptor**
The prostate is dependent on androgens for its normal development and function [15]. Androgen signaling is mediated via the androgen receptor (AR) which is a ligand-dependent transcription factor. The main circulating androgen, testosterone, is mainly produced by Leydig cells in the testis. Testosterone can also be converted into a more potent androgen, dihydrotestosterone (DHT) [16]. AR activation by androgens initiates a process where the AR dimerizes and translocates from the cytoplasm into the nucleus. In the nucleus, receptor dimers bind to promoter regions of specific target genes, such as KLK2, KLK3 (encoding PSA), NKX3-1, STEAP2, and TMPRSS2, and interact with a number of coregulatory proteins, including HOXB13 and FOXA1 [17].

**Treatment of prostate cancer**
Local prostate cancer is treated with curative intentions, either by surgical prostatectomy or radiation therapy. Low risk patients with a long life expectancy can also be put under active surveillance, where treatment is initiated upon signs of tumor progression. Low risk patients with a short life expectancy may instead be subjected to watchful waiting, meaning that they will receive no treatment until symptoms of metastasis appear. In advanced prostate cancer where metastases have formed, the treatment objective shifts from curative to palliative. Patients are treated with androgen deprivation therapy (ADT), either by chemical or surgical castration.

ADT lowers androgen levels, resulting in reduced tumor size, relief of pain associated to bone metastases, and in most patients, a drop in PSA levels [18]. Unfortunately, within 1-2 years, most tumors become castration-resistant and relapse [19]. In castration-resistant prostate
cancer, tumors have an activated AR, despite low serum levels of testosterone [20, 21]. Several mechanisms have been suggested for the reactivation of the AR, including AR amplification and mutation, aberrant activities of AR co-regulators, and activation of AR through other signaling pathways [22]. Activation of the AR can also be a result of intratumoral androgen synthesis. Studies show that even during ADT, intratumoral levels of testosterone and DHT are similar to levels within hormone naïve tumors [23]. In castration-resistant patients with active AR signaling, palliative treatment is continued, usually with ADT in combination with an AR antagonist. Moreover, relapsing patients often receive therapies, such as chemotherapy, radiotherapy, abiraterone (an inhibitor of androgen synthesis) [24], and immunotherapy [25], to slow down disease progression and reduce pain. Therapies specifically aimed at targeting the bone microenvironment are also commonly used and will be described in more detail in a later chapter.

**Normal bone remodeling**

To understand why some tumor cells thrive in bone, it is of essence to understand the natural composition of the bone microenvironment and the processes that occur at these sites. The bones of our skeleton are classified as flat bones and long bones. They are both composed of different proportions of cortical and trabecular bone. Cortical bone is dense and form the outer layer of bone that surrounds the bone marrow. Trabecular bone is a light and porous bone located in the bone marrow compartment [26, 27]. The skeleton remains its structural integrity via constant remodeling of the bone. This process is maintained by the tightly controlled balance between two types of bone cells, osteoclasts and osteoblasts. Old bone is degraded by bone-resorbing osteoclasts, followed by the formation of new bone at these sites, performed by bone-forming osteoblasts.

Bone is composed of an inorganic matrix, consisting mainly of type I collagen, and an inorganic component, made up of mineral crystals, which strengthens the bone matrix [28]. The bone matrix is also rich in osteoblast-produced growth factors, such as insulin-like growth factors (IGFs), bone morphogenetic proteins (BMPs), transforming growth factor-betas (TGFβs), fibroblast growth factors (FGFs), and platelet-derived growth factors (PDGFs) [27, 29]. These proteins are
stored in the bone matrix during the formation of new bone and are released in the bone marrow cavity during resorption.

The balance between osteoclasts and osteoblasts is easily disrupted in various pathological conditions. For example, in patients with rheumatoid arthritis or postmenopausal osteoporosis, osteoclast activity is increased, resulting in excessive bone resorption [26]. In cancer, when metastases are formed in bone, the balance is also shifted. Depending on the origin of the primary tumor the characteristics of the resulting metastases differ widely. Prostate cancer bone metastases are primarily viewed as sclerotic, with an increased bone formation, while bone metastases from breast, lung, and renal cancers are predominantly lytic, with an increased bone resorption [28, 30].

Osteoclasts

Osteoclasts are derived from cells in the monocyte-macrophage hematopoietic lineage. The maturation and activation of osteoclasts is a complex multistep process starting with the differentiation of these precursor cells into inactive osteoclasts, that later fuse together to form multinuclear giant osteoclasts. Two crucial factors in osteoclastogenesis are macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). M-CSF is important in the initial phase of osteoclastogenesis [31], while RANKL is crucial in most steps of osteoclastogenesis and also in the activation of mature osteoclasts [32, 33]. RANKL is mainly expressed by bone stromal cells, including osteoblasts, and binds to its receptor (RANK) on differentiating and mature osteoclasts [28, 34]. Studies that further support the importance of RANK/RANKL in osteoclastogenesis show that RANK and RANKL whole-body knockout mice fail to produce osteoclasts, resulting in severe osteopetrosis, i.e. abnormally dense bone [35, 36]. RANKL production in osteoblasts and stromal cells is stimulated by a number of factors, including prostaglandins, parathyroid hormone (PTH), and 1,25-dihydroxyvitamin D3 (vitamin D3) [28]. The formation of osteoclasts can also be directly stimulated by cytokines, such as interleukin-1 (IL-1) [37].

Under normal conditions, osteoclastogenesis is controlled by the balance between RANKL and its decoy receptor, osteoprotegerin (OPG). OPG is a member of the superfamily of tumor necrosis factor receptors, and is expressed by osteoblasts [34, 38]. Studies have shown that knockdown of OPG in mice results in lowered bone
mineral density (BMD) [39], while overproduction of OPG leads to an increased density [40].

To resorb bone, activated multinuclear osteoclasts form a resorption compartment between itself and the bone surface. The osteoclasts produce acids that lowers the pH within this sealed microenvironment, resulting in the release of matrix minerals from the bone [31, 41]. The organic bone matrix is degraded by osteoclast-secreted enzymes, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), and matrix metalloproteinase-9 (MMP9) [41]. For an overview of the most important factors discussed within this thesis, see figure 1.

![Figure 1](image-url)

**Figure 1** Schematic illustration of key players in normal bone remodeling. Hematopoietic precursor cells can differentiate into multinucleated bone resorbing osteoclasts. Mesenchymal stem cells can differentiate into bone forming osteoblasts. During bone formation some osteoblasts differentiate into osteocytes and become embedded in bone.
**Osteoblasts**

Apart from regulating osteoclastogenesis, the main function of osteoblasts is to produce new bone. Osteoblasts arise from mesenchymal stem cells located in the bone marrow stroma. During the first phase of osteoblastogenesis, when mesenchymal stem cells commit to becoming osteoprogenitors, BMPs and members of the Wnt pathways are important regulators [42, 43]. Another essential factor for osteoblast differentiation is the runt-related transcription factor 2 (RUNX2). Studies show that Runx2 knockout mice have no mature osteoblast and therefore lack the ability to form bone, resulting in a skeleton made entirely of cartilage [44, 45].

The differentiation of these osteoblast progenitor cells into mature osteoblasts starts off with highly proliferating cells, producing alkaline phosphatase (ALP), that later mature into non-proliferating osteoblastic cells. These cells then transform from a flat to a cuboidal appearance and increase their expression of bone matrix proteins, both noncollagenous proteins, such as osteocalcin (BGLAP) and osteopontin (SPP1), and collagenous proteins, such as collagen type I [27, 42]. These bone matrix proteins form the organic matrix, which is later mineralized by the osteoblasts [42].

After the bone forming phase, most osteoblasts undergo apoptosis, whereas the remaining cells either differentiate into inactive lining cells or non-proliferative, terminally differentiated, osteocytes [46]. Osteocytes are embedded into small spaces in the bone matrix, called lacunae, they can function as mechanosensors and regulate the function of both osteoblasts and osteoclasts [47]. Osteocytes are known to express high levels of dickkopf-1 (DKK-1) and sclerostin (SOST), two inhibitors of Wnt-signaling, and by doing so they block Wnt-mediated bone formation [42]. They communicate with each other and other cells, for instance the bone lining cells, via dendritic processes that run through small tunnels in the bone, termed canaliculi.

**Bone metastasis**

**Osteotropism**

Both prostate and breast cancers are osteotropic, meaning that they have a tendency to form metastases in bone. It has been estimated that together they account for over 80 % of patients with metastatic bone disease [48]. It is still not completely understood why some
cancer types almost always disseminate to bone, while others, such as
tumors of the gastrointestinal tract, rarely metastasize to bone [26, 28]. The most popular theory, the ‘seed and soil’ hypothesis, was
proposed already in 1889, by English surgeon Stephen Paget [49]. He
examined autopsy records from 735 women with breast cancer and
found that there was a non-random distribution of metastases to bone
and other organs. From these findings he postulated that certain
tumor cells, the ‘seeds’, will only colonize organs, the ‘soil’, that
provide a favorable environment for the cells. Paget’s theory has been
challenged by many others throughout the years. In 1928 James
Ewing proposed that the anatomical structure of the vascular and
lymphatic channels determines where metastases will form. He
hypothesized that tumor cells from the primary tumor will follow the
blood flow and lodge in the first organ it comes across [50]. Although
Ewing’s theory prevailed for decades it was later shown, by Weiss and
Sugarbaker, that it could mainly account for regional metastasis [51, 52]. For tumor cells to metastasize to distant organs the ‘seed and soil’
theory still persisted [50]. In osteotropic cancers the bone and bone
marrow serve as a congenial soil for tumor cell growth. Red marrow
has a high blood flow and is the preferred site for bone metastases
[53]. Other than the high vascularity, a number of biochemical factors,
such as cell adhesion molecules, cytokines, and chemokines, as well as
physical factors, including acidic pH, hypoxia, and high levels of
extracellular calcium, also support the colonization, survival and
expansion of tumor cells in bone [54].

Mechanisms behind prostate cancer bone metastases
As previously mentioned, bone metastases in prostate cancer are
generally categorized as sclerotic. In other osteotropic cancers, such as
breast, lung, and renal cancers, metastases have a predominantly
osteolytic phenotype [55]. There is however an overlap in bone cell
activities between these two types of metastases. Some osteolytic
metastases have sites with increased osteoblast activities, and some
osteosclerotic metastases show increased osteoclast activities [28, 56–
58]. In fact, bone resorption markers in serum are often higher in
patients with prostate cancer bone metastases compared to patients
without bone metastases, and also compared to breast cancer patients
with bone metastases [59, 60]. Despite prostate cancer bone
metastases having an increased bone formation, the resulting bone is
weaker than healthy bone, and pathological fractures are common in
these patients [61].
Why prostate cancer cells have a preference for the bone milieu is most likely a combination of several factors. The main focus in this thesis is the abundancy of tumor cell stimulating growth factors, in particular IGF-1, in the bone microenvironment. It has been hypothesized that when prostate cancer cells first metastasize to bone they induce bone resorption, followed by a release of growth factors from the bone matrix, which in turn stimulates tumor cells to induce osteoblast activity [53, 58]. Examples of evidence supporting this theory includes the finding described above, that patients with prostate cancer bone metastases have increased levels of markers of both bone resorption and formation, in their blood [59, 60]. Furthermore, it has been demonstrated in mouse models that a decreased bone turnover prior to the introduction of tumor cells significantly delays the formation of metastases in bone [62]. If RANKL is instead activated during the early stages of osteoblastic bone metastasis, the resulting increase in bone resorption facilitates metastatic colonization of prostate cancer cells [63].

A number of tumor-derived factors has been suggested as particularly important in prostate cancer bone metastasis. For example, prostate cancer patients with osteoblastic metastases have elevated serum levels of endothelin-1 (ET-1) [64]. ET-1 stimulates osteoblast proliferation and thereby promote bone formation [65, 66]. One important function of ET-1 is to suppress DKK-1, a negative regulator of Wnt [67], thereby increasing Wnt signaling. Furthermore, overexpression of DKK-1 in prostate cancer cells give rise to osteolytic metastases [68]. Most prostate cancer bone metastases display increased levels of Wnt-1 [69]. Moreover, members of the BMP family are also important for the formation of bone metastases in prostate cancer. They are expressed in both normal and neoplastic prostate tissue at various levels [70]. Studies have for instance shown that prostate cancer cells can promote their osteoblast activity via BMP-6 [71]. Some of the tumor-derived factors found to be associated with formation of bone metastases may also have a connection to the IGF family of proteins. Urokinase-type plasminogen activator (uPA) has been suggested to stimulate proliferation of osteoblasts by hydrolyzing IGF binding proteins (IGFBPs), and in that way increasing free IGF levels [30]. Similar effects can be seen with tumor-derived PSA, which can cleave IGFBP3 [72]. PSA can also cleave parathyroid hormone-related protein (PTHrP), a promoter of osteoclastogenesis, thereby decreasing bone resorption [73].
Effects of sex hormones on bone
Hormones are known to influence skeletal homeostasis during adulthood and to affect both the size and shape of bones during growth [74, 75]. The relatively sclerotic nature of bone metastases in prostate cancer, compared to other cancers, indicates an association with the AR. The AR is expressed in osteoblasts, and its expression has been found to increase as the cells mature into osteocytes [76]. Androgens are known be important for maintaining trabecular bone mass, and it has been indicated that AR signaling can indirectly inhibit osteoclastogenesis by downregulating RANKL expression in osteoblasts [77, 78]. Androgens can also be converted into estrogen and activate the estrogen receptor (ER) in osteoclasts and progenitor osteoblasts [74]. This results in decreased bone resorption, in part by inhibition of RANKL synthesis and stimulation of OPG synthesis in osteoblasts [42] (Summarized in figure 2). Both AR and ERα are known to interact with other nuclear transcription factors, such as RUNX2, to modulate transcription. It has been demonstrated that when RUNX2 binds to either AR, or ERα, this most often results in inhibited, but in some cases activated, transcription of target genes [79–81]. This can affect both bone formation and osteoclastogenesis.

Figure 2 Effects of androgens and estrogens on bone remodeling. Androgens can be converted into estrogens by the enzyme aromatase.

In men, low androgen levels results in bone loss, and the same effect can be observed in women with menopause associated estrogen deficiency [74]. In both cases bone loss is associated with increased
bone remodeling activities. Germline deletion of AR in male mice results in low BMD and high bone turnover [82, 83]. In agreement with these findings, ADT is known to have a negative impact on the skeleton in prostate cancer patients, causing reduced BMD and an increased overall risk of fractures [84, 85].

**Therapeutic strategies for targeting the tumor-bone microenvironment**

Both ADT and bone metastases themselves, can induce bone pain and also increase the risk of skeletal-related events (SREs), such as spinal cord compressions and pathological fractures [13, 14, 84, 85]. Therefore, therapies aimed to specifically target the bone microenvironment are used in patients with bone metastases. Bisphosphonates are a class of drugs that binds to bone surfaces, and are internalized by osteoclasts during bone resorption, resulting in osteoclast apoptosis and reduced bone resorption [86]. Despite prostate cancer bone metastases being predominantly osteoblastic, as previously mentioned, they often have osteolytic components as well, motivating the use of bisphosphonate therapy in these patients. Clinical trials have shown that bisphosphonates, such as zoledronic acid, reduce bone pain and the number of SREs [86, 87], while no effect in overall survival has been achieved [88]. The RANKL antibody denosumab is another inhibitor of osteoclastogenesis and bone resorption, that delays the onset of SREs [89].

Radioisotopes can also be used to specifically target tumor cells in bone. Radium-223 is an alpha emitter, that due to its chemical similarity to calcium, can be incorporated into the bone matrix during bone formation [90, 91]. Since prostate cancer cells induce bone formation, the uptake is supposedly higher at metastatic sites [92]. The high-energy alpha particles emitted from bone-incorporated radium-223 delivers an intense and, given its short range, highly localized radiation dose to surrounding cells [90]. A large phase III clinical trial by Parker et al demonstrated that radium-223 treatment increase the overall survival in castration-resistant prostate cancer patients with bone metastases [92].

In addition to the therapies described here, there are a number of novel bone targeting agents currently tested in clinical trials. Some of these will be discussed in later chapters.
The insulin-like growth factor system

**IGF-1R signaling**
The IGF-1R is a transmembrane receptor tyrosine kinase with a high sequence homology to the insulin receptor (INSR) [93]. Both IGF-1R and the INSR are composed of two α-chains and two β-chains. The extracellular α-subunit binds the ligand while the transmembrane β-subunit contains the tyrosine kinase domain and is responsible for intracellular signaling [94]. Because of the high sequence and structure similarities between IGF-1R and INSR they can form hybrid receptors [93]. The IGF-1R can be activated by three ligands, IGF-1, IGF-2, and insulin. IGF-1 has the highest affinity for the receptor, and insulin the lowest [94].

IGF binding proteins (IGFBPs) are a group of proteins that binds to IGFs and modulates their biological activities. For example, IGFBPs can regulate the half-life of IGFs, and modify their distribution to different tissues and their bioavailability for receptor binding [95]. There are six different IGFBPs; IGFBP1-5 have similar affinities to both IGF-1 and IGF-2, whereas IGFBP6 have a higher affinity for IGF-2 [96, 97]. Insulin can bind to all IGFBPs, but with a very low affinity [96, 98].

Upon ligand binding to the IGF-1R α-subunit, the β-subunit undergoes a conformational change, followed by auto-phosphorylation of tyrosine kinase residues in the β-subunit [99, 100]. This initiates a cascade of downstream signaling via the PI3K/AKT pathway the Grb2/RAS/RAF/MAPK pathway, affecting for example proliferation, differentiation, and transformation [101, 102].

**Physiological functions of the IGF axis**
The IGF axis is important in both embryonic development and postnatal growth. Even though IGF-1 is expressed only at low levels in mouse embryos, the majority of IGF-1 knockout mice die before or soon after birth [103–105]. In addition, those that are born are markedly underweight compared to wild-type mice, and exhibit a delayed ossification and infertility [103–106]. In humans, IGF-1 deficiency results in short stature [107].

Disruption of IGF-1R results in similar, but more pronounced, embryonic defects as seen in IGF-1 knockout mice, and homozygous null mutants die at birth due to respiratory failure [105, 108]. Knockout studies have also revealed a role of the IGF axis in the
development of cardiac and skeletal muscles [105, 109–111], and the nervous system [106, 112].

Serum levels of IGF-1 increases slowly from birth, peaks during puberty, and later declines with age [113]. In adults levels of circulating IGF-1 is determined by both genetic and lifestyle factors [114]. IGFs are mainly synthesized in the liver, in response to somatotropin stimulation [115], but they can also be produced in other tissues such as bone, brain and muscles [116]. Mice with liver-specific disruption of Igf-1 has a 75 % reduction in levels of circulating IGF-1 [117, 118]. However, they exhibit no significant reduction in overall postnatal body growth compared to wild-type mice. Indicating that, despite the liver being the largest producer of serum IGF-1, the local production in other organs is more important for growth.

**The IGF axis in prostate cancer**

Numerous studies have implicated the importance of members of the IGF axis in the development and progression of various cancers. Elevated levels of IGF-1 in serum has been linked with an enhanced lifetime risk of developing prostate cancer [119–123]. However, there has been reports where no association between serum IGF-1 and risk for prostate cancer was found [124–126].

Furthermore, it has been suggested that the IGF-1R has an essential role in transformation. For instance, fibroblasts from IGF-1R knockout mice, cannot transform in response to viral or cellular oncogenes unless IGF-1R is reintroduced to the cells [127]. Besides this, IGF-1R is frequently overexpressed by tumor cells in advanced and metastatic prostate cancer [128–130], although some reports show that levels of IGF-1R is unchanged or even lowered [123, 131, 132]. Nonetheless, disruption of IGF-1R reduces tumor cell growth and survival [133–136].

Under normal conditions, prostate stromal cells secrete factors, such as IGF-1, that stimulates epithelial and vascular cells within the prostate [137, 138]. It has been shown in vivo, that castration causes a rapid decline in IGF-1 signals from the stroma [137]. And in patients there is a correlation between the reduced IGF-1 levels within the prostate and increased tumor cell apoptosis [139].

Of great interest in prostate cancer is the suggested connection between the AR and both IGF-1 and IGF-1R. It has been shown that the AR can upregulate expression of IGF-1R [140–143], the underlying mechanisms are however not fully understood [144]. The upregulation
of IGF-1R in prostate cancer cells results in increased cell proliferation and invasiveness in response to IGF-1 stimulation [141].

There is also evidence that IGF-1 can influence AR signaling. According to some reports IGF-1 can activate the AR in a ligand-independent manner [145, 146], which is of particular interest during ADT. However, Plymate et al. showed that this may only be valid in primary tumors, and that in metastatic cell lines IGF-1 suppress AR activity in response to DHT [140]. Studies also show that IGF-1 may enhance nuclear translocation of AR in the absence of androgens, and that this effect can be blocked by inhibiting IGF-1R [144, 147]. This process might be of importance during prostate cancer progression to an androgen independent disease.

**IGF-1R as a therapeutic target**

Several clinical trials studying IGF-1R inhibition in cancer has been performed, however, many of these trials have reported a poor effectiveness of the drugs as well as problematic side effects (primarily hyperglycemia) [101, 116]. There are several different approaches to target the IGF-1R in patients. Currently two methods have been used on prostate cancer patients in clinical trials; Tyrosine kinase inhibitors and neutralizing antibodies. Tyrosine kinase inhibitors prevent autophosphorylation of the tyrosine kinase domain of the receptor, and thereby the enzymatic activation of downstream proteins [148]. Since the IGF-1R and INSR are highly homologous, tyrosine kinase inhibitors generally inhibit both receptors, although their affinities for the two receptors can vary. The compound used within the work of this thesis, NVP-AEW541, was developed by Novartis and is a small molecule inhibitor 27-fold more potent against the IGF-1R compared to INSR [134]. In tumors that depend on both IGF-1R and INSR signaling the therapeutic effects would most likely be lower with an IGF-1R-specific drug. And preclinical evidence show that tumor cells can deploy INSR signaling to adapt to IGF-1R-specific antibodies [149, 150]. NVP-AEW541 has been used in numerous pre-clinical studies of cancer, where it has been shown to inhibit tumor cell proliferation and survival both *in vitro* and *in vivo*, in a variety of cancer types [151–153].

A number of monoclonal antibodies targeting the IGF-1R has been developed. These antibodies are highly specific for IGF-1R and do not cross-react with the INSR. They bind to the extracellular domain of the IGF-1R, thereby blocking ligand binding, hence, preventing the activation of downstream signaling pathways [102]. Other important
effects of monoclonal antibodies is that they can downregulate IGF-1R over time by inducing internalization and degradation of the receptor [133, 154], and that they might induce antibody-dependent cellular cytotoxicity [148, 155]. Antibodies have a longer half-life than small molecule inhibitors and could result in more severe hyperglycemia [102]. However, Phase I and II clinical trials demonstrate that most of these antibodies are well tolerated [148]. Cixutumumab (IMC-A12) is a human monoclonal antibody that inhibits ligand binding to IGF-1R without blocking insulin binding to the INSR [133]. Several preclinical studies have demonstrated the antitumoral effects of cixutumumab on for example prostate, breast, colon, multiple myeloma and pancreatic cancer cells [133, 135, 136, 144]. In a xenograft model of human prostate cancer cixutumumab was shown to reduce growth rates of both androgen-dependent and androgen-independent cancer cells [156]. Furthermore, cixutumumab, in combination with castration, has been shown to prolong the time to androgen independent recurrence, compared with castration alone. [147] At present, this antibody is tested in several clinical trials, either in combination with other therapies or as a single agent.
Aims

General aims
Skeletal metastases are common in patients with prostate cancer, and at present, there are no curable treatments for these patients. There are, most likely, a number of reasons why prostate cancer cells thrive in bone. It is known that as tumor cells disseminate to bone they alter the activities of cells responsible for bone remodeling, and by doing so they create a more favorable environment for themselves.

The general aim of this thesis was to expand our knowledge about the bidirectional interplay between tumor cells and bone cells, and thereby to identify possible therapeutic targets and predictive markers of therapeutic response.

Specific aims

Paper I
To establish an *in vitro* model system that enables studies of interactions between tumor cells and bone cells

Paper II
To confirm stimulating effects of bone-derived IGF-1 on prostate cancer cells and to examine if IGF-1R inhibition can enhance the inhibitory effects of simvastatin on prostate cancer cell lines co-cultured with bone

Paper III
To study acute tumor-inhibiting effects of IGF-1R inhibition in combination with castration in a rat model of prostate cancer growth in bone

Paper IV
To characterize bone remodeling activities, in relation to tumor cell AR activity, in clinical samples of prostate cancer bone metastases, and thereby identify therapy-predictive markers and possible therapeutic targets
Materials and methods

For detailed descriptions please see the corresponding papers.

In vitro studies

Cell lines (Paper I-III)
Human prostate cancer cell lines PC-3, LNCaP and 22Rv1 were used for in vitro experiments. PC-3 is an androgen-independent cell line, originally derived from a clinical bone metastasis and it is known to induce an osteolytic response in bone [157, 158]. LNCaP is an androgen-sensitive cell line, derived from a clinical lymph node metastasis and it induces a mixed, somewhat osteoblastic, response in bone [158, 159]. 22Rv1 originates from CWR22 cells (derived from a primary prostate tumor) that were serially passaged in mice [160]. After castration-induced regression the CWR22 cells relapsed, giving rise to the 22Rv1 cell line. 22Rv1 cells are androgen-sensitive and induce a mixed response in bone [161].

The rat prostate cancer cell line Dunning R3327-G was used both in vitro and inoculated into rats. The original Dunning R3327 cells were derived from a spontaneous prostate tumor in a Copenhagen rat, and after serial passages these cells gave rise to a number of sublines [162]. The subline used here, Dunning R3327-G, is an androgen-sensitive, cell line with low metastatic potential [163].

All cell lines were cultured according to the manufacturer’s instructions.

Co-culture model (paper I-III)
Tumor cells were seeded in their respective culture medium (for more details, see paper I-III) in 6-well plates. After 24 hours the medium was replaced with bone culturing medium, and cells were incubated for another 24 hours. Thereafter, cells received new bone culture medium and after 3-4 hours calvarial bones on metal grids were placed in the wells (see paper I). These grids separate the bones from the tumor cells, but enables signaling through the medium between the two compartments. The calvarial bones were dissected from 5- to 7-day-old CsA mice, cut into halves, preincubated with indomethacin for 24 hours. Indomethacin prevents initial bone resorption induced by an increased release of prostaglandins as a result of dissection
trauma [164]. Calvariae were subsequently washed before they were introduced to the cell cultures. In the different papers various stimulating factors or inhibitors were added to the co-cultures to study their effect on the calvariae and/or the tumor cells (vitamin D₃ in paper I, an IGF-1 neutralizing antibody (R&D Systems) or NVP-AEW541 (Novartis Pharmaceuticals) and/or simvastatin (Sigma-Aldrich) in paper II, and DHT in paper III).

**Cell viability assay (paper II-III)**

Tumor cell viability was determined by the Cell Proliferation kit I (Roche Diagnostics) according to the manufacturer’s instructions. Briefly, cells were seeded in 96-well plates and 24 hours later culture medium was replaced with phenol-free medium with 2.5 % charcoal stripped FBS. This medium was supplemented with various concentrations of NVP-AEW541 and/or simvastatin in paper II, and rat recombinant IGF-1 (R&D Systems), and/or DHT and/or NVP-AEW541 in paper III. Cell viability was measured at time points between 2-7 days.

**Flow cytometry (paper II)**

Flow cytometry was used to examine the relative levels of apoptotic tumor cells from co-cultures supplemented with NVP-AEW541 and/or simvastatin. Tumor cells from co-cultures were harvested and washed, and apoptosis was assessed using the FITC Annexin-V Apoptosis Detection Kit (BD Pharmingen) according to manufacturer’s instructions. Staining was determined by flow cytometry (FACSCalibur, BD Biosciences FACS) and analyzed using the CellQuest software (BD).

**RNA analyses**

**RNA extraction and cDNA synthesis (paper I-III)**

Total RNA, from cell lines and calvariae, was extracted using the RNaqueous kit (Ambion) according to the manufacturer’s instructions. RNA was DNase-treated with TURBO DNase (Ambion) and RNA concentrations were determined using a Nanodrop ND 1000 (Nanodrop Technologies). RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; paper II) or the 1st Strand cDNA Synthesis kit (Roche) with Oligo(dT)₁₅ primers (paper I, III).
**Quantitative real-time RT-PCR (paper I-III)**

To evaluate bone cell activation in calvariae, mRNA levels of osteoclast activator *Rankl* and its decoy receptor *Opg*, together with osteoclast markers genes *Catk, Mmp9* and *Trap*, and osteoblast marker genes *Alp* and *Oc*, were measured using quantitative real-time RT-PCR. Quantifications were performed using the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) in combination with the TaqMan Universal PCR Master Mix (Applied Biosystems) or the Power SYBR Green Master Mix (Applied Biosystems). Negative controls were always run in parallel. Resulting quantification data was analyzed using the ABI SDS software and the standard curve method (User Bulletin #2, Applied Biosystems). All samples were normalized to the expression levels of housekeeping genes (*Actb* in rodent mRNA and *RPL13* in human mRNA).

**Gene expression array analysis (paper IV)**

Total RNA was extracted from representative areas of fresh frozen bone metastases sections using the Trizol (Invitrogen) or the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) protocols. Nucleic acids were quantified by absorbance measurements using a spectrophotometer (ND-1000 spectrophotometer; NanoDrop Technologies). The RNA quality was analyzed with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and verified to have an RNA integrity number ≥ 6. Gene expression array analysis was performed using the human HT12 Illumina Beadchip technique (Illumina, San Diego, CA). Data from two gene expression studies were combined and samples were included if they contained a minimum of 10 % bone tissue and a tumor cell content of at least 30 %. Beadchip data was included for all probes with average signals above two-times the mean background level in at least one sample per study array. Arrays were individually normalized using the quantile method (GenomeStudio V2011.1, Illumina) and each probe was centered by the median of the samples in the corresponding dataset. Normalized datasets were merged by mapping Illumina ID and Hugo gene symbol, leaving 13846 probes for further analysis.
Protein analyses

Protein extraction (paper II)
Proteins were extracted in lysis buffer containing 1 % Igepal CA-630 (Sigma-Aldrich, ST. Louis, MO), 20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 10 % glycerol, 2 mM EDTA and Complete Protease Inhibitor (Roche Diagnostics). Lysis buffer for cells to be used in the phospho-RTK array also contained 10 μL/mL Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). Samples were mixed and incubated on ice for 30 minutes. Supernatants were isolated following centrifugation and protein concentration was determined by the BCA Protein Assay Reagent kit (Pierce Chemical Co.)

Western blot (paper II)
Proteins were separated by 7.5 % SDS-PAGE under reducing conditions and subsequently transferred to PVDF membranes. Membranes were blocked in 5 % milk prior to incubation with primary antibodies targeting the AR (N20, Santa Cruz Biotechnology), to detect full length AR and AR variants with an intact N-terminal domain, or the IGF-1R (AF-305-NA, R&D Systems). Following incubation with the appropriate secondary antibodies (Dako) protein expression was visualized using ECL Advanced or ECL Plus detection kits (GE Healthcare). Membranes were stripped and re-analyzed with primary antibody against actin (Sigma) and the relative AR and IGF-1R levels were adjusted for the corresponding actin levels.

Phospho-receptor tyrosine kinase array (paper II)
Starved tumor cells were stimulated with calvaria-conditioned media for 10 minutes and then lysed on ice as described above. Phospho-receptor tyrosine kinase arrays (R&D Systems) were performed according to the manufacturer’s instructions and receptor tyrosine kinase phosphorylations were detected using the ECL Plus detection system. Average pixel density was determined using the Quantity-one software (Bio-Rad Laboratories) and average pixel density of duplicate spots was normalized to positive control spots.

ELISA (paper I-III)
For measurements of proteins secreted from murine calvariae or human cell lines during co-culture, conditioned media from the cultures were collected. Tumor cell-secretion of IGF-1 (R&D Systems) was determined using a human-specific ELISA. Bone- and bone cell-
secreted IGF-1 (R&D Systems), RANKL (R&D Systems), and OCL (Demeditec Diagnostics GmbH), was measured using mouse-specific ELISAs. The release of collagen fragments specific for bone resorption (C-telopeptide fragments of collagen type I, CTX-I, Immunodiagnostics Systems) and bone formation (Procollagen type I N-propeptide, PINP, Immunodiagnostics Systems) were also measured in conditioned media. All assays were carried out according to the manufacturer’s instructions.

Immunohistochemistry (paper (III-IV))

Sections containing bone tissue were decalcified in formic acid for 48 hours prior to paraffin embedding. Paraffin sections were stained with hematoxylin-eosin and van Gieson, for morphological analysis. Immunostainings were performed using primary antibodies against bromodeoxyuridine (BrdU, 347580, BD Biosciences), cleaved caspase-3 (9661L, Cell signaling), IGF-1R (AF-305-NA, R&D Systems), IGF-1 (05-172, Millipore), AR (pG21, 06-680, Millipore), RUNX2 (ab81357, Abcam), and TRAP (MABF96, Millipore). Negative control sections were prepared by performing immunostaining procedures without adding primary antibodies.

For a more detailed description of histochemical and morphological evaluations, see paper III and IV.

Animals and treatments (Paper III)

Intra-tibial injections of Dunning R3327-G cells were performed in anesthetized, adult, male Copenhagen rats (Charles River, bred at our animal facility). Four weeks later half of the rats were treated per oral gavage with 80 mg/kg/day of NVP-AEW541 dissolved in 25 mM L(+) -tartaric acid (Sigma-Aldrich), and the other half with 25 mM L(+) -tartaric acid only. On the second day of treatment, half of the treated and half of the control rats were castrated and the remaining rats sham operated. At sacrifice, on the fifth day of treatment, rats were injected i.p. with bromodeoxyuridine (BrdU 50 mg/kg; Sigma-Aldrich) and one hour later animals were sacrificed. Tumor-containing tibias, prostate lobes and lungs were dissected and weighted before fixed in 4 % paraformaldehyde for 48 hours, and subsequently embedded in paraffin. Six rats were injected with RPMI only, and sacrificed 5 weeks later. Animal work was carried out in accordance with protocol approved by the Umeå Ethical Committee for Animal Studies (permit number A5-15).
Patient materials (Paper III-IV)

Bone metastasis samples were obtained from a series of fresh-frozen and formalin fixed paraffin embedded biopsies collected from patients with prostate cancer operated for metastatic spinal cord compression or pathologic fractures at Umeå University Hospital (2003-2015). The study was approved by the local ethic review board of Umeå University and participants gave written or verbal consent (Dnr 03-158, Dnr 04-26M).

Statistics

Univariate statistics (Paper I-IV)
Correlations between variables were investigated using Spearman rank test. Groups were compared using the independent samples t-test, paired samples t-test or Mann-Whitney U test. Kaplan-Meier survival analysis was performed with death due to prostate cancer as event and follow-up time as time between metastasis surgery and the latest follow-up examination. A P-value below or equal to 0.05 was considered statistically significant. Statistical analyses were performed using the latest version of SPSS software.

Multivariate statistics (Paper III-IV)
Multivariate modelling by means of principal component analysis (PCA) and orthogonal projections to latent structures (OPLS), was used to capture variations in whole-genome expression data. Models were based on IGF-1R immunoreactivity score in paper III and bone remodeling activity in paper IV. Multivariate statistical analyses were performed with SIMCA software version 14.0 (Umetrics AB).

Functional enrichment analysis
Enrichment analysis was performed using the MetaCore software (GeneGo, Thomson Reuters) in order to identify enriched process networks in the data. Upstream analysis was used to identify regulators with a probability to be responsible for the observed enriched process networks, based on the P-value for a calculated connectivity ratio between actual and expected interactions with objects in the data.
Results and discussion

Paper I

“Establishment and validation of an in vitro co-culture model to study the interactions between bone and prostate cancer cells”

In order to understand the underlying mechanisms of prostate cancer bone metastases and to find new treatments for these patients we need reliable model systems, both in vitro and in vivo, where interactions between tumor cells and the bone microenvironment can be studied. Here we present a two-compartment co-culture system where tumor cells are cultured together with calvarial explants from mice. The tumor cells are separated from the calvaria by a metal grid that enables signaling between the two compartments via the culture medium. We validated the model using two human prostate cancer cell lines; PC-3, which is known to be osteolytic, and LNCaP, that have a mixed/osteoblastic phenotype [158].

To evaluate the effects on bone remodeling induced by the presence of tumor cells we used a panel of genes expressed by bone cells [42]. Catk, Mmp9 and Trap were used to detect active osteoclasts, and Alp and Oc were used to detect active osteoblasts. Osteoclast activator Rankl, and its decoy receptor Opg, were also included in the panel. Co-culture of calvariae and LNCaP cells initially induced calvarial transcription of osteoclast marker genes, which was later followed by a shift towards a more osteoblastic response, with upregulation of osteoblast marker genes. This resembles the, above described, tumor cell-induced bone response observed in patients, where increased osteolytic and osteoblastic activities can be observed simultaneously, and will be further discussed in paper IV. It also resembles the sequence of events seen in normal bone remodeling where resorption, performed by osteoclasts, causes a release of factors that in turn initiates bone formation.

PC-3 cells induced calvarial transcription of genes associated with the activation and function of osteoclasts, and a decreased transcription of osteoblast associated genes. This gene expression profile was similar to that induced by vitamin D3, which was used as a positive control for bone resorption. However, the inhibitory effect on transcription of osteoblast associated genes was more pronounced
with PC-3 cells compared to vitamin D₃. To examine the actual biological resorption of bone the levels of collagen fragment CTX-I, which is released during degradation of bone matrix, was measured in the co-cultures. Both PC-3 and vitamin D₃ stimulated the release of CTX-I, while LNCaP had no effect on CTX-I levels. This further supports the observed gene expression profiles. Interestingly, vitamin D₃ was found to have a stronger effect on CTX-I release, compared to PC-3, and furthermore, gene expression profiles were initially more lytic in vitamin D₃ stimulated calvariae. We speculate that this could be due to the direct addition of vitamin D₃ when initiating the experiments, compared to the accumulation of resorption stimulatory factors in the media when PC-3 cells are used.

Gene expression analysis of the prostate cancer cell lines revealed that PC-3 cells express high levels of genes associated with bone resorption, such as DKK-1 and PTHRP. These genes were only expressed at very low levels in LNCaP cells. Moreover, IGF-1R was highly expressed in both cell lines, even though PC-3 had non-detectable, and LNCaP only very low levels of its ligand, IGF-1. This indicates that in order to activate this receptor the tumor cells require IGF-1 produced by other cells. Most likely, PC-3 cells acquire IGF-1 by inducing bone resorption, which results in degradation of bone matrix, and thereby a release of IGF-1 and other growth factors from the matrix [26, 165, 166]. The LNCaP cells might be able to acquire IGF-1 in the same way, but since they activate bone formation it is possible that they can be stimulated by IGF-1 produced by osteoblasts before it is incorporated into the newly formed bone matrix [26, 167, 168]. IGF-1R signaling is further studied in paper II and III.

One of the main concerns when performing research on bone metastases is the complexity of the bone microenvironment and how to reproduce this in vitro. Many studies are based on co-cultures of tumor cells and one, or several, types of bone cells, including bone marrow endothelial cells or osteoblasts, cultured for instance as monolayers, in collagen matrices, or in direct contact with each other [169–172]. These models have advanced our knowledge of for example what stimulates tumor cells and bone cells, however, they cannot provide us with information on how tumor cells and bone cells interact with each other, and the rest of the bone microenvironment, at the same time. To overcome this limitation tumor cells can instead be cultured together with bone tissue explants. This provides a more natural setting, including the extracellular matrix together with all bone cell types. In our co-culture model presented in this paper the
calvarial bone is separated from the tumor cells by a metal grid. Since not all factors that can stimulate tumor cells or bone cells are secreted, but require cell-cell or cell-extracellular matrix interactions, it is possible that by culturing the tumor cells in direct contact with the bone even more processes can be activated in tumor cells and bone cells. Furthermore, the extracellular matrix makes bone remodeling possible which is a very important process in bone metastases, facilitating the release of different factors important both for tumor cells and bone cells.

In conclusion, when performing in vitro studies of bone metastases it is important to keep in mind the benefits and limitations of the different model systems when selecting a method suitable for the type of questions that are asked. In this work we present a co-culture model that can be used to study tumor cell-mediated activation of bone cells and bone remodeling, and the effects of the bone microenvironment on tumor cells. The model is simple to use and the experimental settings can easily be manipulated. The use of this model in comparison to other available models will be discussed further in the general discussion.

Paper II

“Inhibition of the insulin-like growth factor-1 receptor enhances effects of simvastatin on prostate cancer cells in co-culture with bone”
Within this paper we found that tumor cells were stimulated by co-culture with calvarial explants from mice. To understand which bone-derived factors could be responsible for this, we stimulated prostate cancer cell lines (PC-3 and 22Rv1) with calvaria-conditioned medium and examined the phosphorylation status of 42 receptor tyrosine kinases. The IGF-1R was found to be one of the most highly activated tyrosine kinase receptor in both cell lines. In addition to this, we found that, during co-culture, both cell lines induced calvarial release of IGF-1 into the media. Only very low levels of human IGF-1 from the prostate cancer cells were found in the co-culture media, and neither the absence, nor the presence, of calvariae had any effects on these levels. We also found that by adding a neutralizing antibody targeting murine IGF-1 to the co-cultures the calvariae-stimulated increase in PC-3 proliferation could no longer be observed. No significant change was seen in number of 22Rv1 cells, indicating that there are additional
calvaria-derived factors that can affect their growth. Possible candidates will be further discussed in the general discussion. Taken together, these results show that tumor cells can induce the release of bone-derived IGF-1, resulting in IGF-1R activation, and stimulation of tumor cell growth. Tumor cell-response to ADT is generally less pronounced in bone metastases compared to primary tumors. One reason for this might be that the bone milieu is rich in tumor stimulating growth factors, such as IGF-1. We hypothesize, that by blocking IGF-1 tumor cell-stimulation the tumors would have a stronger response to apoptosis-inducing therapies, in this case simvastatin.

A previous study from our group showed that prostate cancer bone metastases contain high levels of cholesterol [173]. And it has been proposed that cholesterol targeting drugs, namely statins, can induce apoptosis in prostate cancer cells in vitro [174–176]. Here we used simvastatin in combination with a small molecule IGF-1R kinase inhibitor, NVP-AEW541. The drugs were tested on tumor cells only, but also added to the co-cultures. We found that tumor cell numbers decreased when cells were treated with either drug alone, and that when the drugs were combined the inhibitory effect was enhanced. The effects on apoptosis were also more pronounced when a combination of the drugs was used. The fact that 22Rv1 cells were unaffected by neutralization of calvaria-derived IGF-1 in the co-cultures, but inhibited by NVP-AEW541, could indicate a possible autocrine stimulation of IGF-1R, or that IGF-1R is transactivated by other ligands.

Statins are one of the most frequently prescribed drugs worldwide, mainly used to prevent cardiovascular diseases [177]. Due to the widespread use of statins, it has been possible to perform large epidemiological studies, revealing that statins might be associated with a reduced risk of developing prostate cancer, in particular a more aggressive prostate cancer [178–180]. The results from these reports are however controversial, since other studies have shown that there is no direct association between statin use and risk of developing prostate cancer [181, 182]. To further elucidate this potential relationship more studies has to be performed, both to understand the mechanisms behind the potential anti-cancer properties of statins, and to learn the optimal timing for administration of the drug, and what type of statins to use [183]. For the work presented in this paper it is also interesting to note that statins and IGF-1R inhibitors might, at least in part, affect the same intracellular pathway, the PI3K-Akt
pathway [175]. Statins have also been shown to downregulate expression of IGF-1R in tumor cells [184, 185]. We found that, in PC-3 cells, both IGF-1R mRNA and protein levels were reduced in response to simvastatin alone, and in combination with NVP-AEW541. This effect could not be observed in 22Rv1 cells, where the only significant change found, was increased IGF-1R mRNA levels after NVP-AEW541 treatment.

It has been indicated that statins can affect expression and degradation of the AR in some prostate cancer cell lines [186]. We investigated this in 22Rv1 cells co-cultured with calvariae and treated with simvastatin. Protein levels of full-length AR were unaffected, while mRNA and protein levels of the constitutively active AR-V7 splice variant were reduced in response to simvastatin. It has been reported that expression of ligand-independent AR variants, such as AR-V7, promotes the development and growth of castration-resistant prostate cancer [187–190], supporting the idea that statins can reduce the development of aggressive prostate cancer and suggesting that statins might be of benefit to patients expressing AR variants. Another interesting aspect of statin use in prostate cancer patients is the connection to steroidogenesis. One mechanism by which the AR can be reactivated, as prostate cancer becomes castration-resistant after ADT, is de novo intratumoral androgen synthesis [23, 191]. Cholesterol is a precursor in the multi-step pathway of androgen synthesis [192], and it has been shown that cholesterol levels are higher in bone with prostate cancer metastases compared to bone without tumors [173]. Therefore it has been suggested that administration of cholesterol-lowering drugs, such as statins, may be able to reduce intratumoral steroidogenesis, and thereby delay the development of a castration-resistant cancer [193].

In summary, we show that prostate cancer cells induce the release of growth factors such as IGF-1 from bone, which can in turn stimulate tumor cell growth. Simvastatin induced apoptosis and reduced proliferation of prostate cancer cells co-cultured with bone, and by combining simvastatin with IGF-1R inhibition the therapeutic effects were further enhanced. Thus, inhibition of the IGF-1R might be a way to improve effects of therapies that induces apoptosis in prostate cancer cells in a bone milieu. Simvastatin effects in patients with AR-V7 positive metastases deserve further evaluation.
Paper III

“Inhibition of the insulin-like growth factor-1 potentiates acute effects of castration in a rat model for prostate cancer growth in bone”

The theories proposed in paper II were further tested in paper III, advancing from an *in vitro* model to an *in vivo* rat model. Within this study the acute effects of NVP-AEW541 in combination with castration, the conventional treatment for advanced prostate cancer, were investigated. Treatment response (proliferating and apoptotic tumor cells) was evaluated 4 days after castration, when the maximum effects on apoptosis are normally found. The rat prostate cancer cell line, Dunning R3327-G, was found to be a suitable model since we demonstrated that these cells were stimulated by IGF-1 and DHT, responded to NVP-AEW541 therapy, and were found to induce an osteoblastic response in bone *in vitro* and an overall increase in bone remodeling *in vivo*.

To evaluate the response of these treatments, Dunning R3327-G cells were injected into the tibia of male rats. Five weeks after injection, established tumors were found in the bone marrow cavity and, occasionally, also outside the bone in the knee joint cavity and in muscles. Interestingly, we found that the effects of castration differed depending on where the tumor cells were located. Outside the bone, castration resulted in decreased proliferation and increased apoptosis in tumor cells. In contrast, proliferation and apoptosis in tumor cells within the bone marrow cavity was unaffected by castration. Importantly, NVP-AEW541 was able to reduce proliferation both alone, and even further, in combination with castration, independent of tumor site. Apoptosis was however only induced outside tibia in response to NVP-AEW541. Comparing the basal apoptotic rates, we found that tumor cells located in the bone marrow had a significantly higher apoptotic rate compared to tumor cells outside the bone. We speculate that this might affect the response to treatment, and that it could be a result of the hypoxic environment in the bone marrow and its low androgen levels [194, 195]. Recently, Halin et al [194], reported similar findings using Dunning R3327-G in rats. Castration had no effect on tumor cells growing in the bone marrow cavity, while tumor cells growing orthotopically, in the rat ventral prostate, displayed a decreased proliferation and increased apoptosis in response to castration. Castration-induced apoptosis has also been observed in an AR-negative subline of Dunning R3327, AT-1, growing in rat ventral
prostate [196]. Thus, it is evident that the tumor microenvironment plays a large role in response to castration. In the prostate, ADT affects epithelial-derived tumor cells both directly, via reduced stimulation of the AR, and indirectly by altering stromal cells, resulting in for example reduced IGF-1 levels [137, 197]. The reasons why we observe castration-induced effects in Dunning R3327-G cells outside the tibial bone, but not within the bone, is not known. It is possible that the microenvironment of the knee joint cavity and the skeletal muscles is androgen-sensitive and thereby influence the tumor cells in a similar way as described in localized prostate cancer. It could also be caused solely by the direct effect on AR signaling in the tumor cells. The lack of therapy-induced effects within the bone might in addition to the reason discussed above be explained by the presence of tumor-stimulating factors, other than IGF-1. This needs to be further examined in the Dunning model, and whether or not this is also true in patients.

The hypothesis investigated in this paper was recently tested in a phase II clinical study where prostate cancer patients were treated with a combination of ADT and the IGF-1R targeting cixutumumab [198]. The treatment was well tolerated, however, the primary endpoint of the study, a difference in the rate off undetectable PSA after 28 weeks, was not reached, and therefore the trial was terminated. In our study we show that tumor cell IGF-1R immunoreactivity varies between patients, indicating that not all patients can be expected to respond to this therapy. Therefore, we believe that there is a need for predictive markers that can identify the subgroup of patients that are most likely to benefit from inhibition of IGF-1R signaling. This subject will be further debated in the general discussion.

Gene expression profiles and measurements of calvaria-released collagen fragments revealed that Dunning R3327-G cells were able to induce a sclerotic bone response in vitro. However, in rats, bones with Dunning R3327-G cells were found to have increased activities of both osteoclasts and osteoblasts. This resembles the findings we make in prostate cancer metastases from patients described in paper IV. It is also in line with other studies, where elevated serum levels of bone resorption markers can be found in patients with sclerotic bone metastases [28]. We currently don´t know the reasons to why resorption is induced by Dunning R3327-G in vivo, but not in vitro. As discussed above, the use of a rat cell line with a murine calvaria in vitro may hinder some of the communication between the tumor cells.
and the bone, but several other explanations might exist for this finding. There was no increase in bone mass in rats even though osteoblast activity was highly stimulated. The mechanisms behind this might be an increased number of osteocytes differentiating from osteoblasts. Osteocytes produce high levels of RANKL [199], which in turn activates osteoclasts and consequently increases bone resorption. These results support the ‘seed-and-soil’ theory previously described, and results in a release of IGF-1 and other growth promoting factors yet to be identified from the bone matrix.

In summary, using the Dunning R3327-G model we show that inhibition of pro-survival IGF-1R signaling has the potential to enhance the acute therapeutic effects of castration in prostate cancer. We also reveal that tumor cell response to both castration and anti-IGF-1R therapy is greatly dependent on the tumor cell microenvironment, and tumor-protecting factors present in the bone needs to be identified. Moreover, we found that Dunning R3327-G cells induce bone remodeling by activation of both osteoclasts and osteoblasts in rat tibia, demonstrating the relevance of using this model for studies of tumor cell and bone cell interactions and also to investigate the effects of treatments aimed at prostate cancer bone metastases.

**Paper IV**

**“Bone remodeling in relation to androgen receptor activity in prostate cancer bone metastases”**

The osteoblastic nature of prostate cancer bone metastases in comparison to other cancers, such as breast, lung and renal cancers, where bone metastases are generally classified as osteolytic, indicates an association to the AR. In this study we aimed to characterize the ongoing bone remodeling activities in prostate cancer bone metastases, and to investigate how bone remodeling relates to AR activity.

Using whole-genome expression analysis, together with histological evaluations, we studied bone remodeling activities in bone metastases from prostate cancer patients. We defined a set of genes, similar to the gene profiles used to follow bone remodeling in calvariae in co-culture (Paper I, II), to represent osteoclasts (ACP5; encoding the TRAP protein, CTSK, and MMP9), osteoblasts (ALPL, BGLAP, and RUNX2), and osteocytes (SOST) [42]. The transcript levels of these genes revealed high osteoblast activity to be coupled to high osteoclast
activity. In other words, patients either had high or low overall activity of bone remodeling. This was further verified using immunohistochemistry. The fraction of TRAP-positive osteoclasts and RUNX2-positive osteoblasts lining the bone surface was positively correlated to the expression of their respective genes. The correlation was, however, relatively weak, which could be a result of heterogeneity between samples. Throughout the histological examinations performed within this work we found a large heterogeneity, both between different tissue specimens taken from the same metastasis, but also within samples taken from the same piece of tissue. For example, areas of high bone remodeling were often found in the same tissue section as areas with old bone and no apparent bone cell activity. This is important to keep in mind when comparing the results from the various analyses performed here. One should also note that the metastases used in this study are taken from one site, after spinal cord compression, and may not represent the whole tumor burden. Measuring markers of bone remodeling in serum may provide a better picture of bone remodeling activities in patients.

To investigate what distinguishes patients with high bone remodeling activity from patients with low activity, we compared RUNX2 and TRAP immunoreactivity to a number of clinical variables. Results showed that RUNX2 and TRAP immunoreactivity was associated to patient age at diagnosis, implying that older patients have more active bone remodeling. It has been shown that androgen levels decrease with age [200], however, it is not known if this influences our results since most of the included patients have received ADT. Out of 35 patients, four had undergone chemotherapy, and interestingly, we found that these patients had significantly lower RUNX2 immunoreactivity, while TRAP immunoreactivity was unaffected. This indicates that chemotherapy might select for development of osteolytic metastases, and needs to be further explored, both functionally in animal models, and in a larger cohort of patients.

Moreover, serum PSA levels at metastatic surgery was found to be inversely correlated to RUNX2 and TRAP immunoreactivity. To further investigate this finding, we analyzed the whole-genome expression data and found that bone remodeling activity was inversely correlated to genes indicating tumor cell AR activity [201], specifically, the AR itself, AR co-regulators FOXA1 and HOXB13, and AR regulated genes KLK2, KLK3, NKK3-1, STEAP2, and TMPRSS2. As previously described, osteoblasts have both AR and ERα, and activation of these
receptors results in increased bone formation, and an indirect inhibition of osteoclast formation. Estrogens can also inhibit osteoclastogenesis directly, by binding to the ERα found on osteoclasts. In addition, androgens can be converted to estrogens and thereby stimulate the ERα. When levels of these sex hormones drop, bone remodeling increase, resulting in bone loss. It is known that increased levels of bone remodeling markers can be found in serum of patients undergoing ADT, and that a side effect of this treatment is reduced bone density [85]. Most of the metastases included in our study were taken from castration-resistant patients, thus they were likely to have increased bone remodeling activity as a consequence of ADT. From this, we further hypothesized that some metastases may have regained AR activity via intratumoral steroidogenesis and then also have regained the protective effects exerted by androgens on bone. The net effect of bone remodeling in some metastases included in the study might then be predominantly osteoblastic metastases, as expected in prostate cancer patients, and it would be interesting to compare these findings in relation to scintigraphy examinations as well as to intratumoral androgen and estrogen levels. Based on our results, however, this does not seem to the result of an overall high tumor AR activity, but might as discussed above, still be depending on direct stimulation of androgens or estrogens on bone cells, something that could be verified by immunohistochemical evaluation of AR and ERα in bone cells. Aromatase, the enzyme converting androgens to estrogens [202], could also be evaluated by immunohistochemical staining, although expression of CYP19A1, the gene encoding aromatase, was not detected in our data. Furthermore, we speculate that other tumor-secreted factors may be responsible for shifting the balance between bone formation and bone remodeling towards a more osteoblastic phenotype.

By secreting factors that stimulate osteoblasts or osteoclasts, tumor cells can initiate a vicious cycle, where tumor cells and bone cells stimulate each other. Since tumor cell AR activity was not found to be the driving force behind the increased bone remodeling seen in a subgroup of metastases, we focused on pathways that were enriched in these metastases. Ontology analysis revealed that the most enriched process in metastases with high bone remodeling was associated to ossification and bone remodeling, which can be expected since the model is based on a number of bone cell markers. Other highly enriched processes involved epithelial-to-mesenchymal transition, cell adhesion, proliferation, and BMP and GDF signaling. Interestingly,
inflammation, was also found among the most enriched processes. Bone traumas, such as fractures, are known to induce inflammation followed by bone repair [203].

Next, we identified probable upstream regulators that could be responsible for inducing the increased bone remodeling activities, and found that BMP signaling is a possible driver. Expression of BMP2, BMP4, and other factors involved in BMP signaling was highly enriched in bone metastases with high bone remodeling activity. BMP signaling involves specific SMADs that translocate to the nucleus to activate transcription. SMADs can form complexes with RUNX2, and thereby stimulate the transcription of genes involved in osteoblastogenesis and bone formation [204]. Other than being involved in bone formation, BMPs have also been found to be involved in several developmental processes that in the end have the potential to stimulate tumor cells. SPP1 was another interesting gene whose expression was found to be correlated to increased bone remodeling. BMP2 signaling, via the RUNX2-SMAD complex, can activate transcription of SPP1. It has been reported that osteopontin, the protein encoded by SPP1, can stimulate tumor cells proliferation and invasiveness, and that it can instigate the growth of otherwise quiescent metastases [205–207]. Whether BMP2, BMP4, and SPP1 are expressed by the tumor cells cannot be determined from gene expression analyses performed within this work, but will need immunohistochemical evaluation. It would also be interesting to evaluate the expression of these factors in relation to the total tumor burden of the patients.

Revealing the ongoing bone remodeling processes within bone metastases can be useful when selecting therapy for patients with bone metastatic cancer. As previously described, therapies targeting the bone microenvironment have been proven beneficial for patients with prostate cancer bone metastases. Bisphosphonates inhibits osteoclasts and successfully reduce the number of SREs. The same effects can be obtained with the RANKL inhibitor denosumab. In this study, expression of the gene encoding RANKL was undetectable in the array data in most metastases and it was therefore excluded from analysis. RT-PCR and/or immunohistochemical evaluation of RANKL in the studied metastasis cohort might still be done to evaluate RANKL expression in relation to the observed bone remodeling activity and if it could aid in determining a subgroup of patients likely to respond to denosumab treatment. Another interesting therapy for bone metastatic cancer is radium-223 which is incorporated into bone in
areas with a high bone turnover. It seems plausible that the bone remodeling activity within metastases may be of importance for patient response to radium-223 [208].

As indicated in this work, using therapies directed at BMP signaling may also be a way of targeting bone metastases. Studies that further signify the use of BMP inhibition show that the natural BMP inhibitor noggin can inhibit the expansion of PC-3 cells in vitro, and that inhibition of BMP receptors can inhibit ovarian cancer cell growth in vivo [209, 210].

In conclusion, we demonstrate that high osteoblast activity is coupled to high osteoclast activity in prostate cancer bone metastases, and that high bone remodeling activity is inversely related to tumor cell AR activity. All of these variables might be of use when selecting therapies for patients, especially when bone-targeting targeting therapies are considered. Our results also provide some insight when searching for possible therapeutic targets in bone metastases. We found that BMP levels were high in metastases with high bone remodeling activity, and suggest that its importance in osteoblastic metastases and its suitability as a therapeutic target should be further examined.
Conclusions

• The co-culture system described in this thesis provides an easily manipulated, simple to use, *in vitro* model to study interactions between tumor cells and bone cells.

• Prostate cancer cells induce the release of IGF-1 from bone, which can in turn stimulate tumor cell growth.

• Inhibition of the IGF-1R has the potential to enhance the therapeutic effects of both castration and simvastatin.

• The microenvironment is important for tumor cell-response to both castration and IGF-1R inhibition.

• Dunning R3327-G cells induce an osteoblastic response mouse calvariae *in vitro* and increased bone remodeling in rat tibial bone.

• A high osteoblast activity is coupled to a high osteoclast activity in prostate cancer bone metastases, in the experimental Dunning R3327-G model and in clinical samples.

• There is an inverse relation between bone remodeling activity and tumor cell AR activity in clinical bone metastases that may be of importance for patient response to conventional therapies.
General discussion and future perspectives

At present, there is no cure for patients with prostate cancer bone metastases. This highlights the importance of expanding our knowledge of the molecular processes occurring within these metastases, and thereby finding therapy-predictive markers and novel potential therapeutic targets. The work presented within this thesis was in part aimed at finding model systems where tumor cell interactions with bone cells and the bone itself can be studied. We also utilized the models presented here, to further investigate the potential of IGF-1R inhibition in bone metastases as a therapy to be used in combination with other treatments. Moreover, our characterization of bone remodeling activities in clinical samples of prostate cancer bone metastases might be useful when searching for novel therapies and markers predicting therapy-response to already available treatments.

Limitations of experimental models for prostate cancer bone metastasis

The metastatic cascade is a complex, multistep process and it would be ideal to have a pre-clinical model of prostate cancer bone metastasis where all of these steps occurred spontaneously. In such model primary tumors would form in the prostate, and after some time intravasate into the blood and/or lymph vessels, followed by extravasation from the circulation and formation of distant metastases with growth starting in close contact with bone marrow. Unfortunately, there are no such animal models and instead researchers have to use models that replicate a chosen stage of the metastatic cascade [211–214]. Dogs develop prostate cancer at a relatively high frequency and they occasionally develop metastases to the bone [212]. Their disease is most often hormone-insensitive and tumors frequently arise in castrated dogs, therefore they might be more similar to castration-resistant prostate cancer in humans [215]. Performing large studies on dogs would be difficult and very expensive since it is a disease that affects elderly dogs and only a few percent of dogs develop spontaneous cancers [212, 216]. Spontaneous bone metastases can also arise in rats. The Dunning cell line used in our
study was originally isolated from a spontaneous tumor in the prostate of a Copenhagen rat. Multiple sublines, with various phenotypes, that can form metastases at different sites, have later been developed [162]. Unfortunately, none of the Dunning sublines are able to spontaneously metastasize to bone.

A number of animal models can mimic the pathology of human primary prostate cancer, but there are no animal models that adequately mimics the formation of bone metastases of human prostate cancer [213]. Most studies are performed in mice and rats since they can be genetically modified, are relatively inexpensive, and share a high organ homology with humans [211–214].

In the work presented within this thesis we used a syngeneic rat model, meaning that tumor cells are implanted into the same strain of rat as the tumor cells were originally derived from. With this type of model, the cancer cells and the host animal are immunologically compatible and therefore the rats have an intact immune system. This is a major advantage, since it is known that the immune system plays an important role in cancer progression [217]. One drawback with using a model where the tumor cells are not of human origin is that drugs designed to be directed at a specific target in human tumor cells may not be as efficient in rodent tumor cells. Using xenograft models, human cancer cells can be inoculated into rodents, most commonly, mice [211, 212]. These animals have to be immunocompromised, otherwise the host’s immune system would reject the tumor cells, meaning that the effects of immune cells will be lost in these animals. With this kind of model there is also the uncertainty of whether some of the signaling between tumor cells and bone cells is lost because of interspecies signaling incompatibilities [218]. To overcome this potential problem, xenograft models where either human bone fragments, or tissue-engineered bone constructs consisting of human cells and matrix proteins, are implanted subcutaneously into immunodeficient mice [219–222].

Since spontaneous metastases are uncommon in rodents, tumor cells have to be injected, either via tail vein, intracardiac, orthotopic, or, as in our study, intraosseous injections [214]. Direct injections into bone, in this case tibia, is a reproducible model system. Since the tumor cells are forced into the bone marrow cavity, this model cannot be used to study early metastatic events. Instead it represents already established metastases, and it allows for studies of interactions between cancer and bone cells and also the bone microenvironment. The fact that rats have larger bones than mice makes them easier to
work with, both regarding the precision work of injecting the cells, and also the subsequent analyses of the bones. A drawback with rats is however that the number of transgenic and knockout rat strains is much lower than with mice. And there are far less analytic reagents for cancer research available for rats.

Taken together, there is no ideal animal model for studies of prostate cancer bone metastases. There are, however, a variety of animal models available that can be used for studies of different stages of the metastatic cascade. Hence, it is important that researchers are familiar with the strengths and weaknesses of the specific models to design appropriate strategies to address their specific questions.

In the co-culture model presented in this paper we used human prostate cancer cell lines and murine calvariae. The use of different species within the same model system can be viewed both as an advantage and a disadvantage. By using different species, the subsequent analyses can be performed using species-specific primers for gene expression analysis and species-specific ELISAs to study factors secreted into the media from the different compartments. In paper III we used a rat prostate cancer cell line, Dunning R3327-G, for co-cultures with calvariae. Because of the species similarities between rat and mouse we could not measure the release of IGF-1 from the calvaria without detecting rat IGF-1. Thus, in that specific case, using species that are highly related was unfavorable. A more critical drawback with the use of two different species is that there might be lack of species-specific osteotropism. Since not all proteins are identical amongst all species, it is possible that this could impair some interspecies communication. Using a mouse model of breast cancer, Kuperwasser et al [222] demonstrated that orthotopic inoculation of human breast cancer cell lines into the mammary fat pads of mice can result in spontaneous metastasis to ectopically implanted human bone plugs. Metastases were not formed in the skeleton of the mice, highlighting the species-specific osteotropism. In another, recently published, study, human breast- and prostate cancer cells were co-cultured with human bone tissue collected from patients undergoing total hip replacement [223]. This is a very promising model, not only because one single species is used, but also since bones from patients with, for example, an appropriate age for prostate cancer studies can be used. Even though a lot can be learned by using calvarial explants from mice that are only up to one week of age, the use of bones from elderly men might provide researchers with even more knowledge
about the disease. Another interesting feature of this model is the presence of bone marrow cavities and viable bone marrow since this is where tumor cells normally form metastases. There is, however, the difficulty of finding suitable patients and the fact that every patient is different. Factors such as age, lifestyle, and diseases might therefore make it difficult to reproduce studies. Using calvarial bones from inbred mouse strains yields less variation and might be more appropriate for certain studies.

Another factor that should be considered when studying bone metastases in vitro is hypoxia. The bone marrow is normally highly hypoxic [195], and furthermore, hypoxia is known to affect tumor cell progression [224]. Despite this, most studies using, the above described, in vitro models are carried out under normoxic conditions [169–172], with only a few exceptions [223, 225]. It would be fairly easy and very interesting to test how hypoxia affects both tumor cells and bone cells in our model system.

In conclusion, there are a number of in vivo and in vitro models available for studies of tumor cell and bone cell interactions. No model is ideal and the strengths and weaknesses of the models needs to be considered when designing specific experiments.

Bone-derived factors stimulating and/or protecting metastatic tumor cells

The relatively low response to therapies targeting bone metastases suggests that there are factors present in the bone milieu that stimulates the tumor cells. In paper II, we found that one of the most highly activated tyrosine kinase receptor after stimulation with bone-conditioned medium, was IGF-1R. We were able to show that prostate cancer cell line PC-3 was stimulated by IGF-1 from the bone and that this effect could be diminished by neutralization of IGF-1. Prostate cancer cell line 22Rv1 was also found to be stimulated by bone-conditioned medium. However, neutralization of IGF-1 was not sufficient to reduce this effect, suggesting that other bone-derived factors are stimulating the tumor cells.

To discover factors that in addition to IGF-1 may originate from the bone microenvironment and play a role in protecting and/or stimulating metastatic tumor cells we will make use of the phospho-array data presented in paper II, and which we also have available for prostate cancer cell line LNCaP (unpublished data). In agreement with
PC-3 and 22Rv1 cells, LNCaP cells also showed phosphorylation of the IGF-1R by stimulation of calvaria-conditioned medium (data not shown), but as can be seen in figure 3 other tyrosine kinase receptors were highly activated as well.

ErbB2, also known as HER2, was found to be activated by bone-conditioned medium in all three cell lines. ErbB2 has no known ligand and is instead activated via heterodimerization with other receptors of the ErbB family, for instance EGFR and ErbB4. The EGFR was the most highly phosphorylated receptor in all three cell lines before stimulation, and only a small increase was seen after stimulation. ErbB4 phosphorylation increased in both 22Rv1 and LNCaP cells upon stimulation. In PC-3 cells, however, phosphorylation was low prior to stimulation, and it even decreased after. This indicates that, at least in PC-3 cells, ErbB2 must be activated by other means. One possibility is crosstalk between ErbB2 and other receptors, such as EphA2 [226].
Interestingly EphA1 and EphA2 were also among the most highly activated receptors after stimulation with calvaria-conditioned medium. EphA receptors are activated by membrane-bound ephrinA ligands, and play an important role in the interplay between osteoclasts and osteoblasts. However, the tyrosine kinase receptors of tumor cells in this experiment were not activated by direct cell-cell contact with bone cells, but by something found in the medium. It is possible that calvarial bone cells release exosomes with ephrin ligands into the medium, however, so far only ephrinBs have been found in exosomes [227]. There is also evidence that ephrinAs can be released from the cell surface and thereby activate its receptors [228, 229].

MCSF-R was highly activated in PC-3 and 22Rv1 cells. This receptor is activated by CSF-1 that has been found to be expressed by several cells in the bone, including osteoblasts. M-CSF is, together with RANKL, the two major regulators of osteoclastogenesis [41]. In prostate cancer, increased expression of both MCSF-R and its ligand in primary prostate cancer is associated with metastatic potential [230]. Our transcriptomic studies on bone metastases revealed that expression of CSF1R, the gene encoding this receptor, was highly associated to bone remodeling activity (supplementary tables, Paper IV).

MuSK and ROR1 are closely related receptors [231]. MuSK is activated by LRP4 and ROR1 by WNT5A [232, 233]. ROR1 is expressed in prostate cancer tissue but not in normal prostate [234], and its involvement in Wnt signaling makes it an interesting target in cancer therapy [235].

Tie-2, which was found to be activated in 22Rv1 cells, is normally expressed by osteoblasts, endothelial cells, and hematopoietic stem cells. The receptor is activated by Angoipontin-1 (Ang-1), which is secreted large amounts by osteoblasts. It has been demonstrated that osteoblasts can regulate the stemness of hematopoietic stem cells by secreting Ang-1 and thereby activating Tie-2 [236]. Interestingly, Tie-2 overexpression in prostate cancer cells has been found to enhance their chemoresistance and increase their ability to adhere to stromal cells such as osteoblasts [237].

One approach to further investigate the potential importance of the receptors presented in figure 3, could be to neutralize the ligands or to use inhibitors targeting the specific receptor, as done in paper II with IGF-1R and its ligand. Using an animal model, for instance the Dunning rat model presented in paper III, the effects on tumor cells could be further evaluated.
In summary, understanding what bone-derived factors that are able to stimulate tumor cells might provide more insight into the biology behind prostate cancer bone metastasis, and possibly provide us with potential therapeutic targets. However, it need to be kept in mind that in some signaling pathways, activation is dependent on a direct contact between tumor cells and bone cells, or tumor cells and the bone itself.

**The need for predictive biomarkers and individualized treatment plans**

The concept of cancer treatment is gradually changing from the broad use of agents that are systemically toxic, to individualized, targeted and hopefully more effective therapies. To better guide the therapeutic decision making, we need suitable predictive markers to find the subgroups of patients that are most likely to respond to a specific therapy.

A previous study from our group, where the same metastatic samples as here (paper III & IV) were analyzed, identified two subgroups among castration-resistant bone metastases. One subgroup with high AR and metabolic activities, but low cellular immune responses, and another subgroup with low AR and metabolic activities, but high cellular immune responses [201]. In paper III, when analyzing gene expression in relation to tumor cell IGF-1R immunoreactivity, we found similar subgroups. Metastases with high IGF-1R levels showed a high similarity to the previously identified group with high AR activity. We speculate that the AR-driven metastases are more likely to respond to the wide range of AR-targeting agents available, and furthermore, that they might benefit from adding IGF-1R targeting therapies.

The group of non-AR-driven metastases are, unfortunately, not likely to respond to AR-targeting therapies, and therefore have less treatment options. It is, however, possible that since they were found to have a high cellular immune response; including tumor cell expression of MHC class I expression and metastasis infiltration of cytotoxic T cells, but also of immunosuppressive macrophages and myeloderviced suppressor cells, they may be susceptible to immune-strengthening therapies. Furthermore, in paper IV, we found that the non-AR-driven subgroup of patients had increased bone remodeling activities, signifying the use of therapies targeting the bone
microenvironment in these patients. There are assays available for measuring levels of bone turnover markers, such as ALP, PINP and OC indicating osteoblastic activity, and CTX-I, TRAP and osteopontin, indicating osteolytic activity, in patient serum or urine. It is possible that these could function to mirror the overall ongoing bone remodeling in a patient and, thus, as predictive markers for response to bone targeting therapies.

Reviewing the literature on IGF-1R-targeted therapies in prostate cancer bone metastasis, it becomes very clear that a marker to predict response to IGF-1R inhibition is urgently needed. The number of pre-clinical studies presenting evidence of the importance of the IGF axis in tumor development and progression, led to the initiation of several phase II and III trials targeting IGF-1R signaling. Most clinical trials have sadly reported disappointing results [101], and it is of course possible that the findings from in vivo and in vitro experiments does not translate to human disease. However, the fact that a subset of patients show beneficial response to anti-IGF-1R treatments indicates that we should not discard IGF-1R inhibition as an interesting option for prostate cancer patients. Instead, we need to find predictive biomarkers that can identify the subgroup of responding patients. To the best of our knowledge, no such markers have been used for selecting therapy or even for retrospectively evaluating response in prostate cancer patients. However, higher levels of IGF-1 in plasma prior to treatment has been indicated to predict therapy response in non-small cell lung cancer and pancreatic adenocarcinoma [238, 239]. Expression of the IGF-1R has also been suggested as a predictive marker in, for example, non-small cell lung cancer and osteosarcoma [240, 241].

The shift towards individualized treatment plans has made it more important than ever to find methods for up-front, prospective monitoring of biomarkers, and liquid biopsies could be a realistic substitute to more invasive tissue biopsies. As discussed above, serum and plasma samples could be useful for monitoring markers of bone remodeling. Other markers as the membrane-bound IGF-1R will be better assessed at the RNA level in [100]. Furthermore, exosomes, thrombocytes, and even free circulating RNA and/or DNA may also carry tumor-derived markers and important information from the tumors [242–246].
Acknowledgements

Här kommer det äntligen! Mitt tacktal till alla som stöttat och pepat under åren. Eftersom jag varit sångliggande i feber i flera dagar nu har jag kommit på en väldigt lång lista på människor att tacka.

Först och främst vill jag tacka min fenomenala huvudhandledare Pernilla Wikström som tog sig an mig när Jonas bestämde sig för att fly landet för en herrans massa år sen. Du är inte bara en fantastisk forskare, mentor och förebild, utan även en väldigt omtänksam person som jag lärt mej så otroligt mycket av under denna tid. Att vi dessutom numera jobbar i skift gör oss till ett grymt effektivt lag!

Min biträdande handledare Anders Widmark, ”if you’v got the time, I’ve got the money, honey!” och precis så blev det, en våldans massa tid tog det och jag har säkerligen kostat en del också! Tack att du alltid är så positiv!

Min andra biträdande handledare Jonas Nilsson, som kanske gjorde sin största insats inom det här projektet när jag började på labbet för 10 år(!) sedan. Tack för allt du lärt mig! Du är en idépruta utan dess like. Tack också för allt lunchsällskap (även om jag ibland surnat till när du ”ska bara”….Alfons!).

Anders Bergh och Ulf Lerner, er tankar kring mina projekt är alltid lika värdefulla och man skulle kunna säga att jag ser er som mina två inofficiella biträdande handledare! Anders din fantastiska förmåga att ta ett, eller flera, steg bakåt och se helheten är något som vi alla borde bli bättre på! Ulf, jag vet ingen annan som med sådan inlevelse kan berätta om vad som helst som är benrelaterat (eller fågelrelaterat för den delen) som du. Du är en sann inspiration!


Håkan, för att du hela tiden uppmuntrar oss att bredda vår kunskap inom allt som har med cancer att göra! Camilla, tack för all pepp och alla samtal om allt möjligt här i livet! Samuel, en större nörd får man leta efter. Tack för allt onödigt vetande du lär oss! Mikael J, min partner in crime när det kommer till att sätta ihop fula bildspel till disputationsspex. Lotta, du ska bara veta hur många av dina middagstips jag faktiskt
Daniel tack för att du fyllde mitt skrivrum med så mycket lådor att ingen annan fick plats att sitta där! Lee-Ann I’m sorry for abandoning you at the office like that, several years together, and then I suddenly leave without a warning! Thanks for all the nice long chats. Tack också till övriga på labbet eller med anknytning till labbet som hjälper till att göra det till den fina arbetsplats det är: Mahmood, David, Britta, Henrik, Martin, Markus, Simona, Carl, Charlotte, Anna C, Agneta, Parviz, Kjell, Roger och Beatrice och alla andra som kommit och gätt.

Maria, Pia, Karin, Clas, Terry, Carina, Åsa, Monica. Ni är många som på olika sätt hjälp till med det administrativa. Stort tack till er alla!

Mina doktorandkollegor i Pernillas grupp: Erik, Erik och Helena Ni är så sköna människor alla tre och lycka till med resten av er tid som doktorander! Emma tack för att du skrev en så bra bok som jag har kunnat ta lärdom av nu när jag själv skrivit.


Personalen på Ulfs lab, framförallt Anita, Inger och Ingrid för allt ni är ett så trevligt gäng och för att ni alltid tagit er tid att svara på mina frågor. Tack också till personalen på djurhuset som gjort sitt bästa för att försöka könsbestämma en vecka gamla möss. Jag vet att det inte var enkelt!

Alla ni som under dessa år på olika sätt förgyllt livet utanför jobbet!

Tack till David & Britta, Linda, Mattias & Sofia, Henke & Vickan, Åsa & Frasse, Kräftskivegånget, Knatti, Benny & Carina, som på olika sätt underhållit med middag, festligheter, lunchsällskap, golfrundor, hemmagjorda musikquiz, allsång långt in på småtimmarna
och ett stort antal afterworks på Rött. **Johan & Leonora**, it is always fun to see you and your wonderful girls! Please come here more often now that you have the house.

Ett extra stort tack till:

Den så kallade ”Umeåfamiljen”: **Annasara, Andreas, John, Marie, Reine, Sofia.** Ni är anledningen till att jag gick från att känna att flytten till Umeå var ett av de dumaste beslut jag tagit till att det var ett riktigt bra beslut. Vi har hunnit med så många roliga upptäg genom åren och våra mysiga pysselkvällar är bland det bästa jag vet!


Jag är så glad att Calle kom med en så fin släkt på köpet! Tack för all tid vi får spendera med er, alla middagar, semesterresor, och alla högtider och kalas vi får fira med er! Mina fantastiska svärföldrar **Marre** och **Göran!** Vad skulle vi göra utan er?! Tack för att ni alltid finns där för oss och för att ni drar ihop familjen så att vi alla får träffas så pass ofta som vi gör! **Carin**, älskade Carin! Tack för alla kvällar du och jag planlöst vandrat runt på Berghem, det är som balsam för själen! Team win for life ☺ Och tur att du skaffat dig en så toppenbra familj!!! **Erik**, nu när jag har slutat upp med att vara gravid/ha en pyttelelen bebis/skriva avhandling så är jag äntligen redo för den där Liverpool resan! **Wille och Isac**, bröderna som lärt mej mer om pokemons än jag någonsin trodde att jag
skulle kunna. Tack för att ni är sånna fina grabbar och att ni tar hand om era småkusiner på ett sånt fint sätt!

**Emelie**, väääärlends bästa systör, och hon är min! Trots att du är några timmar bort så muntrar du upp min vardag nästan varje dag. Och **Emil** du är grym du med! Tack för allt du lärt oss om viktiga saker som ellära och hur man ska fota småfiskar för att få dem att se större ut 😊 Finaste **Isabelle**, moster Annos lilla älskling som snart ska bli stortjej, hur galet är inte det?! Önskar jag fick krama på dig varje dag! **Mormor** Starka, fina, underbara mormor! Det är synd att avståndet är så långt, annars skulle vi komma och fika hos gammelmormor ”gung-gung” mest hela tiden! **Mamma** och **Pappa**, för att ni alltid låtit mig gå min egen väg och alltid stöttat vad jag än gjort! Ni är helt enkelt BÄST!

Mina älskade flickor, **Olivia** och **Thilda**! Underbara ungar! Ni är anledningen till att jag gick in i ”maximum efficiency”-läge under dagarna så att jag kunde åka hem och krama på er på kvällarna! Men det allra största tacket måste ändå gå till dig **Calle**! Du har som vanligt varit mitt allra största stöd! Du har peppt mig, påmint mej om ”tänk Niva!”, gått på rekordsnabba lunchdejter, tagit hand om allt som har med barnens överlevnad att göra, läst igenom en stor del av det jag skrivit och kommit med bästa kritiken. Jag vet att det varit en tuff period, men du kämpade dig igenom den! Jag älskar dig!
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