Pathogenesis and treatment of skeletal metastasis : studies in animal models

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Chapter 1

General Introduction

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General introduction

1 Clinical Problem of Skeletal Metastasis

Cancers account for over 20% of deaths in Western countries and these are primarily due to the spread of cancer to distant organs [1]. It has long been recognized that primary cancers spread to distant organs with characteristic preference [2], and the skeleton is one of the most common organs to be affected by metastatic cancer [3-6]. Breast and prostate cancer are osteotropic tumors, i.e., carcinomas that have a special predilection to form bone metastases. At postmortem examination ~70% of patients dying of these cancers have evidence of metastatic bone disease (Table 1) [5,7]. Carcinomas of thyroid, kidney and bronchus also commonly give rise to bone metastases, with an incidence of 30% to 40%, but tumors of the gastrointestinal tract rarely (<10%) produce bone metastases. In these routine autopsies, the true incidence of metastases in the skeleton is most likely underestimated, and more accurate assessments may be determined by scintigrams. Together, breast and prostate cancer probably account for more than 80% of cases of metastatic bone disease [6].

Table 1 **Incidence of skeletal metastases at autopsy**
Based on all autopsy studies listed by Galasko [5] + study from Walther [7]

		Incidence (%) of bone metastases	
Primary tumor site	**Number of studies**	**Median**	**Range**
Breast	13	67	47– 85
Prostate	8	66	33 – 85
Thyroid	5	38	28 – 60
Lung	6	36	30 – 55
Kidney	4	34	33 – 40
Esophagus	3	6	5 – 7
Gastro-intestinal tract	5	7	3 – 11
Rectum	3	11	8 – 13
Uterine/ cervix	1	50	x
Ovaries	1	9	x
Liver	1	16	x

Breast and prostate cancer are the most commonly diagnosed malignancies [1] and the second leading cause of cancer death in the western world in women and men, respectively [1]. At time of diagnosis, most patients with breast [3] and prostate [8] cancer do not have clinicopathologic signs of overt distant metastases. Thus, after resection of the primary tumor and all positive lymph nodes, these patients are in complete clinical remission. However, disseminated tumor cells (DTCs) can already be present in bone marrow (BM) [9-13], a clinical situation called *minimal residual disease (MRD)*. The DTCs that are still present in BM are frequently resistant to current treatment (chemo-/hormone therapy), and can stay

dormant for many years [10,14,15]. So, only the very small subfraction of the DTCs that have acquired the abilities of metastasizing to, surviving in, and colonizing the bone/BM microenvironment, can eventually result in the development of an overt bone metastasis. Only this subpopulation of DTCs can, therefore, be regarded as true *metastasis-initiating cells* (MICs). Even though, the general presence of DTCs in BM at time of diagnosis is significantly associated with the formation of distant metastases, particularly to bone [9,10].

The clinical courses of patients with breast and prostate cancer with a first recurrence in bone are relatively long, with a median survival of 24 and 40 months [4]. This is in marked contrast to those with first recurrence of breast cancer in the liver (3 months)[3]. Moreover, patients with disease that remains confined to the skeleton have a better prognosis than those with subsequent visceral involvement [16]. In these patients, the decline in quality of life and eventual death is due almost entirely to skeletal complications and their subsequent treatment [17,18]. The prognostic indicators that metastatic breast cancer remains confined to skeleton are low histological grade (well-differentiated), lobular subtype (vs. ductal), postmenopausal state and a low number of positive lymph nodes at time of surgery. For patients with breast cancer, good prognostic factors for survival after the development of bone metastases are low histological grade (well-differentiated), positive estrogen receptor status, a long disease free interval, and increasing age [16].

Bone pain is the most common complication of metastatic bone disease, resulting from structural damage, periosteal irritation, and nerve entrapment [19]. In addition, hypercalcemia occurs in 5–10% of all patients with advanced cancer but is most common in patients with breast cancer, multiple myeloma, and squamous carcinomas of the lung [20,21]. Pathologic fractures are a relatively late complication of bone involvement.

Bone metastases in prostate cancer patients are predominantly osteoblastic (osteosclerotic), characterized by increased bone formation due to exaggerated osteoblastic activity. In contrast, patients with breast, lung, and kidney cancers have predominantly osteolytic bone metastases, characterized by increased bone degradation resulting from enhanced osteoclastic activity [18,21,22]. However, patients can have both osteolytic and osteoblastic bone metastases, or mixed lesions containing both elements (see paragraph 5.2, 'Bone Metastatic Phenotype').

In this chapter, a brief introduction into the multistep process of metastasis will be given. Subsequently, this chapter will give an overview of our current understanding of the molecular and biological mechanisms involved in the process of bone metastasis formation, including a summary of normal bone physiology and therapeutic opportunities. Finally, the outline of this thesis will be presented. But first, to set the scene, the next paragraph will describe the well-established 'seed and soil' hypothesis as postulated by Stephen Paget in 1889.

2 Paget's 'Seed and Soil' Hypothesis

Over a century ago Stephen Paget was the one of the first who observed a non-random pattern of metastasis to certain organs by analyzing autopsy records of 735 women who had died of breast cancer [2]. He proposed the 'seed and soil' hypothesis in which he compared the seeding of cancer cells to the dispersal of the seeds of plants. Accordingly, circulating cancer cells ('seeds') disperse in all directions, but can accomplish metastases only in the organs where the microenvironment ('soil') is permissive for their growth, i.e., osteotropic cancer cells posses certain properties that enable them to grow in bone, and the bone/bone marrow microenvironment provides a fertile soil on which to grow. Ever since, the hypothesis holds forth. Based on this hypothesis, the 'seed' (see paragraph 'The Seed: Tumor Progression and Metastasis'), the 'soil ('The Soil: Bone/Bone Marrow') and their interactions ('Seed–Soil Interactions') will be specifically addressed in this chapter.

3 'The Seed': Tumor Progression and Metastasis

Since Paget postulated the 'seed and soil' hypothesis, our understanding of the metastatic process has increased tremendously. It has now been well-established that tumor progression and metastasis is a multistep process characterized sequentially by carcinogenesis and growth of the primary tumor, angiogenesis, cell invasion, access to the systemic blood circulation (intravasation), survival in circulation, arrest in microvasculature and subsequent extravasation, and growth at distant organs, (reviewed in [23]) (Fig. 1). These processes will be discussed in more detail in the next paragraphs.

3.1 Carcinogenesis

The great majority of cancers (>80%) occur in epithelial tissues, yielding carcinomas [1,24]. Epithelial tissues are generally built according to a common set of architectural principles; relatively thin sheets of epithelial cells are separated from complex layers of stroma by a basement membrane. By definition, carcinomas begin on the epithelial side of the basement membrane as hyperplastic and dysplastic growth progressing to a carcinoma *in situ,* and are considered to be *benign*. Nevertheless, carcinoma *in situ* may develop into an invasive malignancy as it breaks through the basement membrane and, by then, is classified as *malignant*, and commonly called a cancer [24].

In the normal, healthy situation tissue fibroblasts regulate the proliferation and differentiation of epithelial tissues [25]. Likewise, tumor–stroma interactions also play a critical role in development and progression of carcinomas [26,27]. For example, transformed stroma can induce malignancy in lung [28] and mammary epithelia [29], and conversely, normal fibroblasts have been reported to convert malignant epithelia to morphologically benign lesions

[30]. Cancer-associated fibroblasts (CAFs) could also induce tumorigenesis in non-malignant prostatic epithelial cells, mediated via CAF-secreted stromal-derived factor (SDF-1) and transforming growth factor-ß (TGF-ß) [31]. This is in line with the dual role that is implicated for TGF-ß in carcinogenesis. In normal and non-malignant epithelial cells TGF-ß actc as a potent growth inhibitor [32,33]. However, different types of carcinomas (e.g., Ras-transformed cells) can become refractory to growth inhibition. In fact, TGF-ß can potentiate tumorigenesis and contribute to invasiveness by stimulating an epithelial-to-mesenchymal transition (EMT) [34-38] (see paragraph 3.3 'Acquisition of an Invasive Phenotype: Epithelial-to-Mesenchymal Transition')

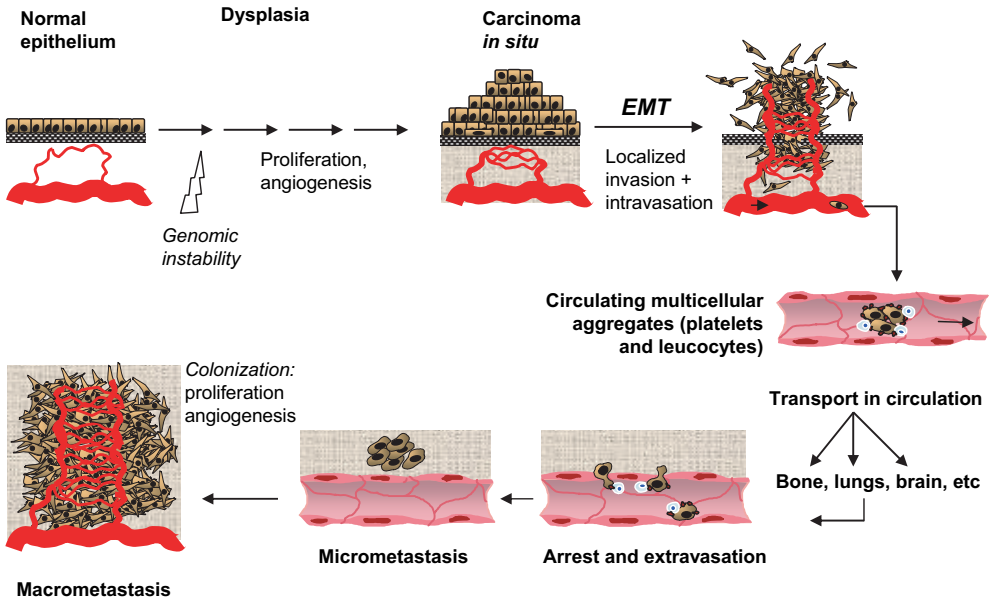

Figure 1 **Main steps in tumor progression and metastasis**. Cellular transformation due to genomic instability and changes can result in dysplasia, which may eventually result in a carcinoma *in situ*. At the same time, new blood vessel are formed (angiogenesis). This will facilitate cancer cells to enter into the systemic circulation (intravasation), after they have undergone an epithelial-to-mesenchymal transition (EMT). The EMT confers an invasive phenotype to the cancer cells, including loss of cell-cell adhesion, increased motility and matrix degradation. Aggregates of cancer cells with platelets and leukocytes may form cell embolis that may consequently be protected from the immune reaction. Arrest in the capillaries may be facilitated by mechanical mechanism and by adhesion to endothelium-specific cell adhesion molecules. Eventually some of the micrometastases may acquire the ability to colonize the tissue in which they have landed, enabling them to form a macrometastasis. The small probability of successfully completing all steps of this cascade explains the low likelihood that any single cancer cell leaving a primary tumor will succeed in becoming the founder of a distant, macroscopic metastasis.

Many other tumor–stroma interactions are also mediated by members of the TGF-ß superfamily [39], e.g., the involvement of TGF-ß signaling in fibroblasts was determined by conditional inactivation of the TGF-ß type II receptor (TßRII) gene in mouse fibroblasts ($TßRII^{fspKO}$) [40]. The loss of TGF-ß responsiveness in fibroblasts resulted in intra-epithelial neoplasia in prostate and invasive squamous cell carcinoma of the forestomach, both associated with an increased abundance of stromal cells. TGF-ß and other members of the TGF-ß superfamily will be discussed in greater detail in the section 'TGF-ß superfamily members'.

For over 150 years, it has been anticipated that only a minority of cells within a tumor are responsible for tumor growth and development (reviewed in [41]). Over the past few years it has become possible to isolate and characterize these tumor-initiating cells, first from hematological malignancies [42,43] and recently also from many solid tumors, including breast [44] and prostate cancer [45,46]. Cell fractions of as few as 100 to 200 sorted cells with a particular phenotype from human primary breast [44] and prostate cancer [46] could successfully be orthotopically transplanted in immune-deficient mice, whereas much larger numbers (10^4–10^5) of the rest of the cells depleted of that particular phenotype could not. Many of these isolated tumor-initiating cells share (surface) markers with somatic stem cells and both share the characteristics of 1) self-renewal, 2) an indefinite proliferative potential, 3) differentiation along one or several diverse lineages, and 4) homing to allow cells to migrate and seek their niche. For these reasons, tumor-initiating cells have been given the popular name 'cancer stem cells (CSCs)' [47,48]. The concept of CSCs underscores the importance of targeting the correct cells for cancer therapy. Eliminating only the more differentiated, rapidly dividing cells by chemo- or radiotherapy is not likely to result in successful long term remission if the less differentiated and slower proliferating CSCs remain to repopulate the tumor. At present, much remains to be learned about the identification, molecular signature and functional plasticity of the CSCs [49].

3.2 Angiogenesis

Angiogenesis, also referred to as neovascularization, is the process of new capillary formation from pre-existing vessels [50,51]. Angiogenesis is a complex multistep process that involves dissolution of the basement membrane of the vessel, extracellular matrix degradation, migration and proliferation of endothelial cells, capillary differentiation, stabilization and anastomosis. These processes are tightly regulated by inducers and inhibitors of endothelial proliferation, migration and differentiation [52].

In malignancy, tumor cells can switch from an angiogenesis-inhibiting phenotype to an angiogenesis-stimulating phenotype, the so-called *'angiogenic switch'* [53]. This angiogenic switch is essential for tumor growth beyond 1–2 mm^3 without neovascularization [54]. In breast cancer, tumor-induced angiogenesis is already evident at the pre-invasive stage of ductal carcinoma *in situ*, characterized by a rim of microvessels formed around the ducts that are filled with proliferative epithelial cells. So before carcinoma cells breach the base-

ment membrane, they often succeed in stimulating angiogenesis on the stromal side of the membrane, by dispatching angiogenic factors through this porous barrier to endothelial cells within the stroma [55]. Angiogenesis is not only required to meet the growing metabolic demands of the tumor by supplying nutrients and oxygen, but also provides routes for tumor dissemination and metastasis [54,56]. Not surprisingly, high blood vessel counts or production of factors that stimulate angiogenesis are independent predictors of poor prognosis in many primary solid cancers [57-61]. A variety of factors, including hypoxia and genetic changes in the tumor cells, contribute to the increase in production of angiogenic factors. Furthermore, cells within the activated tumor stroma also play an important role in increasing the production of vascular endothelial growth factors (VEGFs) and other angiogenic factors, including basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) [26,62].

VEGF-A is a specific endothelial cell mitogen and regarded as the most important inducer of angiogenesis. It is constitutively expressed in many cancers [62,63]. Its importance was underlined when it was demonstrated that monoclonal antibodies against VEGF-A inhibited the growth of various tumors in animal models [64]. Angiogenesis is not only essential for the primary tumor to grow and metastasize, but is required for the outgrowth of a micrometastasis into an overt (bone) metastasis as well [65,66].

Besides weakly stimulating angiogenesis, VEGF-C and VEGF-D are the main inducers of tumor lymphangiogenesis, and are overexpressed in breast and prostate cancers as well [67]. Accordingly, VEGF-C and VEGF-D expression levels have been correlated with increased number of lymph vessels in cancer patients, and have been shown to promote metastatic spread via the lymphatics [68,69].

In addition to (lymph)angiogenesis, evidence is accumulating that malignant tumors are often capable of inducing other structures that may contribute to (invasive) growth and dissemination (reviewed in [70]). Networks that stain positive with periodic acid-Schiff (PAS) have been observed in different types of cancers [62,71-73]. In both uveal and cutaneous melanoma, these structures have been shown to be of prognostic significance [73-75].

The PAS reaction is a non-specific indicator for polysaccharides, which are present in basement membranes, including those of blood vessels. The PAS-positive patterns may represent (a mixture of): blood vessels/vascular network [72,74], fibrovascular tissue [76,77], tumor cells [78], or a fluid conducting meshwork [71]. Maniotis and co-workers have shown that highly aggressive, but not non-aggressive, melanoma cells are capable of forming highly patterned vascular channels *in vitro* that are composed of a PAS-positive basement membrane in the absence of endothelial cells and fibroblasts [78]. These channels formed *in vitro* are morphologically identical to PAS-positive channels in histological preparations of highly aggressive primary and metastatic uveal and cutaneous melanomas. The generation of microvascular channels by aggressive tumor cells was termed "vasculogenic mimicry" (VM) to emphasize their *de novo* generation without participation by endothelial cells [78-80]. Since then, VM has

been identified in several cancers, including prostate [81] and inflammatory breast cancer [82]. However, the and co-workers remains a debatable subject, and
Although some investigators state that the original findings of VM by Maniotis are not convincing [83,84], or lack novelty [85], VM is becoming increasingly accepted as a concept for tumor cell plasticity

3.3 Acquisition of an Invasive Phenotype: Epithelial-to-Mesenchymal Transition

In the pre-invasive stage of carcinoma *in situ*, with or without increased neovascularization in the underlying stroma, the first step leading to dissemination is the acquisition of local invasiveness [23]. The organization of the epithelial cell layers in normal tissue is incompatible with the motility and invasiveness displayed by malignant cancer cells. Therefore, in order to acquire motility and invasiveness, cancer cells must shed many of their epithelial characteristics, detach from epithelial sheets, and undergo a drastic alteration, which is referred to as the epithelial-to-mesenchymal transition (EMT) [37,86].
In order to invade adjacent cell layers carcinoma cells are required to remodel the nearby tissue environment by excavating passageways through the extracellular matrix (ECM) and pushing aside any cells that stand in their path [87]. The most important effectors to create space in the ECM are matrix metalloproteinases (MMPs)(reviewed in [88]). In carcinomas, the great bulk of these proteases are secreted by recruited stromal cells, notably macrophages, mast cells and fibroblasts, rather than neoplastic cells [26,89]. During the course of degrading ECM components (e.g., fibronectin, tenascin, laminin, collagens, and proteoglycans), MMPs can also activate certain growth factors that have been tethered in inactive form to the ECM or to cell-surfaces [88].

The EMT displayed by cancer cells is reminiscent of the highly conserved and fundamental process of EMT that occurs during early embryonic development [90-93]. During gastrulation, embryonic epithelial cells need to undergo an EMT in order to migrate to a new environment. Eventually these embryonic cells may regain a fully differentiated epithelial phenotype via a mesenchymal-to-epithelial transition (MET) (Fig. 2) [94-98]. The transition to a more mesenchymal, motile cellular phenotype is the result of a complex physiological process that includes dissolution of adherens junctions, loss of cell polarity, a change to spindle-like cell morphology, cytoskeletal reorganization, increased cell motility, loss of epithelial markers and induction of mesenchymal markers [90-93,99]. Increased vimentin expression and perturbation of E-cadherin-mediated cell adhesion appear as hallmarks of this process [37,100-102]. Accordingly, it has been shown that once E-cadherin expression is suppressed, other cell-physiologic changes associated with the EMT seem to follow suit. Additionally, re-expression of E-cadherin in different types of cancer strongly suppressed the invasiveness and metastatic dissemination [103,104].

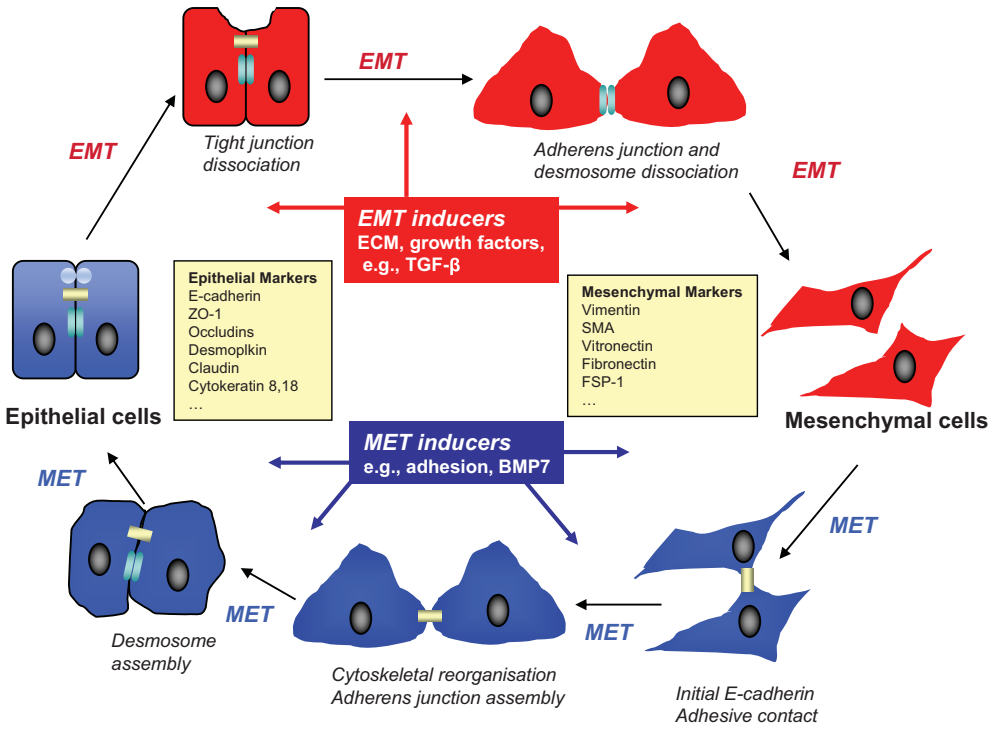

Figure 2 **The cycle of epithelial-cell plasticity as observed in embryonic development during gastrulation.** The diagram shows the cycle of events during which epithelial cells are transformed into mesenchymal cells and vice versa. The different stages during EMT (epithelial–to-mesenchymal transition) and the reverse process MET (mesenchymal–to-epithelial transition) are regulated by effectors of EMT and MET, which influence each other. Important events during the progression of EMT and MET, including the regulation of the tight junctions and the adherens junctions, are indicated. ECM, extracellular matrix; ZO-1, zona occludens 1; SMA, smooth muscle actin; FSP-1, fibroblast-specific protein-1; adapted from [97].

Under non-pathological conditions, EMT and MET are both non cell-autonomous processes, and thus require an external stimulus to be initiated [92,93,105]. In malignancy, both genetic alterations and tumor environment may be able to induce EMT in tumor cells. Transformed mammary epithelial cells that are in direct contact with the surrounding stromal cells can undergo an EMT, indicated by expression of vimentin and an elongated, fibroblastic shape [105]. However, established metastases of most cancers recapitulate the differentiated phenotype of their primary tumors including re-expression of E-cadherin [106,107]. Therefore, the important steps that enable metastasis can be reversible, and are not explained solely by irreversible genetic alterations, indicating the existence of a dynamic component to human tumor progression [108,109].

TGF-ß is one of the main inducers of EMT as was demonstrated in Ras-transformed mammary and other epithelial cells [110-112] (Fig. 3). These mammary epithelial cells were even able to maintain the mesenchymal, fibroblast-like state through autocrine TGF-ß signaling [110,111]. However, when TGF-ß was removed (by adding fresh medium or neutralizing antibodies) they reverted back to their epithelial appearance [110], indicating that they had undergone an MET. Another example of a self-sustaining positive feedback loop for EMT is the expression of αvß6 integrin by tumor cells, which can activate the latent form of TGF-ß produced by the stroma cells [113].

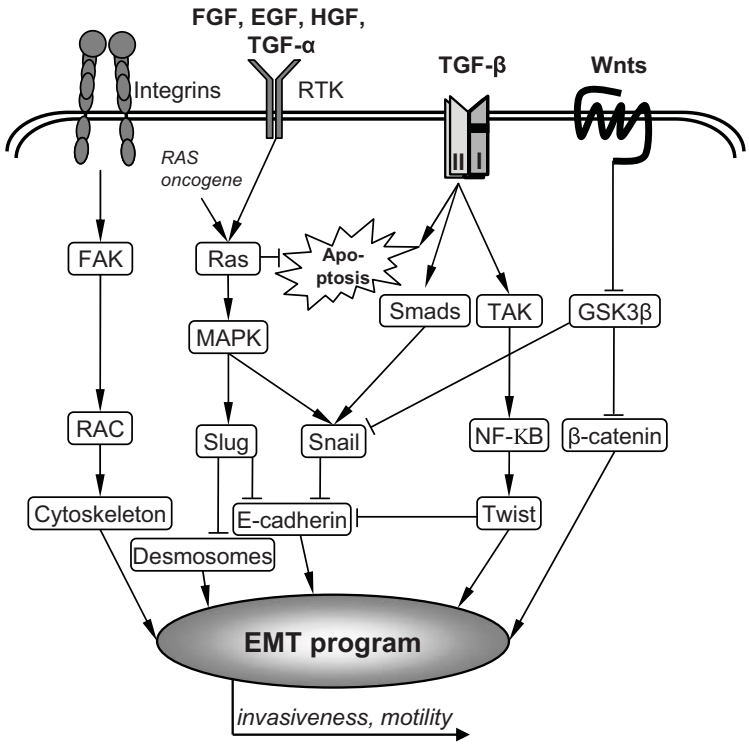

Figure 3 **Overview of the molecular networks that regulate EMT.** A small selection of EMT regulators, and a limited representation of their crosstalks are illustrated. TGF-ß is one of the main inducers of EMT. However, several normal and transformed epithelial cell lines need co-activation of Ras-signaling, to overcome transforming growth factor-ß (TGF-ß)-induced apoptosis. It is likely that the EMT is normally triggered in response to a mixture of signals that carcinoma cells receive from the stroma together with intracellular signals, e.g. the signal released by the Ras oncogene. FGF, fibroblast growth factor; HGF, hepatocyte growth factor; EGF, epidermal growth factor; RTK, receptor tyrosine kinase; FAK, focal adhesion kinase; GSK3ß, glycogen-synthase kinase-3ß; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TAK1, TGF-ß-activated kinase-1.

Additional microenvironmental signals that can induce EMT are ECM components and soluble factors, including other members of the TGF-ß superfamily, fibroblast growth factor (FGF) family, epidermal growth factor (EGF), hepatocyte growth factor (HGF, alias scatter factor) [114], insulin-like growth factor (IGF)-II [115] and proteins of the wingless (Wnt) and Hedgehog (Hh) families (Fig. 3) (reviewed in [86,97]). It is important to take into account that besides the tumor–stroma interactions at the edge of tumor, infiltrating cells (e.g., lymphocytes, macrophages) might also be able to induce EMT in tumor cells. Moreover, accumulation of irreversible mutations could also trigger invasive behavior, particularly resulting in poorly differentiated anaplastic cancers, in which no re-differentiation is detectable in primary cancers and metastases [116,117].

Both the EMT concept and the CSC concept cover distinct aspects of tumor progression and metastasis, but can be converged into a single concept, which subdivides CSCs in the *stationary cancer stem cell* (SCS cell) and the *migrating cancer stem cell* (MCS cell) [109]. SCS cells, which are still embedded in the epithelial tissue, are already active in benign precursor lesions, such as carcinoma *in situ*, and persist in differentiated areas throughout all steps of tumor progression; however SCS cells cannot disseminate. MCS cells, which are located predominantly at the tumor–stroma interface, are derived from SCS cells through the acquisition of a (transient) EMT in addition to stemness. This concept takes into account two important requirements — CSCs that have undergone EMT can disseminate, and DTCs that retain stem-cell functionality can form metastatic colonies. So, the MCS cell concept combines the important current tumor initiation and progression concepts — the cancer stem cell and EMT concept — and it integrates both genetic alterations and the tumor environment as combined driving forces of malignant progression. However, the concept still awaits verification in different models of tumor progression for various types of cancer.

3.4 Intravasation, Circulation and Extravasation

Once tumor cells detach from the primary tumor via EMT, they can intravasate into the blood or lymphatic system. Via the lymphatic system, tumor cells can form lymph node metastases in close proximity of the primary tumor, or eventually enter the blood circulation in the left and right subclavian veins. Tumor cells that directly, or indirectly, enter the blood vessels can spread via the pulmonary circulation throughout the whole body, where they may found new colonies. However, the event of a successful development of a distant metastasis is very rare, and most cancer cells are not able to by-pass the capillary bed of the lungs. This is substantiated by the fact that in animal models, it has been estimated that 3–4 10^6 cancer cells/g of tumor can reach the bloodstream per day [118], but that only a very small minority of cancer cells reaching the blood will survive and grow at the distant sites.

In the blood circulation, cancer cells may interact with platelets [119,120] and leucocytes, to create aggregates or emboli. These may increase resistance to shear stress and protect from immune-cell–mediated tumor cell clearance [23,121]. These aggregates may also

facilitate mechanical trapping in the capillaries of different organs, and promote extravasation [122]. Once the tumor cells have exited the circulation, the activated platelets are a source of factors that are able to induce angiogenesis [123], stimulate tumor cell proliferation, and indirectly enhance osteoclastic activity in the bone environment [119,120].

3.4.1 Vertebral Venous System

As first demonstrated by Batson, there exists a *vertebral venous system*, which is composed of three freely communicating thin-walled valveless networks: intraosseous vertebral veins, epidural and paravertebral venous complexus [124,125]. The pressure in the intraosseous veins is always 35–50% higher than on the caval side of the venous circulatory system, enabling blood to flow along this pressure gradient from the vertebrae to the inferior vena cava. However, the absence of valves makes it possible that an increase of abdominal pressure, such as caused by coughing, lifting or palpation, could cause retrograde flow. Moreover, Batson showed in human cadaver and animal experiments that venous blood from both the pelvis and the breast flowed not only into the vena cava, but also directly into the vertebral venous system [124,125]. This system, in which blood is thus continuously subjected to arrest and reversal of the direction of the flow, could enable cancer cells from the pelvic region and breast to by-pass the pulmonary circulation, providing an explanation for the predilection of breast and prostate cancer to produce metastases in the axial skeleton. This hypothesis was tested by Coman and De Long, who performed an inoculation with cancer cells in the femoral veins during a moderate temporarily increase in intra-abdominal pressure in experimental animals. Indeed, an increased abdominal pressure enabled cancer cells to directly enter, and form metastases within the vertebral venous system [124,126].

Although the system of blood flow in the *vertebral venous system* can, at least in part, explain the high incidence of bone metastases in the vertebrae, it cannot explain that human cancers, particularly breast and prostate cancer, metastasize with high frequency to other bones [5]. For this explanation, we may need to have a closer look at the interactions between the 'seed' and the 'soil'. But before these interactions will be discussed, the next paragraph will first comprehensively describe the properties of the soil.

4 'The Soil': Bone/Bone Marrow Microenvironment

In addition to anatomy, the pathophysiology of bone metastasis is determined by multiple direct and indirect interactions between metastatic cancer cells and the bone/BM microenvironment (including BM cells, bone cells, and bone matrix). First, the normal physiology of the bone/BM microenvironment will first be dilineated below.

4.1 Bone

Bone is a highly mineralized tissue that provides mechanical support and metabolic functions to the skeleton. It can be formed by either intramembraneous ossification or endochondral ossification. Intramembranous ossification occurs when mesenchymal precursor cells differentiate directly into bone-forming osteoblasts, a process employed in generating the flat bones of the skull as well as in adding new bone to the outer surfaces of long bones. In contrast, endochondral bone formation entails the conversion ofan initialcartilage template into bone and is responsible for generating most bones of the skeleton (reviewed in [127]).

4.1.1 Bone Cells

The formation and degradationof bone matrix is regulated by normal bone cells, namely the osteoblast and osteoclast, respectively. In normal bone, there is a balanced remodeling sequence: first, osteoclasts resorb bone matrix, and then osteoblasts form bone matrix at the same site (reviewed in [127]).

4.1.1.1 Osteoclasts

Osteoclasts are specialized cells derived from the monocyte–macrophage hematopoietic lineage that adhere to and, once activated, degrade bone matrix (reviewed in [128,129]). Activated osteoclasts resorb bone by secreting the protease cathepsin K that dissolves the matrix, and produce acid that releases bone mineral into the extracellular space under the ruffled border of the plasma membrane of osteoclasts, which faces bone (reviewed in [129]). The adherence of osteoclasts to the bone surface is critical for the bone resorptive process, since agents that interfere with osteoclast attachment block bone resorption [130]. Although osteoclasts can be influenced by various locally produced cytokines as well as systemic hormones, two specific hematopoietic factors — macrophage colony stimulating factor (M-CSF, alias CSF1) and receptor activator of nuclear factor κB ligand (RANKL) — are both necessary and sufficient for the formation and activation of osteoclasts. M-CSF is produced by stromal cells and osteoblasts and interacts with its receptor c-fms expressed on macrophage precursors to stimulate proliferation. Accordingly, op/op mice that lack expression of M-CSF due to a gene mutation are deficient in osteoclasts (and macrophages), resulting in enhanced bone formation [131-133]. Substitution of M-CSF reversed the osteopetrotic phenotype in op/op mice [134]. While M-CSF is crucially important in the early steps of osteoclastogenesis, RANKL — expressed on osteoblasts and stromal cells — is critically involved in maturation and activation of the osteoclasts. Interaction of RANKL with the membrane receptor RANK on osteoclast precursors induce — by signaling through the nuclear factor-κB (NF-κB) and Jun N-terminal kinase (JNK) pathways — commitment of the monocyte–macrophage precursor to the osteoclast lineage [135-139]. The importance of RANK–RANKL binding in the formation of osteoclasts has been demonstrated clearly by *RANKL* [140,141] and *RANK* [142] knock-out mice. Both knock-out mice lack osteoclasts, and

as a result, severe osteopetrosis develops. In addition, the development of B cells and T cells is defective in these animals. Likewise, mice bearing mutations or deletions in intracellular signal molecules critically involved in RANK signaling, such as c-Fos [143], c-Jun [144] and NFATc [145], also develop osteopetrosis. Various locally produced cytokines as well as systemic calcitropic hormones — including parathyroid hormone (PTH), 1,25-dihydroxyvitamin D3, and prostaglandins — indirectly stimulate osteoclastogenesis by upregulation of RANKL expression on marrow stromal cells and osteoblasts [139,146,147]. In addition, many cytokines, such as interleukin (IL)-1 and tumor necrosis factor-α, are also able to directly affect osteoclasts [128].

Osteoprotegerin (OPG) was identified as the decoy receptor for RANKL, and is normally present in the BM [139,148]. OPG is a member of the superfamily of tumor necrosis factor receptors and inhibits the differentiation and resorption of osteoclasts *in vitro* and *in vivo* [136,148,149]. The ratio of RANKL to OPG regulates the formation and activity of osteoclasts [150]. Accordingly, OPG-deficient mice display marked osteoporosis [151,152], whereas overproduction of OPG in these mice causes severe osteopetrosis [153].

4.1.1.2 Osteoblasts and Osteocytes

Osteoblasts are the bone-forming cells, responsible for the production of the matrix constituents. They are always found lining the layer of bone matrix that they are producing before it is calcified, referred to as osteoid tissue. Osteoid tissue exists because of a time lag between matrix formation and its subsequent calcification (the osteoid maturation period), which is approximately 10 days [127,154]. Osteoblasts never appear to function individually, and are always found in clusters of cuboidal cells along the bone surface (~100 to 400 cells per bone-forming site). Osteoblasts arise from local mesenchymal stem cells (MSCs), which are precursor cells for many cell types involved in bone formation, such as osteoblasts, osteocytes and chondrocytes, and for other mesenchymal cell lineages, such as fibroblasts, myoblasts, adipocytes, and neuronal cells [155]. For differentiation towards an osteoblast, MSCs first undergo proliferation, become committed and then differentiate into a pre-osteoblast — producing alkaline phosphatase — and subsequently into a mature osteoblast, producing increasing amounts of osteocalcin and calcified matrix [156]. Runt-related transcription factor 2 (Runx2; alias core-binding factor α1) and Osterix are crucial transcription factors that drive the expression of most genes associated with osteoblast differentiation [157,158]. Accordingly, bone does not develop in mice that lack the *Runx2* gene [159,160]. The commitment of MSCs to the osteoblast lineage is regulated by at least three major morphogenetic pathways, the BMP [161,162], the Hh [163], and the Wnt [164] signaling pathway. These pathways can still be influenced and fine-tuned by factors, such as PTH, PTH-related protein (PTHrP), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), TGF-β, sex steroids and other hormones (reviewed in [154]). When osteoblasts become embedded in the bone matrix, they differentiate into *osteocytes*, the most abundant cells in

mature bone. Because they can function as mechanosensors, osteocytes are considered to modulate bone remodeling in response to bone loading (reviewed in [127,165]).

4.1.2 Bone Matrix

Bone is composed of an organic matrix that is strengthened by deposits of calcium salts. Type I collagen constitutes approximately 90-95% of the organic bone matrix, whereas noncollagenous proteins comprise the remaining 5-10%. Crystalline salts deposited in the matrix are primarily calcium and phosphate in the form of hydroxyapatite [127]. Noncollagenous proteins can be subdiveded in 1) cell attachment proteins, 2) proteoglycans, 3) γ-carboxylated (gla) proteins, and 4) growth factors (e.g., IGFs, TGF-ßs, FGFs, PDGFs, and BMPs) (reviewed in [166]). Any of the attachment proteins, e.g., osteopontin (OPN), bone sialoprotein (BSP), and vitronectin and collagen type I facilitate interactions with integrins that are expressed by specialized bone and HSC cells as well as osteotropic cancer cells [167,168]. The mineralized bone also stores a variety growth factors, including IGFs, TGF-ßs, FGFs, PDGFs, and BMPs. Since bone continuously remodels, bone-stored growth factors, such as IGFs, TGF-ßs, FGFs, PDGFs, and BMPs [169,170], are constantly released in the BM cavity by osteoclastic bone resorption, affecting growth of bone metastatic cells as will be discussed in detail in paragraph 5: 'Seed-Soil Interactions'. IGF-I is the growth factor that is stored most abundantly in the bone matrix, and upon release, it can play an important role in stimulation of (cancer) cell proliferation and chemotaxis, and inhibition of apoptosis [171]. TGF-ßs and BMPs will be discussed in detail in paragraph 4.4.

4.1.3 Bone Turnover

Adult bone is continuously remodeled by bone resorption and subsequent bone formation in temporary anatomic structures, called the basic multicellular units (BMUs), described by Frost more than forty–years ago [172] (Fig. 4). These two phases are in a balanced sequence and the net result is replacement of old bone with new bone, thus maintaining structural integrity of the skeleton throughout adult life [172-174]. The actual number and activity of these BMUs determine the bone turnover rate (or status) and they are under the control of mechanical stress, cytokines and hormones. Upon resorption, local mitogenic factors, such as TGF-ß, that are embedded within the calcified matrix are released and activated [175]. These local factors, along with the systemic factors PTH, estrogen, and prostaglandin, recruit new osteoblasts at the BMU to fill the gap created by osteoclasts.

4.2 Bone Marrow

Bones are not entirely compact, and the center of the bone generally consists of spongy bone or a bone cavity, which are lined by endosteal cells. These spaces are occupied by red or yellow bone marrow (BM). Red BM facilitates active hematopoiesis, but with increasing age, the rate of hematopoiesis diminishes and more and more of it is converted to yellow BM —

consisting almost entirely of fat cells. However, under appropriate stimuli, such as extreme blood loss, yellow BM can convert to red BM again. In adults, red marrow is found mainly in the spongy bone of flat bones, such as iliac creast and sternum, and in the proximal ends of the long bones femur and humerus. Interestingly, these bones are relatively often affected by cancer metastasis, suggesting that red BM might be somehow involved [2,5].

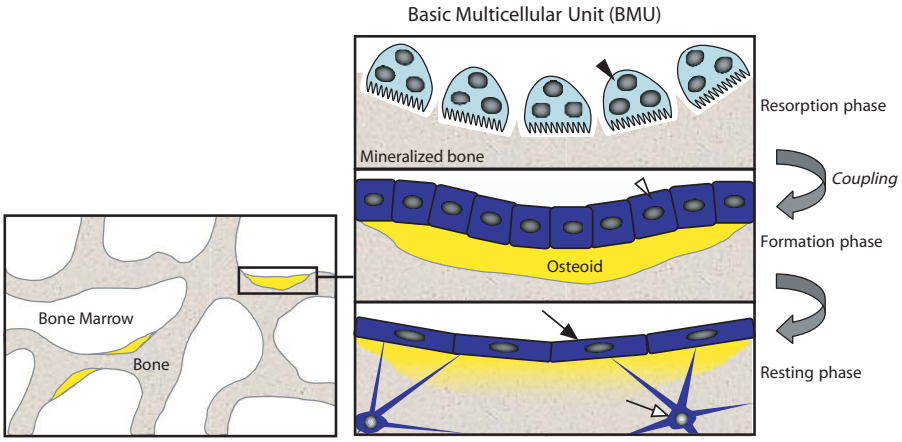

Figure 4 **The basic multicellular unit.** Bone is continuously remodeled by basic multicellular units (BMUs), the temporary anatomic structures that resorb bone and subsequently induce bone formation. Multinucleated osteoclasts (arrowhead) resorb the calcified matrix of a bone trabecula, the *'resorption phase'*. In a tight coupling, pre-osteoblasts migrate and differentiaite into osteoblasts (empty arrowheads). These lay down new bone matrix, which is not yet calcified and is reffered to as osteoid, the *'formation phase'*. Subsequently, the osteoid becomes mineralized. At the end phase of this remodeling, the surface of new bone is covered by flattened osteoblasts or 'lining cells' characterized by low/absent bone forming activity, the *'resting phase'*. Osteoblasts that are trapped in the matrix become osteocytes (arrow with empty arrowhead); others die or form new, flattened osteoblast or 'lining cells' (arrow). The number of BMUs can be modulated by mechanical loading, hormones and cytokines, marrow hematopoiesis and drugs (bone resorption inhibitors); adapted from [344].

Hematopoietic stem cells (HSCs) can reside in BM in two niches, the osteoblastic niche and the vascular niche (reviewed in [176-178]). The osteoblastic niche is formed by endosteal osteoblasts that provide a quiescent environment for HSCs maintenance, support expansion of HSCs into the different hematopoietic lineages, and control HSC numbers [179,180]. In contrast, the vascular niche has been identified in association with fenestrated endothelium of sinusoids, and facilitates HSC transendothelial migration during mobilization or homing, dependent on endothelium-derived factors (Fig. 5) [177,178,181].

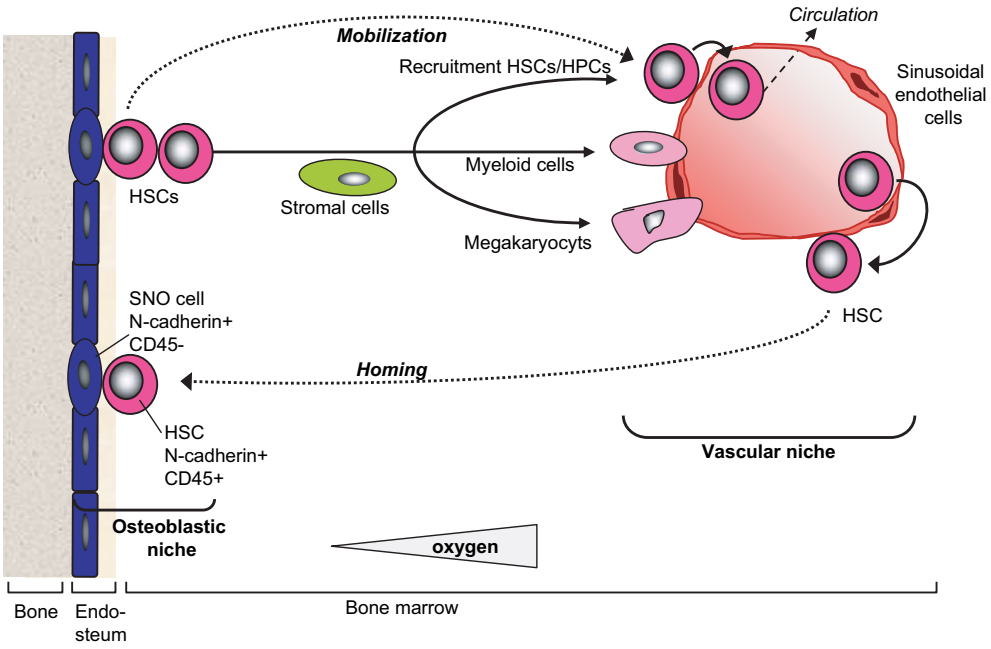

Figure 5 **Osteoblastic and vascular niche in bone.** Under normal physiological conditions, HSCs reside in either the osteoblastic or vascular niche. In response to low levels of SDF-1 in the bone marrow, a portion of HSC daughter cells will leave the bone marrow and begin to mobilize and circulate. HSC homing is the reverse of mobilization, occurring in response to higher levels of SDF-1 in the bone marrow, particularly produced by the immature osteoblasts that align the periosteum. The osteoblastic niche may provide a quiescent microenvironment for HSC maintenance. In contrast, the vascular niche facilitates HSC transendothelial migration during mobilization or homing and may favor HSC proliferation and further differentiation. Higher oxygen concentration gradients as the cells progress from the osteoblastic niche to the vascular niche might play a role in recruitment, proliferation, and differentiation of HSCs/HPCs. Stress such as thrombocytopenia can induce HSCs to enter into the cell cycle, mobilize to the vascular niche, and differentiate. SNO, spindle shaped N-cadherin+ osteoblast; adapted from [178].

Endothelial cells, osteoblasts, and other stromal cells constitutively express the chemoattractive cytokine, or chemokine, SDF-1 (alias CXCL12), while HSCs express its receptor CXCR4 [182]. SDF-1 generated by endothelial cells induces HSCs from the circulation to undergo transendothelial migration mediated by E- and P-selectins [183,184]. Subsequently, HSCs could migrate toward the osteoblastic niche, where HSCs have been shown to adhere only to a subset of immature osteoblasts, the spindle-shaped N-cadherin^{+}CD45^{-} osteoblastic (SNO) cells [180]. Accordingly, an increase of these SNO cells correlated with an increase in HSCs, suggesting that SNO cells function as key components of the osteoblastic niche [180]. Little is presently known about the molecules that define the vascular niche, and its relationship with the osteoblastic niche.

Granulocyte-macrophage colony stimulating factor (GM-CSF, alias CSF2) induces HSC and progenitor cell mobilization and is widely used clinically during stem cell-based transplantation procedures. The mechanism involved is a reduced concentration of SDF-1 in BM, whereas SDF-1 in the peripheral circulation is less affected after GM-CSF treatment [185]. In bone marrow, GM-CSF induces proteolytic enzymes such as elastase, cathepsin G, MMP-2, and MMP-9, which inactivate SDF-1 and are required for cells to penetrate the endothelium [186]. In addition, GM-CSF could regulate SDF-1 expression in osteoblasts at the transcriptional level [187,188].

4.3 TGF-ß Superfamily

The TGF-ß superfamily consists of more than 30 proteins in mammals, including TGF-ßs, activins, BMPs, growth/differentiation factors and anti-Müllerian hormone (Fig. 6). These growth factors, and their antagonists, control many different biological processes such as proliferation, differentiation, apoptosis, and invasiveness in many different epithelial as well as non-epithelial cells [189-192]. In mammals, three isoforms of TGF-ß, i.e., TGF-ß1, TGF-ß2 and TGF-ß3, and more than 20 BMP–related proteins have been identified [193]. In general, TGF-ß isoforms have highly comparable structures and *in vitro* biological activities [194]. In contrast, different BMPs often exert different, and even opposing, effects [161]. Therefore, they should be regarded as individual proteins rather than be classified as a group in respect to cell function.

TGF-ß1 is the most abundant isoform with the largest sources in platelets (20 mg/kg) [195] and bone (200 mg/kg) [196,197]. In comparison, the sources of BMP2, BMP4, and BMP7 in bone are 21 mg/kg, 6 mg/kg, and 84 mg/kg, respectively [170].

The source of TGF-ß/BMPs in the bone/BM microenvironment may be acellular, released by osteoclastic resorption of the extracellular bone matrix, or cellular, derived from cells that reside in bone (e.g., osteoclasts, osteoblasts, and osteocytes) or in bone marrow (e.g., megakaryocytes) (reviewed in [198]).

4.4.1 Signaling Pathways

Members of the TGF-ß superfamily mediate their pleiotropic effects by signaling through transmembrane serine/threonine kinase type I and type II receptors (Fig. 7) [39,193,199]. The type II receptor kinases are constitutively active without ligand stimulation. Upon ligand–induced heteromeric complex formation between type II receptors and type I receptors, type I receptors become phosphorylated by the type II receptors and activate downstream signaling components among which Smad (Sma-Mad related protein) molecules proteins play a pivotal role [193,200]. Unlike other members of the TGF-ß superfamily, BMPs have a higher affinity for the type I receptor, rather than for the type II receptors [201,202]. In mammals, five type II receptors and seven type I receptors have been identified [39,161,203] (Table 2). The type II receptors include activin type II and type IIB receptors (ActRII and ActRIIB), TGF-ß type II receptor (TßRII), BMP type II receptor (BMPRII) and anti-mullerian hormone type II recep-

tor (AMHRII). Type I receptors are termed activin receptor-like kinases (ALKs) 1 through 7. It is theoretically possible to form more than 30 different combinations of type II and type I receptors. However, under physiological conditions the combinations of type II and type I receptors appear to be limited by the variety of ligands that converge at the receptor level.

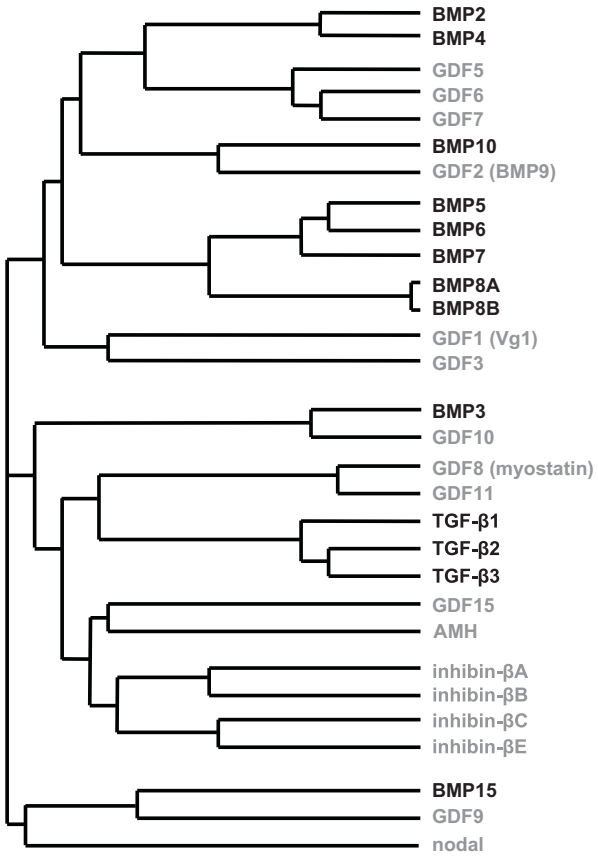

Figure 6 **Phylogenetic tree of TGF-ß superfamily**. The phylogenetic tree is derived from protein alignment of the human, putative mature and fully processed forms. The length of the horizontal lines connecting one sequence to another is proportional to the estimated difference between the proteins. BMPs and TGF-ßs are shown in black, other members of TGF-ß superfamly in grey. AMH, anti-Müllerian hormone; BMP, bone morphogenetic protein; GDF, growth and differentiation factor; TGF-ß, transforming growth factor-ß; based on [415].

In most cell types, ALK5 (TGF-ß type I receptor) is the predominant type I receptor that is activated by TGF-ß through TßRII (Fig. 8) [204]. ALK3 and ALK6 (BMP type IA and IB, respectively) are structurally highly comparable to each other and function as BMP type

I receptors for BMPs. In addition, BMP6 and BMP7 have also been shown to bind ALK2 [201,205]. Different BMPs bind with different affinity to the type I receptors, e.g., BMP6 and BMP7 binds with higher affinity to ALK2 and ALK6 than to ALK3 [201,206] (Fig. 8). The expression of ALK3, ALK6 and BMPRII was observed in normal and benign prostate tissue, and was found to correlate with low tumor grade (well-differentiated) in prostate cancer. In addition, loss of BMPRII expression correlated with poor prognosis in prostate cancer patients [207,208].

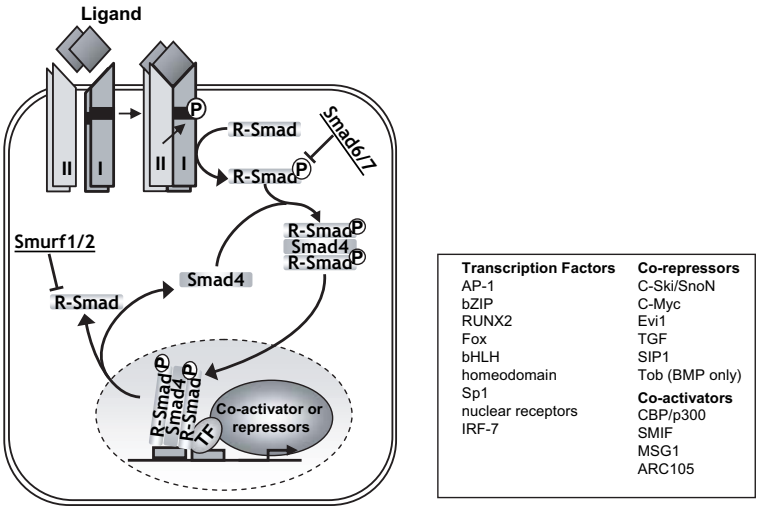

Figure 7 **General Mechanisms of TGF-ß/BMP-induced Smad activation.** At the cell surface, a ligand (homo- or heterodimer) binds a complex of transmembrane receptor serine/threonine kinases (types I and II) and induces transphosphorylation of the GS segments (black) in the type I receptor by the type II receptor kinases. The consequently activated type I receptors phosphorylate selected Smads at C-terminal serines, and these receptor-activated Smads (R-Smads) then form a complex with the common Smad, Smad4. Activated Smad complexes translocate into the nucleus, where they regulate transcription of target genes, through physical interaction and functional cooperation with DNA-binding transcription factors (TF) and coactivators or repressors. Activation of R-Smads by type I receptor kinases is inhibited by Smad6 or Smad7. The E3 ubiquitin ligases Smurf1 and Smurf2 mediate ubiquitination and consequent degradation of R-Smads; based on [199].

Table 2 **Type I and II receptors for TGF-ß superfamily members in mammals.** Alk, activin receptor-like kinase; ActRII and ActRIIB, activin type II and IIB receptors; TßRII, TGF-ß type II receptor; BMPRII, BMP type II receptor.

Type I receptor	*alias*	Type II receptor
Alk1		BMPRII
Alk2	ActR1A	ActRII
Alk3	BMPR1A	ActRIIB
Alk4	ActR1B	TßRII
Alk5	TßR1	AMHRII
Alk6	BMPR1B	
Alk7		

Besides the signaling type I and type II receptor, several accessory type III receptors, are known, e.g., betaglycan (TβRIII) [209] and endoglin (CD105) [210]. While endoglin inhibits TGF-ß1-induced ALK5–Smad3 signaling, it promotes BMP7–Smad1/Smad5 signaling [211,212]. On the other hand, betaglycan acts as a co-receptor for TGF-ß that modulates the binding of TGF-ß to its receptors, thus enhancing signaling via ALK5 [213]. Moreover, it can also function as a co-receptor for inhibin, disrupting activin and BMP signaling [214].

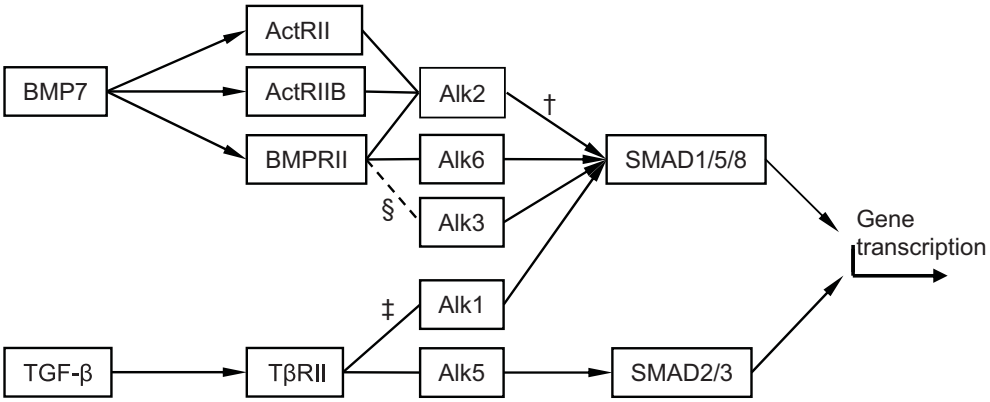

Figure 8 **Type I and II receptors, and R-Smads involved in BMP7 and TGF-ß signaling.** It is important to note that the presence of particular type I and II receptors, and R-Smads greatly differs in various cell types. † = activates only SMAD1 and -5, but not -8; § = BMP7 binds with low affinity to BMPRII/Alk3 complex; ‡ = pathway known in endothelial, but not epithelial, cells. Alk, activin receptor-like kinase; ActRII and ActRIIB, activin type II and IIB receptors; TßRII, TGF-ß type II receptor; BMPRII, BMP type II receptor; based on data from [201,205,416-419].

Smads, which are intra-cellular substrates for the TGF-ß superfamily receptors can be subdivided into: 1) receptor-activated Smads (R-Smads) which include Smad2 and Smad3 for TGF-ß and activin signaling, and Smad1, Smad5, and Smad8 for BMP signaling, 2) the common mediator Smad (Co-Smad), commonly refeered to as Smad4, and 3) inhibitory Smads (I-Smads), which include Smad6 and Smad7 [39,193]. R-Smads contain a conserved SSXS phosphorylation motif in their very C-termini, of which the last two serine residues become phosphorylated following interaction with activated type I receptors [39]. Phosphorylation of R-Smads results in their heteromerization with Smad4 followed by nuclear translocation and regulation of gene transcription in association with other transcription factors [161,200].

Smads are not equally activated by their cognate receptors. For example, ALK3 and ALK6 activate all three BMP R-Smads, whereas ALK2 can activate only Smad1 and Smad5, but not Smad8 [205,206]. Although Smad2 and Smad3 are structurally very similar to each other, Smad3 can directly bind DNA, whereas Smad2 signaling requires a specific combination of other transcription factors [215]. In fibroblasts, TGF-ß-mediated induction of matrix metalloproteinase-2 (MMP-2) was selectively dependent on Smad2, whereas induction of c-fos,

Smad7, and TGF-ß1 autoinduction relied on expression of Smad3 [216]. Moreover, in tubular epithelial cells it was found that TGF-ß-induced increases in CTGF and decreases in E-cadherin expression were Smad3-dependent, whereas an increase in MMP-2 expression was Smad2–dependent [217]. In NMuMG mammary epithelial cells it was reported that complexes of Smad4 with both Smad2 or Smad3 can induce full EMT, however, the Smad3–Smad4 complex was most efficient [36]

Non-Smad pathways may also play an important role in understanding the diversity of signals generated by the TGF-ß superfamily proteins. MAP (mitogen-activated protein) kinases, including ERK (extracellular-signal-related kinase), JNK (JunN-terminal kinase), and p38MAPK, can, among many other growth factors, also be activated by BMPs and TGF-ßs in certain cell types. Moreover, signaling by Smads is modulated by various other signaling pathways allowing TGF-ß superfamily ligands to elicit different biological responses in target cells (reviewed in [193,199,200]).

4.4.2 Regulation of Activity

The level of activity of the TGF-ß superfamily members is determined by the regulation of 1) bioavailability and 2) cellular signaling.

The bioavailability of TGF-ß superfamily members is regulated by several types of mechanism, including proteolytic processing, secretion, interaction with ECM components, and extracellular binding proteins, and each step in the activation pathway is tightly controlled. For TGF-ß and BMP7 post-translational processing is shown in figure 9 [198,218-222]. During transit through the rough endoplasmic reticulum, the signal peptide is removed from the pre-pro-protein and, following dimerization, another cleavage occurs by the convertase family of endoproteases [219]. Within the secretory vesicles or in the extracellular space, these proteases cleave the precursor into a C-terminal mature peptide and an N-terminal precursor remnant, referred to as latency associated peptide (LAP) for TGF-ß, and prodomain for BMP7. Thus control and/or localization of convertase activity may represent an important level of regulation. After cleavage, mature TGF-ß and LAP remain attached via non-covalent bonds to form the small latent complex (SLC). LAP shields the receptor interacting epitopes in the mature protein and this keeps TGF-ß in its latent form [223]. The SLC can covalently attach to the large latent TGF-ß-binding protein (LTBP) to form the large latent complex (LLC) [224,225]. Most cell types secrete TGF-ß as part of LLC, although some cells (such as the bone cell line UMR-106) secrete SLC [226]. Four different LTPBs have been identified, of which LTBP1, LTBP3 and, to a lesser degree, LTBP4 covalently bind to LAPs of all three TGF-ß isoforms [227]. After secretion, LLC is targeted to ECM molecules, e.g., in the bone matrix. LTBP show remarkable homology with fibrillins, which are extracellular glycoproteins that are required for elastic fiber formation. Besides binding to ECM molecules, the C-terminal region of LTBP1 can also bind to the N-terminal region of fibrillin-1, linking LLC also to elastic microfibrils [228].

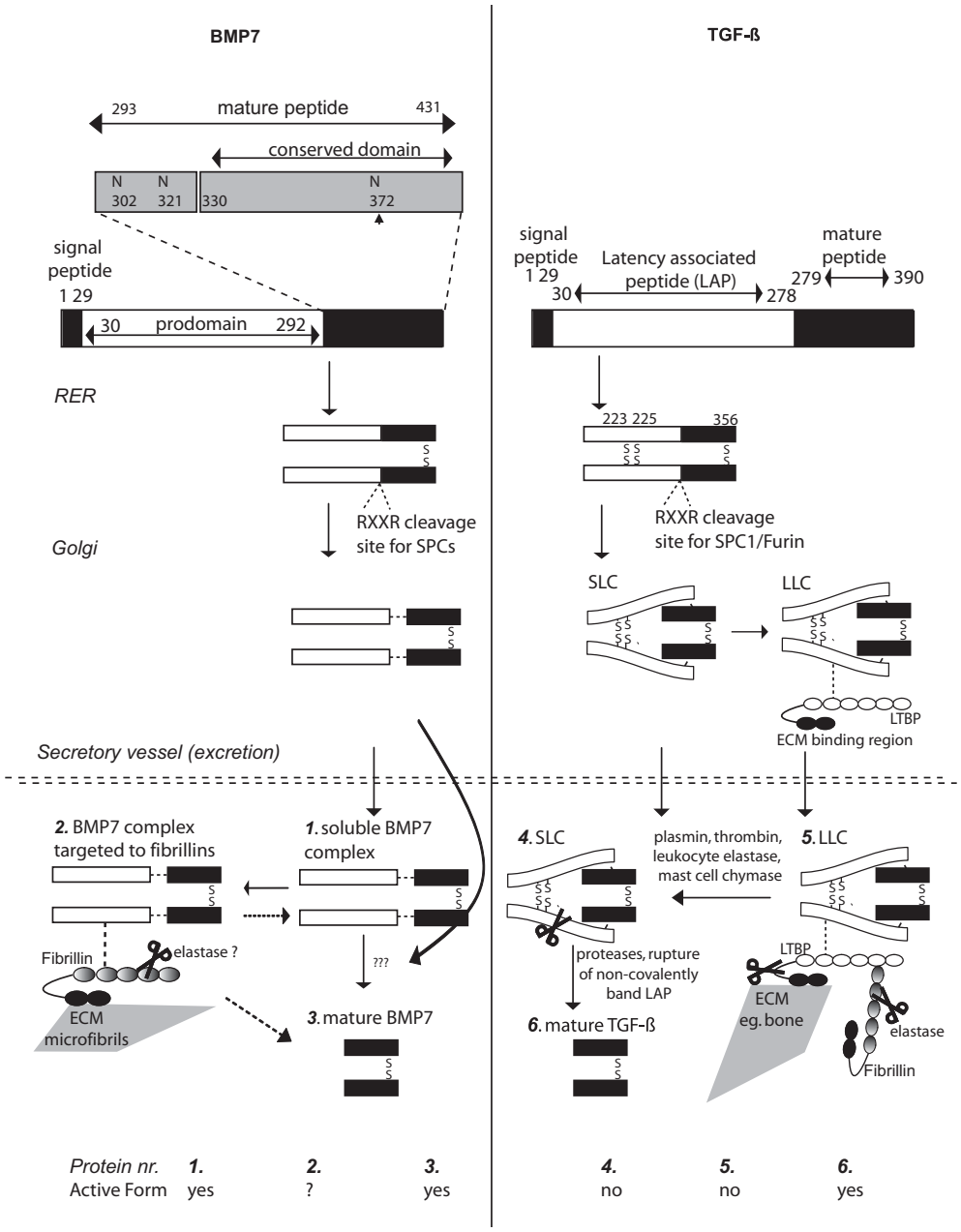

Figure 9 **TGF-ß and BMP7 posttranslational processing.** The mature BMP7 has three potential N-linked glycosylation sites, from which only the glycosylation sites at residue 372 in the TGF-ß conserved domain region is heavily or completely glycosylated (*top of figure:* arrowhead). The signal peptide, which targets the proteins to the secretory pathway, is cleaved off during transit through the *rough endoplasmatic*

reticulum (RER). Subsequently, two monomers dimerize by way of disulfide bridges between cysteine residues. The protein is cleaved by subtilisin-like proprotein convertases (SPCs) at the RXXR motif. This yields the prodomain, or latency associated protein for TGF-ß, and the mature peptide. This mature petide stays noncovalently associated with its prodomain, forming the soluble BMP7 complex for BMP7 or the small latent complex (SLC) for TGF-ß. The noncovalent association prevents the premature activation of mature TGF-ß. The SLC can become covalently attached to a Large Latent Binding Protein (LTBP) to form the Large Latent Complex (LLC), and the SLC to LLC ratio depends on the cell type. After its secretion, the LLC is directed to fibrillin-1 and/or the ECM and stored through binding of the LTBP with these components. Inflammatory proteolytic enzymes, including elastase, release LLC from fibrillin-1, and Several proteases, such as plasmin, mast-cell chymase and thrombin, release LLC from the ECM. The SLC is more readily available for activation, and can be activated by proteases or conformational change in the LAP induced by interaction with, e.g., thrombospondin or integrins. The pro-domain of BMP7 targets the soluble BMP7 complex to the fibrilin-1 and -2 in the extracellular matrix. The precise mechanisms of posttranslational processing are less well understood for BMP7 and remains to be elucidated. Based on data from [219-221] (BMP7) and [198,222] (TGF-ß).

In order to exhibit their biological activity, the latent TGF-ß complexes need to be released from microfibrils and ECM. A recent study revealed that LLC release can be initiated by the displacement of LTBP bound to fibrillin-1 [218]. Degradation of microfibrils by inflammatory proteolytic enzymes (such as elastase) releases fragments of fibrillin-1, including an internal fibrillin fragment that efficiently binds to the N-terminal region of fibrillin-1 and displaces LTBP, releasing LLC from microfibrils [218]. The release of LLC from the ECM in bone and other tissue is mediated by several proteases, including plasmin, mast-cell chymase and thrombin (reviewed in [198,222]).

Subsequently, the truncated LLC and SLC can be subjected to three different mechanisms of *in vivo* activation: 1) degradation of the latency-associated peptide (LAP) by proteases, 2) induction of a conformational change in the LAP, e.g., by interaction with integrins and thrombospondin, and 3) rupture of the noncovalent bonds between LAP and mature TGF-ß [223]. In contrast, the soluble BMP7 complex — consisting of the mature peptide and its noncovalently associated prodomain — is already active, and is targeted to fibrillin-1 and -2 [220]. Therefore, it may be that proteases, such as elastases, are critically involved in the release of the soluble BMP7 complex from the elastic microfibers. However, the precise mechanisms of post-translational processing, secretion and interaction with ECM components are less well understood for BMP7 and remain to be elucidated.

In contrast, extracellular BMP antagonists are relatively well-studied secreted peptides that bind BMPs with high affinity and prevent their interaction with their cognate receptors. These antagonists can be categorized into subgroups, i.e., noggin [229], twisted gastrulation protein [230], the chordin family [231], and the DAN family [232].

TGF-ß/BMP signaling is limited by 1) dominant negative non-signaling membrane pseudoreceptors, 2) I-Smads, 3) ubiquitination and proteasomal degradation of signaling effec-

tors, 4) corepressors, and 5) phoshorylation of the linker region of R-Smads (reviewed in [233,234]). Many of the negative regulators involved are potently induced by BMP/TGF-ß signaling, and may thus participate in a negative feedback loop to control the intensity and duration of TGF-ß signaling [235-239]. The pseudoreceptor BAMBI (BMP and activin membrane-bound inhibitor) is a truncated, kinase-deficient, type I receptor that binds to ligand competitively and thereby inhibits BMP and TGF-ß signaling [240].

The I-Smads, Smad6 [237,241] and Smad7 [242], inhibit signal transduction by interference with the activation of R-Smads and prevention of heterodimerization between R-Smads and Smad4. In addition, I-Smads can also interact with type I receptors through Smurfs (Smad ubiquitin regulatory factors), inducing ubiquitination and degradation of the activated receptor complexes [244-247]. The Smurf1/2–Smad7 complexes play an important role in regulating ubiquitination of TGF-ß receptor complexes [248-250], Smurf1–Smad6/7 complexes appear to target BMP receptor complexes for degradation [247]. So, whereas Smad7 acts as a general inhibitor of TGF-ß superfamily member signaling pathway (BMP and TGF-ß), Smad6 preferentially blocks BMP signaling [236,237,241-243].

TGIF (TGF-ß-induced factor) is a transcriptional corepressor, i.e., a Smad binding protein that interferes with the ability of Smads to bind coactivators. It binds Smad2 and -3, thereby preventing the interaction of Smads with coactivators, resulting in reduced target gene expression. The competition for Smad binding between coactivators and corepressors determines the magnitude of the transcriptional output from a given level of signaling. Sloan-Kettering retrovirus (Ski) and and its relative Ski-related novel gene N (SnoN) [251,252] are also corepressors, which suppress Smad signaling by associating with the MH2 binding domain of Smads. Both Ski and SnoN bind Smad2 and -3, thereby inhibiting TGF-ß signaling [238,251-254]. In addition, Ski, but not SnoN, can also interact with Smad1 and -5 and antagonize BMP signaling [251,255-257]. By interacting with Smad1, -5, -6, -7, and -8, the transducer of ErbB-2 (Tob) also represses BMP signaling [258,259].

Inhibition of the Smad pathway can also occur via growth factor induced phosphorylation of the linker region of R-Smads, reducing ligand-induced nuclear accumulation of R-Smads. Acivation of Erk by EGF and HGF, or activated Ras can induce this phosphorylation [260-262].

4.4.3 Physiologic and Pathophysiologic Functions

BMPs are paradigmatic molecules directly regulating osteoblast generation and were first identified by their ability to form new bone *in vivo* (reviewed in [161,263]). They play a crucial role in skeletal and joint morphogenesis, bone remodeling, and fracture repair by inducing proliferation, lineage determination, differentiation, and apoptosis in chondrocyte and osteoblast precursors [203,264]. Subsequently, they were shown to act as multifunctional regulators of embryonic patterning and organogenesis, tissue remodeling, and repair [265].

BMPs have also been implicated in cancer development. Elevated levels of BMP6 are associated with higher grade primary tumors and advanced prostate cancer with metasta-

sis [266,267], and BMP2 induces migration and invasion in breast [268] and prostate [269-271] cancer cells. BMP7 regulates multiple cellular processes including cell proliferation, migration, and apoptosis. In cells from the osteoblast lineage, exogenous addition of BMP7 can result in inhibition or stimulation of cell proliferation, totally dependent on the cell line type [272,273]. In PC-3 and DU 145 prostate cancer cells, BMP7 inhibited cell proliferation by arresting cells in G1 phase via up-regulation of the cyclin-dependent kinase (Cdk)-inhibitors. However, in C4-2B prostate cancer cells, BMP7 promotes cell survival by inhibiting stress-induced apoptosis via both the Smad/survivin and c-jun NH2-terminal kinase pathways [274].

Yet a different function for BMP signaling was demonstrated when Zhang and co-workers demonstrated that conditional deletion of ALK3 (BMPRIA) lead to increased numbers of SNO cells and subsequently to higher HSC numbers. Thus BMP signaling via ALK3 complex controls the number of HSCs by regulating the niche size [180].

TGF-ß is a potent growth inhibitor in normal and non-malignant epithelial cells [32,33]. However, different types of carcinomas often escape this tumor-suppressing activity and become refractile to growth inhibition [33,111,275]. In fact, TGF-ß can potentiate tumorigenesis and contribute to the progression and invasiveness of various carcinomas by inducing EMT [34-38]. Accordingly, it has been shown that a blockade of TGF-ß (signaling) inhibits tumor cell viability, migration, and metastasis [276-279]. Moreover, TGF-ß stimulates the PTHrP production in metastatic cancer cells, thereby being an important mediator in lytic bone destruction (as discussed in more detail in the paragraph 5.2.1, 'Osteolytic Bone Metastasis').

Although some BMPs, such as BMP2 [280] and BMP4 [281], have also been shown to induce an EMT in certain cell types under specific conditions, the homodimeric protein BMP7 can induce the opposite, a mesenchymal-to-epithelial transition (MET) in normal and non-transformed cells [192,282]. For instance, during kidney development, BMP7 is essential for the condensation and epithelialization of the metanephric mesenchyme in the kidney, resulting in the formation of the tubular epithelium [95,96,98]. In the adult kidney, BMP7 appears to be involved in the preservation of the epithelial phenotype of renal tubular cells [283], and inhibition of fibrosis [284,285] and inflammation [286,287]. Moreover, BMP7 treatment partly inhibited the pathologic effects in experimentally induced acute [287] and chronic [284,288,289] kidney disease, including its complications renal osteodystrophy [290,291] and vascular calcification [292]. In clinical practice, BMP7 is used as an osteoinductive agent in treatment of non-union fractures [293].

5 'Seed–Soil' Interactions

The establishment of a metastasis is dependent on the reciprocal interactions between cancer cells and the microenvironment. In order to form an overt bone metastasis, cancer cells first have to extravasate into, colonize and then grow in, the BM. Thus, the first interaction between the 'seed' and the 'soil', is when circulating cancer cell in blood interact with the endothelium of a specific tissue [294]. In most tissues, the endothelium differs in surface

markers, morphological phenotype and specialized functions [295], and is still susceptible to further modification by specific stimuli, such as inflammation. The specific combination of chemoattractive and adhesion molecules on the endothelium in the BM has been demonstrated to be particularly favorable for the retention and extravasation of circulating cancer cells [294].

As discussed in paragrapgh 4.2, BM endothelium-derived SDF-1 induces CXCR-4 expressing HSCs to undergo transendothelial migration mediated by E- and P-selectins [183,184]. Similarly, metastatic prostate cancer cells that adhere specifically to BM endothelial cells have also been reported to make use of E-selectin ligands for entry into BM [296-298]. Moreovcer, the chemokine receptors CXCR4 and CCR7 are also expressed by a variety of osteotropic epithelial cancer cell types [298-301], also mediating their adhesion to BM endothelium.

OPN, BSP, and type I collagen are major components of mineralized bone. These proteins mediate local adhesion, motility, survival and growth by interactions with matrix adhesion molecules, namely integrins, and are expressed by several types of cells, including HSCs. Similarly, cancer cell adhesion to OPN, BSP and type I collagen is also integrin-dependent [167,168]. So, integrin expression may not only be critical for guiding HSC to hematopoietic sites, but also for BM colonization by cancer cells [302]. Mainly the αvβ3 (alias vitronectin receptor) and αIIbβ3 integrins seem to participate in determining the osteotropism of cancer cells [303-306]. Accordingly, the MDA-MB-231-BO2 breast cancer cell line, which is a subclone of MDA-MB-231 (MDA-231), constitutively overexpresses αvβ3 integrin and only metastasizes to bone [307]. Similarly, *de novo* expression of αvβ3 integrin in 66cl4 breast cancer and CHO ovarian cancer cells that metastasize to lungs, but not to bone, is sufficient to promote their dissemination to bone [307,308]. Moreover, αvβ3 integrin was shown to cooperate with BSP, MMP-2 and -9 in promoting osteotropic cancer cell invasion [309,310]. CD44 is a non-integrin, ubiquitous and multifunctional surface adhesion molecule, which has been identified as a receptor for both the glycosaminoglycan hyaluronan and OPN. It is expressed by various cancer cell types and has a well-established role in skeletal metastasis [311]. Taken together, the bone/BM microenvironment is able to actively allow circulating cancer cells to preferentially arrest in, extravasate into, and colonize BM, adopting similar pathways that are used by the homing of normal HSC to the bone/BM microenvironment [312].

Besides high affinity for bone matrix, breast and prostate cancer cells can also produce bone matrix proteins such as OPN, osteonectin [313], and BSP [314]. This acquisition of bone cell-like properties by tumor cells, is called *osteomimicry* [313,315,316], and improves homing, adhesion, proliferation, and survival in the bone/BM microenvironment.

According to the 'Seed and Soil' hypothesis, interaction between tumor cells and the microenvironment is supposed to occur only after tumor cells reach the microenvironment. However, this classical 'Seed and Soil' hypothesis appears to be too simplistic, since the recent discovery of the so-called *pre-metastatic niche* (PMN) [317]. Kaplan and co-workers showed that VEGFR1$^+$ BM-derived hematopoietic progenitor cells (HPCs) were mobilized by

factors secreted from primary tumors (melanoma and lung carcinoma) to form fibronectin-rich patches in (non-bone) target organs. Remarkably, these niches are formed prior to the arrival of metastatic tumor cells. Blocking the PMN formation by VEGFR1 antibodies significantly inhibited metastasis. Strikingly, the PMN was shown to dictate the metastatic pattern, since conditioned media from a melanoma cell line could redirect lung carcinoma cells to metastatic sites that are favored by melanoma, but not lung cancer. Two cytokines produced by primary tumors, VEGF and placental growth factor (PlGF), were suggested to mediate the formation of the PMN, although additional chemokines or growth factors are certainly necessary to facilitate different organ-specific metastasis [317,318]. In summary, the PMN-model indicates that long range communication between tumor cells and target organs can occur before the arrival of tumor cells at distant sites. Understanding the molecular mechanisms involved in the formation and maintenance of PMNs and normal HSC niches (osteoblastic and vascular) and their role in supporting organ-specific metastasis may provide new approaches for developing novel treatment strategies. However, it remains to be investigated whether the PMN-model is also applicable to other types of cancer, including breast and prostate cancer.

5.1 Bone Turnover and Skeletal Metastasis

The rate of bone turnover is closely associated with the number of sites with increased paracrine availability of growth factors due to osteoclastic bone resorption, and, accordingly, the probability that the cancer cell (the 'seed') will find a favorable 'soil' in the bone/BM environment. The latter part of this hypothesis is based on experimental studies, which have shown that the rate of bone turnover is associated to the occurrence and progression of bone metastases indicating that the growth support provided by the bone microenvironment is active during bone resorption [319-321] (see 'Osteolytic Bone Metastasis'). Recently, this view was further substantiated by clinical studies, showing a strong and highly significant association between the rate of bone resorption and incidence of subsequent skeletal complications in breast and prostate cancer patients [322-325].

Serum alkaline phosphatase (sALP) and urinary hydroxyproline are common and non-expensive markers of bone formation and bone resorption, respectively. They are useful tools for assessing the overall bone turnover rate in cancer patients at risk to develop bone metastasis. Recently, more sensitive and specific biochemical markers have become available. Immunoassays for bone-specific alkaline phosphatase and type I collagen propeptides are currently the most sensitive markers for assessing bone formation. Best indices of bone resorption are the immunoassay for the pyridinoline cross-links and the related peptides that can be measured in urine and serum [326]. These markers for bone turnover may be useful in planning the rational use of preventive treatment with bone resorption inhibitors as will be discussed in more detail in paragraph 7, 'Treatment of Bone Metastasis'.

5.2 Bone Metastatic Phenotype

Whereas prostate cancer predominantly elicits an osteoblastic response resulting in osteosclerotic lesions, breast, lung and kidney cancer preferentially trigger an osteoclastic reaction mainly resulting in osteolytic lesions (reviewed in [18,22]). Although an osteolytic lesion is dominantly destructive, there is usually also a local bone formation response, which presumably represents an attempt at bone repair. Moreover, up to 25% of patients with bone metastases from breast cancer also have blastic lesions that are similar in appearance to those from patients with metastatic prostate cancer, and vice versa, some prostate cancer patients have osteolytic lesions that are similar in nature to those seen in patients with metastatic breast cancer. In other words, the common concept that there are basically two types of bone metastases, should rather be replaced by a view that the type of lesion is the result from a (dis)balance between anabolic and catabolic factors.

With regard to bone integrity, bone regions affected by either osteolytic or osteosclerotic lesions are both more prone to pathological fractures due to architectural distortion and deposition of bone of the woven (immature or embryonic) type, which is much less mechanically competent than the lamellar (mature) type.

An increase in the RANKL to OPG ratio is one of the major determinants of an osteolytic phenotype, and vice versa, a decrease in the ratio is a major determinant of an osteoblastic phenotype (reviewed in [146,327]).

5.2.1 Osteolytic Bone Metastasis

In osteolytic bone metastases, the destruction of bone is mediated by osteoclasts, as could histologically be observed by the numerous osteoclasts eroding the relatively scarce bone matrix [328,329].

PTHrP is regarded as the main mediator of osteoclast activation in bone metastatic breast cancer [18,20]. PTHrP induces RANKL and downregulates OPG in cells of the osteoblast lineage [330]. It is produced by most solid osteotropic cancers [331,332], and plays a major role in the development of the osteolytic features of their bone metastatic lesions [333]. Furthermore, PTHrP is considered to be responsible for the humoral hypercalcemia of malignancy (reviewed in [20]). In breast cancer 90% of metastases in bone were found to express PTHrP, compared to only 17% at non-bone sites and 60% of the primary tumors [331,334]. Initially, expression of PTHrP in the primary tumor appeared to be associated with formation of bone metastases [335,336]. However, a large prospective study of 526 consecutive patients with operable breast cancer demonstrated the opposite, PTHrP expression in primary breast cancer was significantly associated with fewer (bone) metastases [337,338]. Therefore, the most likely explanation for the observed increased PTHrP expression in bone metastases [334,339], is that the bone microenvironment induces cancer cells to express PTHrP rather than cancer cells that metastasize to the bone have an intrinsically higher PTHrP expression.

The hypothesis that PTHrP is induced by the bone microenvironment is also substantiated

by experimental evidence from Yin and co-workers demonstrating that PTHrP production in MDA-231 breast cancer cells was stimulated by TGF-ß [340]. Accordingly, introduction of a TßRII lacking a cytoplasmic domain (TßRIIDcyt) that exhibited dominant-negative effects into these cells prevented an increase in PTHrP production in response to TGF-ß. Moreover, in an experimental model of bone metastasis (see paragraph 6, 'Animal Models of Bone Metastasis'), mice receiving MDA-231 cells lacking TGF-ß signaling had fewer and smaller osteolytic lesions than mice inoculated with parental or empty vector-transfected cells. Taken together, PTHrP increases osteoclastic bone resorption with consequent release and activation of matrix-integrated growth factors, especially TGF-ß and IGFs, stimulating both tumor growth and further secretion of PTHrP, thus establishing a 'vicious cycle' (Fig. 10).

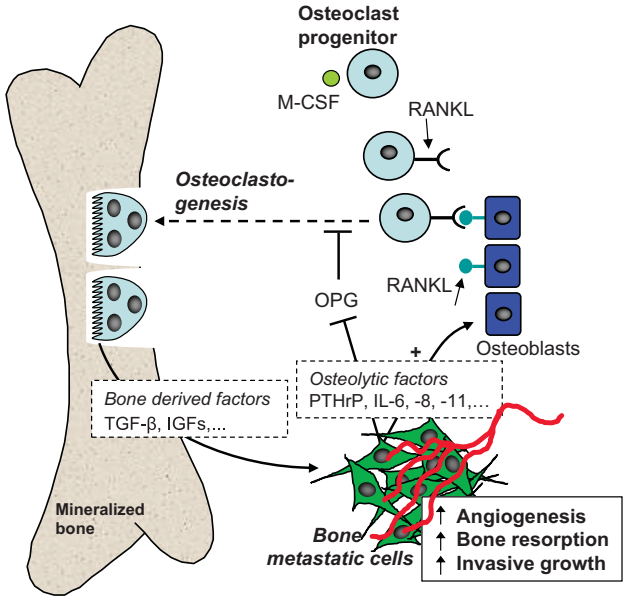

Figure 10 **The vicious cycle of the osteolytic bone metastasis.** Release of osteolytic factors, such as parathyroid hormone-related protein (PTHrP), by bone metastatic breast cancer cells causes nearby osteoblasts to change the mix of signals that they release: they increase RANKL synthesis. RANKL induces osteoclast precursors to mature into functional osteoclasts. The latter undertakes osteolysis, which causes bone demineralization, exposes the extracellular matrix within the bone, and results in liberation of various growth factors, including TGF-ß, IGFs, BMPs, PDGF. These growth factors cause cancer cell proliferation and survival, and the additional presence of TGF-ß stimulates production of PTHrP by the cancer cell, resulting in a self-sustaining positive-feedback loop that has been termed the 'vicious cycle' of osteolytic metastasis.

In addition to PTHrP, RANKL expression on osteoblasts/stromal cells can be upregulated by many other tumor secreted factors such as IL-1, IL-6, IL-8, IL-11, and tumor necrosis factor (TNF)-α. In addition, these osteolytic factors are also capable of modulating osteoclas-

togenesis independent of RANKL (reviewed in [341]). For example, IL-8 was shown to directly bind to CXCR1 present on osteoclast precursors [342]. More recently, the intra-cellular component NF-κB was demonstrated to play a crucial role in osteolytic bone metastases from breast cancer [343]. GM-CSF (alias CSF-2) was identified as the key target of NF-κB that mediates osteolytic bone metastasis of breast cancer by stimulating osteoclast development, thereby identifying new potential targets for prevention and treatment of skeletal metastasis from breast cancer.

The concept of the 'vicious cycle', in which cancer cells express osteolytic factors under influence of growth factors that are released from the bone matrix, has important therapeutic consequences: inhibition of bone resorption may interrupt this vicious cycle and thus may preserve bone mass, prevent pathological bone fractures, and even arrest tumor progression in the bone metastasis.

5.2.2 Osteosclerotic Bone Metastasis

Compared to osteolytic bone metastasis, mechanisms determining the exaggerated response in osteosclerotic bone metastasis are still poorly understood. It is believed that the excess production of mineralized bone matrix adjacent to the metastatic tumor cell deposit is due to an increased secretion of factors inducing proliferation, differentiation, and recruitment of osteoblast progenitors by the metastatic cancer cells (Fig. 11) (reviewed in [18,21,22,344]). Nonetheless, markers of bone resorption are also increased in metastatic prostate cancer, accurately reflecting the extent of disease progession [322-325,345]. In fact, bone resorption markers are even more elevated than in patients suffering from lesions from breast cancer [325], providing the rationale for treatment with bone resorption inhibitors, such as bisphosphonates, in prostate cancer patients (see paragrapgh 7.1.1, 'Bone Resorption Inhibitors').

BMPs are paradigmatic molecules that directly regulate osteoblast proliferation and differentiation (reviewed in [263]). Both normal and neoplastic human prostate tissue have been shown to express a variety of BMPs, namely BMP2, BMP3, BMP4, BMP6, and BMP7 [346]. Therefore, BMPs have been postulated to play an important role in the etiology of the osteoblastic phenotype of bone metastasis. Indeed, prostate cancer secreted BMPs in culture media were shown to promote mineralization *in vitro* (potency from high to low: BMP6 > BMP7 > BMP4) [347]. Moreover, forced overexpression of noggin — a BMP2, -4, -6, and -7 antagonist — in osteoinductive cell lines abrogated the osteoblastic response *in vivo* [270,348], however, tumor progression was not significantly affected [270,348]. Direct experimental evidence that a specific BMP could affect the osteoblastic response was provided by using neutralizing BMP6 antibodies, which inhibited the blastic response [269].

Prostate and breast cancer cells express a variety of Wnt proteins, which can enhance bone mass [349]. Interestingly, in breast cancer and myeloma — characterized by osteolytic lesions with suppression of osteoblastic activity — Wnt signaling is inhibited by the extra-

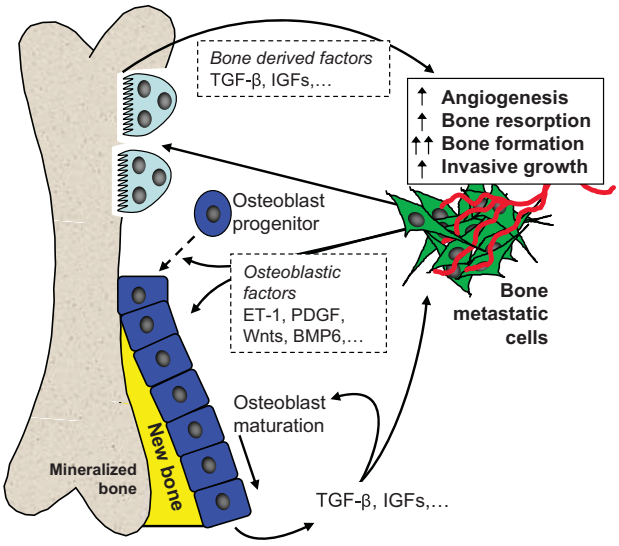

Figure 11 Pathophysiology of osteosclerotic bone metastasis. Release of endothelin (ET-1) and different Wnts by bone metastatic prostate cancer cells causes nearby osteoblasts progenitors to differentiate into osteoblasts. Mature osteoblasts also secrete growth factors such as TGF-ß that may stimulate bone metastatic growth. Therefore, a 'vicious cycle' is also considered to occur in osteoblastic metastasis.

cellular Wnt-antagonist dickkopf (DKK-1) expressed by the tumor cells [348,350,351]. Moreover, *in vitro* differential expression analysis in breast and prostate cancer cell lines showed that the expression of the Wnt and BMP antagonists, DKK-1 and noggin, respectively, rather than expression of Wnts and BMPs determines the manifestation (blastic/lytic) of the bone metastasis [348].

Other factors that have also been implicated as direct stimulators of osteoblast recruitment, proliferation, and differentiation in osteosclerotic bone metastases are IGF-I and -II, FGF-1 and -2, VEGF, platelet derived growth factor (PDGF-BB), and TGF-ß [346,349,352].

Prostate cancer patients with osteoblastic metastases have high serum levels of endothelin-1 (ET-1) [353]. Besides being a potent vasoconstrictor, ET-1 is also a direct mitogen for osteoblast progenitors [354]. A causative role for ET-1 in the pathophysiology of osteosclerotic bone metastasis has been demonstrated in pre-clinical models from prostate [354,355] and breast cancer [356]. The inhibition of bone metastatic but not primary tumor growth by treatment with ET-1-selective receptor antagonists in pre-clinical models suggests that a vicious cycle may also occur in osteoblastic metastasis [356].

In addition to factors that directly affect osteoblasts, prostate cancer cells also produce factors that indirectly affect osteoblast function by modifying the bone microenvironment, such as VEGF, and the proteases urokinase-type plasminogen activator (uPA) and prostate-specific antigen (PSA) [352]. uPA stimulates osteoblast proliferation, probably by hydrolyzing IGF-binding proteins and thereby increasing free IGF levels [357]. PSA is a serine protease of

the kallikrein family and a well-known marker of prostate cancer progression. It can cleave IGF-binding protein (IGFBP)-3, thereby reducing the ability of IGFBP-3 to antagonize the effects of IGF-I [358]. In addition, PSA can cleave PTHrP, thus allowing the osteoblastic reaction to predominate by decreasing bone resorption [359].

In conclusion, tumor cells (the 'seeds') can accomplish metastases only in the organs where the microenvironment (the 'soil') is permissive for their growth, i.e., reciprocal interaction between the bone/BM microenvironment and cancer cells is fundamental for establishing bone metastatic growth. Subsequently, the reciprocal interaction can result in exaggarated stimulation of bone degradation and/or formation, ultimately determining the bone metastatic phenotype.

6 Animal Models of Bone Metastasis

In clinical studies, the possibility to manipulate different variables is, obviously, very limited. Therefore, animal models have become important tools to investigate the pathogenesis of bone metastasis as well as to develop novel therapeutic strategies. In general, the incidence of bone metastases from spontaneous breast and prostate cancer in rodents and non-human primates is extremely rare. So, bone metastases in animal models need to be induced experimentally. These include syngeneic transplantation of spontaneously occurring rodent cancers and xenograft models with tumors or cell lines derived from human cancers into immunodeficient rodents (e.g., nude mice and rats, and severely compromised immunodeficient (SCID) mice). Furthermore, newly developed transgenic mice and immunodeficient mice transplanted with human bone fragments have been actively pursued as alternative models (reviewed in [360,361]).

6.1 Spontaneous Mammary and Prostate Cancer in Animals

Benign and malignant mammary neoplasms frequently develop in rats and mice, and the incidence is dependent on the strain. Unfortunately, these may not be good models for human disease. Most spontaneous mammary carcinomas in mice and rats have mild local tissue invasion, with low incidence of spontaneous metastasis to regional lymph nodes or lungs, and bone metastases are very rare. Moreover, spontaneous tumors can have long latency-periods. Compared to breast cancer, the incidence of prostate cancer in rodents and non-human primates is very rare [362]. However, specific strains of rats exist that have increased incidence of prostate neoplasia, but again, the incidence of bone metastasis formation is very rare [363,364]. Spontaneous prostate cancer occurs most commonly in dogs, including the formation of osteoblastic bone metastases [362].

6.2 Transgenic Induction of Mammary and Prostate Cancer in Mice

Mutations introduced in the genome — e.g., in the tumor suppressor p53 — by genetic engi-

neering have yielded transgenic mice that are prone to spontaneous development of a variety of neoplasms [365]. Tumors arising specifically in the mammary or prostate gland can be accomplished using oncogene expression — such as SV40 large T antigen — driven by tissue selective promoters. The whey acidic protein, C(3)1, and MMTV promoters are often used for the mammary gland [366], whereas the probasin, C(3)1, PSP94 and PSA promoters are used for the prostate gland [367]. Similarly, tissue-specific inactivation of (tumor suppressor) genes using the Cre/loxP site-specific recombination system could also result in tissue-specific carcinogenesis, as was shown by inactivation of p53 and E-cadherin genes [368]. The advantages of transgenic models of cancer include their predictability and the autochtonous development of cancer, i.e., originating where normally found. The disadvantage of the transgenic models is the low incidence of metastasis often due to rapid progression of the primary neoplasm. Moreover, in the transgenic models that do metastasize, metastases are usually found in lungs, but not in other organs such as bone [277].

6.3 Syngeneic and Xenograft Models of Bone Metastasis

Compared to transgenic mice, one of the major advantages of both syngeneic and xenograft models — using cancer cells and mice with an identical or different genetic background, respectively — is that cancer cells can be relatively easily subjected to genetic engineering *ex vivo*. In addition, these models also facilitate selection of cancer cell lines that have specific properties, such as a high propensity to spread to bone. These cell lines can be grown *in vitro* and, subsequently, re-injected intravenously, intracardially, orthotopically, or directly into the BM cavity. It is important to keep in mind that with respect to immunology, the use of immunocompetent mice in the 4T1 and other syngeneic models is preferred over xenograft models, which uses immunodeficient mice. On the other hand, cancer cells in the syngeneic model are not of human origin which may also lead to pitfalls when translating to the human situation.

6.3.1 Routes of Cancer Cell Inoculation

In syngeneic and xenograft experimental bone metastasis models, three major routes of cancer cell inoculation are commonly used, including 1) direct injection into bone microenvironment, 2) systemic injection, particularly intracardially, and 3) injection at orthotopic sites.

Cancer cells can be injected locally adjacent to a bone surface [369] or into a marrow cavity [370,371] (this thesis, chapter 2–6) to create an animal model for tumor growth in the bone microenvironment. However, local injection of tumor cells does not represent the entire process of bone metastasis and there can be distortions from injection caused by the injury to the bone (environment). Therefore, orthotopic or systemic injection is preferred.

Arguello and co-workers were the first who systemically injected cancer cells directly into the left ventricle of the heart [372]. Subsequently, cancer cells are dispersed throughout

the whole body, and metastases only develop in particular organs, i.e., reciprocal interaction between the cancer cell (the 'seed') and the microenvironment (the 'soil') is fundamental for establishing metastatic growth. The original model used immunocompetent mice, but later models used immunocompromised mice. At present, this technique has been adopted by many research groups using different types of cell lines, including the PC-3M-Pro4 prostate cancer cell line [373] (this thesis, chapter 7) and the broadly used MDA-231 breast cancer cell line [66,333,374,375] (this thesis, chapter 4,6). When MDA-231 wild type cells are intracardiacally injected into young (4–6 weeks old) female nude (or SCID) mice, metastases develop in specific organs, including bone. In this model, bone metastases can be clearly monitored by radiography after 3–4 weeks. Histologically, numerous osteoclasts and aggressive tumor colonization can be detected [66,333,374,375]. Bone metastases occur particularly in the metaphyses of the long bones, which are sites of active bone modeling and remodeling in young mice. Active bone turnover, high blood flow, and fenestrated sinusoids at these sites may predispose the metaphyses to the development of tumor growth. Blood vessels at the metaphyses have 180° turns and are common sites for embolization in young animals. Therefore, the anatomic arrangement of blood vessels in metaphyses may predispose to tumor cell embolization and development of bone metastases in growing rodents [376]. One of the disadvantages of the intracardiac injection model is that it lacks the critical early steps occurring between early growth at the primary site and entry into the circulation.

Kuperwasser and co-workers have developed an orthotopic xenograft model in which both the stromal and epithelial component of the reconstructed mammary gland are of human origin, enabling to genetically modify both epithelial and stromal cells *ex vivo*. They demonstrated that an altered stromal environment — stromal cells overexpressing TGF-ß or HGF — promoted breast cancer progression [377]. However, this model as well as other orthotopic models — including syngeneic and xenograft models — rarely show involvement of bone metastases. An exception is the 4T1 syngeneic model, in which orthotopic injection of 4T1 murine breast cancer cells results in formation of a primary tumor that disseminates to lung, adrenal glands, and bone [378,379]. Even more, syngeneic as well as xenograft models facilitate selection of sublines of cancers *in vivo* that have an increased incidence of bone metastasis after injection *in vivo*. For example, the 4T1.2 subclone of the 4T1 subline has an increased incidence of metastasis to bone after re-injection into the mammary fat pad [378].

Alternatively, experimental models can also use tumor tissue instead of tumor cells. Tumor tissue can be generated by implanting human cancer cell lines subcutaneously in nude mice, resulting in hybrid tumors containing a mixture of human cancer cells and murine stroma and blood vessels. Implantation of such a hybrid tumor, containing human MDA-MB-435 breast cancer cells, into the mammary glands of nude mice resulted in formation of bone metastases [380].

Human prostate cancer cell lines have also been transplanted orthotopically in mice, successfully representing different stages of cancer progression *in vivo* ranging from

androgen-dependent growth, androgen-independent growth to metastatic growth, including formation of osteoblastic bone metastases [381-383].

6.4 Subcutaneous Transplantation of Human Bone

Human fetal bone has been transplanted successfully to the subcutis of immunodeficient SCID mice to serve as a preferential site of metastasis of human prostate cancer cells injected into the left ventricle of the heart or as a site of tumor growth after local injection of cancer cells near the viable bone substrate [384]. Similar results were also reported with the use of adult human bone in SCID mice [385]. Similarly, orthotopic injection of a specific human breast cancer cell line, SUM1315, into SCID mice resulted in metastasis to the engrafted human bone, and not to mouse skeleton [386]. Hence, these models demonstrated a preferential selection of human cancer cells to localize and proliferate in human bones compared with mouse bones. Furthermore, it was very recently demonstrated that SUM1315 cells were also able to metastasize to tissue-engineered bone, manufactured by BMP2-stimulated human BM stromal cells on a silk scaffold *in vitro* [387].

6.5 *In Vivo* Imaging

Studying bone metastasis in small animal models has often relied on histological analyses, PCR amplification, and radiography. However, assays that utilize excised tissues are subject to sampling limitations and cannot assess the overall extent of disease in a given animal. To overcome this limitation, *in vivo* detection of bone metastases using high-resolution Faxitron radiography has been used in an attempt to provide temporal information. However, it does not detect micrometastatic lesions, the desired targets for therapeutic intervention, or the actual tumor burden. Therefore, newer imaging modalities — e.g., based on the optical imaging — need to be developed and utilized to improve detection and quantification bone metastases in animal models [388,389] (this thesis, chapter 3).

7 Treatment of Bone Metastases

Once patients show evidence of bone metastasis, the disease is incurable with currently available therapies. At present, therapies focus on symptomatic management and limiting the progression of established disease (reviewed in [17,18]). Therefore, the ultimate goal is to develop new and better treatment strategies that can either prevent or treat bone metastases. For this we need a better understanding of which processes are crucially important in the formation and development of bone metastases.

7.1 Treatment Strategies

The selection of the appropriate systemic anti-tumor treatments, hormone and cytotoxic treatments, that are presently given are very much dependent on the type of cancer. In addition,

external beam radiotherapy can provide excellent palliation for localized metastatic bone pain, which can be achieved with a short treatment schedule of one to five fractions in most clinical situations [390]. Radiopharmaceuticals are now also available for the palliation of metastatic bone pain, with Strontium-89 as an effective wide-field radiotherapeutic in prostate cancer [391].

7.1.1 Bone Resorption Inhibitors

Other treatment strategies for bone metastatic disease are based on the interference with the bone microenvironment and the concomitant release of growth factors from mineralized bone during bone resorption that may support bone metastatic growth. Differently from most other tissues, drugs that can limit local turnover are available for bone. Suppression of bone turnover should interfere with the growth support for the tumor. Consequently, bone resorption inhibitors are becoming an attractive strategy for preventive and adjuvant therapies in patients with bone lesions.

7.1.1.1 Bisphosphonates

Bisphosphonates (BPs) are non-hydrolysable pyrophosphate analogues, and effective pharmacological bone resorption inhibitors that bind preferentially to bone at sites of active bone metabolism. They are released from the bone matrix during bone resorption and taken up by osteoclasts. BPs potently inhibit osteoclast activity and survival, thereby reducing osteoclast-mediated bone resorption [392-394]. In clinical practice, BPs have been widely used to control skeletal complications in various neoplastic diseases. The bisphosphonates zoledronic acid, ibandronate, clodronate and pamidronate are currently given to breast and prostate cancer patients with already established bone metastases to efficiently reduce the number of skeletal-related events (SREs) [392,395-398]. In addition, BPs may delay the progression and prevent the development of new bone metastatic foci in patients either established or without bone metastasis at the beginning of the treatment [399,400]. However, a similar study failed to demonstrate a reduction in the number of metastases [401]. Subsequently, three clinical trials primarily focused their investigation on the possibility that BP may prevent the development of metastases in women affected by primary breast cancer, but free of bone metastases at the moment of diagnosis. In two of these studies the preventive administration of BP significantly reduced the number of patients developing bone metastases and the number of bone metastases per patient [397,402]. In one study there was also a significant reduction of the number of visceral metastases [402]. However, in the third study BP treatment did not prevent the development of bone metastases and even seemed to increase the development of non-skeletal metastases [403]. To resolve the apparent contradiction, two large phase III trials using BPs as adjuvant therapy for primary breast cancer, each enrolling more than 3000 patients with early-stage breast cancer, are underway. The National Surgical Adjuvant Breast Project B-34 trial will determine whether orally administered clodronate (1600 mg daily for 3 years) alone or in combination with chemotherapy

and/or hormonal therapy reduces the incidence of skeletal and non-skeletal metastases or improves overall or relapse-free survival. The Southwest Oncology Group 0307 trial will compare zoledronic acid (4 mg administered intravenously every 4 weeks for 6 months, then every 3 months for 2.5 years), clodronate (1600 mg taken orally daily for 3 years), and the BP ibandronate (50 mg taken orally daily for 3 years) as adjuvant therapy for primary breast cancer. The first results from these clinical trials are expected to be available in 2008. For prostate cancer similar prevention trials are ongoing.

7.1.1.2 Osteoprotegerin

Although the current therapy with BP results in a significant reduction in morbidity, an unmet medical need remains for a more convenient, effective, and safe therapy. Parental BPs must be administered by i.v. infusion. They are not effective in all patients, and renal toxicity and osteonecrosis may limit the dose and use of these agents in certain patients.

OPG is a potent natural bone resorption inhibitor that antagonizes the ability of RANKL to bind its receptor RANK [139,147,148]. Using the model of intracardiac injection with MDA-231 cells, treatment with Fc-OPG inhibited bone metastases formation from breast cancer [374]. In addition, in murine models of hypercalcemia, the Fc-OPG was shown to have a greater effect on reduction of bone turnover and osteoclast numbers than BP (pamidronate or zoledronic acid) treatment [404]. Moreover, in a phase I trial a single s.c. dose of OPG (AMGN-0007) suppressed bone resorption in patients with multiple myeloma and bone metastases from breast cancer at least as potently as with i.v. infusion with pamidronate [405]. Furthermore, Denosumab — a fully humanized, neutralizing monoclonal antibody to RANKL — decreased bone turnover markers in patients with multiple myeloma and bone metastases from breast cancer by a similar magnitude but more sustained than with pamidronate (i.v.) [406]. Phase III clinical trials are now focusing their investigation on whether or not denosumab is able to prevent formation of bone metastases from breast cancer and inhibit bone loss induced by hormone ablation therapy in prostate and breast cancer.

7.1.1.3 Cathepsin K Inhibitors

Another class of bone resorption inhibitors that also target osteoclastic bone resorption are cathepsin K inhibitors. In a recent preclinical study, it was shown that a cathepsin K inhibitor reduced breast cancer-induced osteolysis and skeletal tumor burden [407].

7.1.1.4 Other Treatments

Atrasentan is a small molecule that blocks the functional binding of ET-1 to ETA receptor [408]. ET-1 is a potent mitogen for both prostate cancer cells and osteoblasts, and it is considered to be involved in the generation of pain [409]. Prospective randomized placebo-controlled Phase II and III trials showed that atrasentan (10 mg/day, orally) provided adequate analgesia and a consistent trend demonstrating a delay in disease progression in patients with

metastatic hormone-refractory prostate cancer [408,410,411]. In theory, ET receptor antagonists seem better suited to act in the early phases of the cancer cell-bone interaction. Therefore, atrasentan is under investigation in a maturing phase 3 study in >900 men with non-metastatic hormone refractory prostate cancer to test the hypothesis that it delays the onset of metastatic disease.

7.2 Future Perspectives

Advances in structural deciphering of biomolecules that are involved in the pathogenesis of bone metastasis — such as OPG, integrins and MMPs — have allowed scientists to design molecules that mimic these critical targets. These artificially designed molecules can be used to block or increase the activity of a particular therapeutic target, and have significant potential in the treatment of bone metastasis. Measurements of bone turnover markers may be beneficial for the planning of a preventive treatment with bone resorption inhibitors, either newly designed molecules or more conventional ones. In the near future, gene signatures from primary cancers that predict bone metastatic potential are also very promising tools to plan adequate treatment [412-414].

8 Aim and Outline of Thesis

Once a bone metastasis has developed, patients cannot be cured. From then on, their quality of life is often adversely affected by the frequency and morbidity of skeletal-related events (SRE). Therefore, new and better treatments need to be developed, both to inhibit SRE as well as to inhibit the initial formation of bone metastases. To determine the efficacy of such treatments, more sensitive and less invasive methods to directly detect and monitor *in vivo* minimal residual disease (MRD) in preclinical cancer models are required.

In **chapter 2** of this thesis, the possible contribution to metastasis in breast cancer of periodic-acid Schiff (PAS)-positive structures in the primary tumor and its lymph node metastases is studied. In **chapter 3**, whole-body bioluminescent reporter imaging (BLI) is described as a new and better method to detect, monitor and quantify microscopic bone metastases in experimental models. Therefore, optical imaging is used in the following chapters to detect and quantify the effects of different kinds of treatments. In **chapter 4**, clinically relevant doses of bisphosphonate, given in a preventive or treatment protocol, are used to investigate the effects on progression of bone metastases. Subsequently, in **chapter 5**, high-dose bisphosphonate (BP) and osteoprotegerin (OPG) are given to maximally inhibit bone resorption using two different mechanisms of action to study the effects on established bone metastases. In **chapters 6** and **7**, bone morphogenetic protein (BMP7) is tested as a novel therapeutic molecule for repression of local and bone metastatic growth of breast and prostate cancer. Finally, general conclusions and discussions are described in **chapter 8**.

9 References

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