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Pathogenesis and treatment of skeletal metastasis : studies in animal models

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Chapter 8

General Discussion

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The skeleton is one of the most commonly affected organs in metastatic disease, and it is a major cause of morbidity, characterized by severe pain and high incidence of fractures, spinal cord compression and bone marrow aplasia requiring hospitalization. However, only a restricted number of solid cancers, especially those of the breast and prostate, are responsible for the majority of the bone metastases [1-4]. Despite the high frequency of skeletal metastases, the molecular mechanisms underlying the predisposition for tumors to colonize bone are poorly understood and treatment options are often unsatisfactory. Therefore, the focus of this thesis is to better understand the processes that contribute to the formation of distant metastasis (**chapter 2**), particularly to bone (**chapter 4–7**), as well as to explore new treatment strategies with conventional (**chapter 4** and **5**) and novel therapeutic molecules (**chapter 6** and **7**) using new and better animal models (**chapter 3–7**).

PAS+ Structures as Predictor of Clinical Outcome in Breast Cancer Patients

Although current clinicopathologic parameters are widely used to predict outcome, the clinical behavior of prostate and breast cancer can still be quite different [5]. In order to improve breast and prostate cancer management, better and reliable markers are needed that can distinguish patients with good and poor prognosis.

In **chapter 2**, we demonstrate that, in addition to the tumor blood vessels, different thin periodic acid-Schiff-positive (PAS+) meshworks may exist in invasive ductal carcinoma (IDC) of the breast and their lymph node metastases. More importantly, the complexity of the PAS+ meshwork in the lymph node metastases was related to poor prognosis. Moreover, we found that the presence of the most complex thin PAS+ pattern — the PAS+ network — in lymph node metastases and stage classification of the tumor were the only two independent risk factors associated with the occurrence of a distant metastasis. Additionally, the presence of a PAS+ network in a positive lymph node is the factor most strongly associated with poor prognosis.

Immunohistochemically, these PAS+ meshworks showed continuous expression of SMA and patchy expression of CD31. The presence of lumina in the PAS+ meshwork might be indicative for septa that may facilitate perfusion and/or dissemination of cancer cells. Indeed, tumor cells were frequently present in the lumina, whereas, erythrocytes were only occasionally found in these structures.

In 63 % of the patients, thin PAS+ patterns were present in both the primary tumors and in their respective lymph node metastases. Some tumors may, therefore, have the capability to form such PAS+ patterns; however, none of the thin PAS+ patterns scored in primary tumors appeared to be of prognostic significance. The non-prognostic value of these patterns in primary tumors can be explained by the coexistence of other PAS+/SMA+ structures in primary tumors or normal breast tissue, that hamper the interpretation. Most probably, myoepithelial cells and myofibroblasts interfere with scoring of the PAS+ patterns in the primary breast cancer.

Foss and co-workers provided evidence that PAS+ extracellular septa could be particularly favorable substrates for the in-growth of angiogenic, and possibly lymphatic, vessels [6]. Consequently, tumors that contain these PAS+ structures might be more aggressive, as these structures provide better means for (blood) vessel in-growth than tumors lacking these structures. The lack of continuous flt-4 (alias VEGFR-3) expression in the PAS+ networks of breast cancer patients may, therefore, indicate that these structures do not entirely represent lymphatic vasculature. Indeed, the differences in distribution of flt-4+ and CD31+ patterns within the PAS+ networks in our tissue specimens from the patients, support the notion that PAS+ meshworks are complex and consist of multiple types of vasculature (blood vessels, and possibly lymphatics). If the septa consist of two layers of ECM elaborated by opposing layers of tumor cells, the layers could separate, forming channels and laminar openings. Such openings could particularly lead to capillary growth. The patchy expression of the endothelial marker, CD31, is in agreement with this hypothesis. Finally, it has been suggested that the open ends of the growing capillaries could feed blood into the channels. This may explain the presence of erythrocytes in the channels, reconciling different hypotheses [7].

In both uveal and cutaneous melanoma the presence of networks that stain positive with PAS has also been described [8-11] and was shown to be of prognostic significance as well [11-13]. These cancers could also generate such PAS+ networks *in vitro* without participation of endothelial cells and thus independent of angiogenesis. This was termed 'vasculogenic mimicry' (VM) to emphasize their *de novo* generation [6,14-19]. In VM, the PAS+ interconnected loops are the tumor-lined vessels that might be perfused indicated by the presence of erythrocytes. Tumor cells that form the PAS+ loops express a variety of endothelial markers, such as VE-cadherin. It was reported that vasculogenic mimicry may also exist in ovarian [20], prostate [21] and inflammatory breast cancer [14,22]. It is tempting to speculate that tumor cells that align hollow tubular structures are phenotypically distinct from other tumor cells due to epithelial cell plasticity. Accordingly, epithelial cell plasticity might be a prerequisite for a tumor to form such a network [21]. The absence of expression of VE-cadherin, an endothelial marker that was shown to be a crucial molecule for melanoma VM [23], in both the MDA-MB-231 (MDA-231) and PC-3M-Pro4 cell lines (unpublished results), makes it less likely that VM is also present in our models.

Although the exact nature and origin of the PAS+ network in different carcinomas remains to be elucidated, the presence of PAS+ patterns, at least in breast cancer (**chapter 2**), uveal and cutaneous melanoma [11-13], is a strong and independent risk factor for the occurrence of a distant metastasis. Therefore, by examining IDC patients for the presence of PAS+ networks in their lymph node metastases, the clinical behavior of this heterogeneous group of breast cancer patients can be better predicted, which may ultimately lead to improved management of the disease.

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A Mouse Model of Bone Metastasis using Bioluminescent Imaging

Animal models have become important tools to study the molecular mechanisms that are involved in the formation of bone metastases, and have adequately served as pre-clinical models to test new drug compounds and strategies. The formation of bone metastases after injection of breast cancer cells into the left cardiac ventricle of immunodeficient (*nu/nu*) mice, has been one of the most widely used model of bone metastasis [24-26]. This experimental setting closely resembles the clinical situation of *minimal residual disease* (MRD) after removal of the primary tumor. However, radiographic analysis for detection of experimentally-induced bone metastases is far from ideal. Firstly, radiologically evident osteolytic/osteosclerotic metastases are a late and macroscopic event in the evolution of metastatic bone disease. Thus, radiography lacks the sensitivity that would be necessary to investigate initial processes, such as the 'angiogenic switch', which may be critical for tumor progression [27]. Secondly, radiography does not detect actual tumor burden but reveals only changes in bone mineral density, which is an indirect, late, and inconsistent sign of the presence of a tumor. In fact, determination of an osteolytic area depends on the ability of the cancer cells to secrete bone-resorbing cytokines rather than on tumor mass itself. Furthermore, the determination of the osteolytic area does not take into account the extension of the tumor burden in the surrounding soft tissues. Therefore, more sensitive and direct methods of detecting bone metastatic growth in experimental animals are needed.

As described in **chapter 3**, we are now able to directly monitor the distribution and growth of luciferase-transfected cancer cells in intact animals using whole-body bioluminescent reporter imaging (BLI). BLI is based upon photon signals emitted by luciferase-expressing cancer cells after addition of the substrate luciferin, and provides a direct indication of tumor viability and growth. In comparison with the indirect detection using radiography, the BLI method yields higher sensitivity, reliability, and statistical power. The remarkable sensitivity of the optical imaging in detecting microscopic bone marrow (BM) metastases allows the detection of bone micrometastatic deposits of 0.5 mm^3 volume, preceding the appearance of radiological evident osteolysis by ~2 weeks. In fact, newer optical imaging modalities with even better resolution have already been developed. In **chapters 4–7**, BLI was used both to study the molecular mechanisms in bone metastasis, as well as to develop and evaluate conventional and novel treatments.

Treatment of Bone Metastases with Bone Resorption Inhibitors

Interference with the microenvironmental growth support has been proposed as an attractive strategy for decreasing metastatic tumor growth [28]. Bone is a dynamic tissue that is continuously remodeled by bone resorption and subsequent bone formation [29]. Animal and human studies have shown that increased bone remodeling promotes the formation of bone

metastases through the release of factors from bone matrix that support metastatic growth in the BM [30,31]. Differently from other tissues, agents that specifically interfere with the tumor microenvironment are available for bone and bisphosphonates (BPs) are used clinically for the protection of bone destruction by metastases from different primary tumors [32-36].

In **chapter 4**, we have shown that treatment with clinically relevant doses of BPs before the establishment of micrometastases (preventive protocol) reduced the number of developing bone metastases by ~70%. This level of inhibition is consistent with the observation that in the experimental animal not more than 70% of the bone resorption can be inhibited by high doses of BPs [37]. Possibly, this persistently low level of bone resorption is sufficient to provide growth support to a minimal number of micrometastatic foci. BP treatment also inhibited the early progression of tumor growth in the small number of still developing metastases. However, at later stages, when bone metastases have already developed, BP treatment did not inhibit tumor growth.

So, when BPs were given to animals with established bone metastases they did not inhibit the growth of breast cancer cells in the bone microenvironment, despite a substantial anti-osteolytic effect. There are at least three hypotheses to explain these data, the lack of tumor growth response was due 1) to the magnitude of suppression of bone resorption and, hence, the potency of the BPs given, 2) to the mechanism of inhibition of bone resorption by the BPs, or 3) that the initial growth phase of cancer cells in bone is dependent upon the interaction with the bone microenvironment, but eventually becomes independent of the bone microenvironment and can progress autonomously.

To address these issues, we used an experimental model of intraosseous growth and compared the effects of very high doses of the most potent BP currently available [38,39], zoledronic acid (ZOL), with the effects of osteoprotegerin (Fc-OPG), which inhibits bone resorption by a different mechanism of action (**chapter 5**). As expected, both Fc-OPG and ZOL treatment markedly suppressed bone resorption, protected the integrity of bone trabeculae and prevented the development of osteolytic lesions. Intraosseous tumor burden was reduced, however, the total tumor burden (intra-bone and extramedullary) still progressed. Although at the end of the experiment the total tumor burden in treated groups appeared less compared to controls, this difference was not significant.

Previous studies of the effects of BPs on bone metastatic breast cancer growth in animal models have shown contradictory results. BPs were reported to reduce the tumor burden in established bone metastases but not in soft tissue and visceral metastases. This effect seemed to be mediated by induction of apoptosis of the cancer cells [25,40]. Treatment with BP, given before the development of evident metastasis, resulted in a marked reduction of the number of bone metastases [25,41,42]. However, another preventive study showed that BP treatment resulted in an increased tumor burden in the developing bone metastases but not in visceral metastases [43]. In these investigations tumor burden has been quantified by indirect measurements of the tumor-induced osteolytic area on bone radiographs and end-stage

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histology. In contrast, BLI allowed us to detect the total tumor burden directly and more sensitive, yielding more reliable data. Our BLI data favor the hypothesis that metastatic cancer cells in bone — after an initial growth phase that depends on their interaction with the BM stroma and extracellular bone matrix — become increasingly independent of microenvironmental growth factor support and is self-maintained by autocrine mechanisms.

How can the therapeutic effect of inhibition of bone resorption/turnover on early stages of bone metastasis development be explained? Bone remodeling does not occur throughout the entire bone surface but takes place at microscopic patches of bone resorption and subsequent bone formation. The number and activity of so-called basic multicellular units (BMUs) determine the rate of bone turnover. We hypothesize that the probability that a micrometastasis becomes a clinically evident bone metastasis should be related to the chance that metastasis-initiating cells (MICs) are in close proximity with an active BMU. MICs may extravasate near active BMUs, or dormant MICs may encounter active BMUs. In either case, a higher number of active BMUs (high bone turnover rate) increases the probability that MICs come in close proximity of active BMUs. This hypothesis is substantiated by clinical data demonstrating that high bone turnover in cancer patients with metastatic bone disease is associated with skeletal-related events, disease progression and death [44-47], and by experimental evidence showing that high bone turnover is causally associated with establishment and progression of bone metastases [31,48]. Accordingly, inhibition of bone turnover before the generation of a self-maintaining, autocrine cancer cell growth may arrest the vicious cycle underlying the initial growth support by the BMU and ultimately prevent the outgrowth of a micrometastasis into an overt bone metastasis.

Based on our preclinical data (**chapter 4**) and that of others, one may predict that BP therapy as adjuvant treatment in breast cancer patients decreases the risk of formation of new bone metastases. However, clinical studies have shown mixed results with regard to the development of new bone metastases. Clodronate as adjuvant therapy significantly reduced [49], non-significantly reduced [50], or did not reduce [51,52] the development of new bone metastases. Nevertheless, the largest reported randomized controlled study to date of BP use in patients with breast cancer demonstrated that clodronate significantly improved bone relapse-free survival [52,53]. Results from the National Surgical Adjuvant Breast and Bowel Project B34 trial, an even larger randomized placebo-controlled trial of clodronate versus placebo in women with early-stage breast cancer who have received standard adjuvant systemic therapy, are awaited.

Although current clinicopathologic parameters are widely used to predict clinical outcome, the clinical behavior of breast (and prostate) cancer can still be quite different. Therefore, more specific and more reliable markers that can distinguish between patients with high and low risk for bone metastasis formation are needed, and may become the rationale to start adjuvant BP treatment even before the onset of bone metastatic disease.

At present, the most promising approaches that may lead to a better identification and stratification of the cancer patients at risk to develop bone metastases are the 1) assessment of bone turnover status, 2) use of microarray technology, and 3) detection of disseminating tumor cells (DTCs) in BM (and blood).

Although high bone turnover rate in bone metastatic cancer patients is associated with bone disease progression [45-47], to date, it has not been specifically addressed whether high bone turnover in non-metastatic cancer patients is associated with subsequent formation of new bone metastases. Interestingly, many cancer patients with a resected primary tumor display a high bone turnover rate, which is either induced at a basal level (e.g., induced by menopause) or induced after surgical or chemical gender steroid deprivation. Especially these patients — who probably harbor micrometastatic MICs in the BM at the moment of diagnosis and/or surgery — may benefit from prophylactic bone resorption inhibition by BPs.

Microarray analysis is a new and promising technique that may also identify which patients are at increased risk for developing bone metastases. Using this technology, Kang and co-workers generated a 122-gene bone metastasis signature by comparing gene expression among sublines of the human breast cancer cell line, MDA-231, that are highly vs. weakly metastatic to bone in nude mice [54]. Moreover, Woelfle and co-workers reported a 73–gene signature capable of identifying lymph node-negative patients with or without BM micrometastasis [55]. Furthermore, Smid and co-workers have studied lymph node-negative primary breast cancer and reported a putative molecular signature capable of predicting metastatic recurrence to bone vs. recurrence elsewhere in the body [56]. A 31-gene signature was selected to predict which patients developed bone metastases with 100% sensitivity and 50% specificity. The low specificity may reflect that microarray analysis only describes global mRNA obtained from the tumor, whereas all genes that are necessary to metastasize to bone have to be coordinately expressed in an individual tumor cell. Surprisingly, genes that were previously identified to promote bone metastasis such as IL-8 [57], PTHrP [58] and αvβ3 [59], were not members within any of these bone signatures. Although the bone signatures from Kang and Smid display a common increase in the FGFR-p42/44 MAPK pathway, it is still disappointing that just one single gene (*BENE*) is shared between any two of the three signatures. Besides differences in experimental approaches and patient population selected, the lack of overlap may also be reflected by the low reproducibility between studies using microarray technology. This was demonstrated by the comparison of two studies which both generated a gene signature capable to predict distant metastasis in lymph node negative breast cancer patients [60,61]. Although both studies included a similar number and type of patients, had comparable experimental design, and used similar statistical methods of validation, the resulting 70 and 76 gene predictors overlapped only by three genes.

Another approach to predict which patients are at high risk to develop a bone metastasis is to screen BM for the presence of DTCs. Although not only true MICs might be identified,

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the presence of DTCs in BM at time of diagnosis is significantly associated with the formation of distant metastases, particularly to bone [62,63].

In conclusion, these different approaches are very promising and may lead to a better stratification of patients at high risk for the *de novo* formation of bone metastasis, and therefore, may become the rationale to start adjuvant BP treatment even before the onset of bone metastatic disease. However, these approaches need first to be extensively validated in large confirmatory studies by independent groups.

BMP7 in Progression and Metastasis in Osteotropic Cancers

The increased motility and invasiveness of cancer cells are reminiscent of the epithelial-to-mesenchymal transition (EMT) that occurs during embryonic development [64]. The EMT is associated with decreased expression of epithelial markers such as E-cadherin, and increased expression of mesenchymal markers such as vimentin. By signaling through the Smad mediators, transforming growth factor (TGF)-ß is a well-known inducer of EMT in both embryogenesis and its' counterpart in cancer progression. However, in organogenesis, 'mesenchymal' cells may possess remarkable plasticity and may regain a fully differentiated phenotype via a mesenchymal-to-epithelial transition (MET) under the influence of bone morphogenetic protein (BMP) 7 [65-67]. Under pathologic conditions, particularly in malignant disease, the role of BMP7 has remained largely elusive.

Expression of BMPs during Cancer Progression

In **chapter 6** and **7**, we described that BMP7 expression in breast and prostate cancer cell lines *in vitro* correlated with expression of E-cadherin, and inversely correlated with aggressiveness and expression of vimentin. In addition, human prostate cancer cells from the primary tumor had a decreased BMP7 expression when compared with matched non-carcinogenic prostate epithelium as assessed by laser-microdissection and immunohistochemical staining. Overall, BMP6 is overexpressed in aggressive prostate cancer when compared with normal prostate tissue [68-70], and BMP2, BMP4, [70] and BMP7 (**chapter 7**) are expressed predominantly in normal prostate tissue, and their expression tends to be lower or absent during progression of prostate cancer (reviewed in [71]). In this regard, it should be mentioned that it seems to have become a general misconception that BMP7 was found to be significantly increased in prostate cancer bone metastases. This is probably due to the misleading title "Increased expression of BMP7 in bone metastatic prostate cancer" of an article [72], in which BMP7 mRNA expression levels in bone metastatic prostate cancer from autopsies were only found to be significantly increased when compared to non-tumor bearing bone, not when compared to primary tumor. Therefore, this first needs to be further investigated, including immunohistochemical staining for BMP7 on autopsy material.

BMP7 expression in prostate cells is regulated by androgens because orchidectomy decreased BMP7 expression [73], while testosterone and dihydrotestosterone increased it [74] (Fig. 1). These observations are in line with our data, showing that BMP7 expression is reduced in prostate cancer cell lines that are androgen-insensitive. These data, consequently, raise the question that evolution of prostate cancer to metastatic dissemination after hormonal therapy may be linked to the loss of androgen-induced BMP7 expression

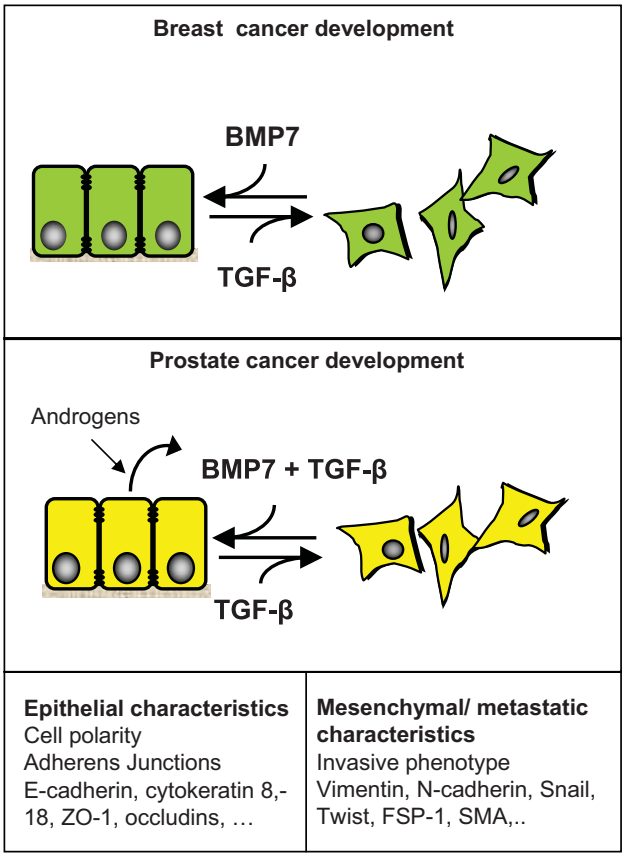

Figure 1 **Role of BMP7 in cancer progression.** Top: In breast cancer, TGF-ß and BMP7 exert opposing effects. Whereas TGF-ß induces epithelial-to-mesenchymal (EMT), BMP7 induces the opposite process of mesenchymal-to-epithelial transition (MET). Bottem: Similarly, BMP7 induces MET, and TGF-ß induces EMT in prostate cancer cells. Remarkably, BMP7 induces MET more prominently in the presence of TGF-ß in prostate cancer cells. In the prostate gland, androgens stimulate the production of BMP7 by prostate epithelial cells. In the later stage of the disease, BMP7 ceases to act on cancer cells, potentially because of the loss of androgen-induced production of BMP7 after anti-androgen therapy. Subsequently, TGF-ß alone induces EMT, including repression of E-cadherin expression and stimulation of the acquisition of an invasive phenotype. Consequently, the cancer cells with an acquired metastatic potential can disseminate through the bloodstream to other organs, including bones.

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and loss of epithelial phenotype maintenance. In other words, androgen deprivation therapy may directly result in perturbation of the epithelial homeostasis.

The expression of different BMPs in breast cancer progression is less well-studied, and only a very limited number of studies have explored the consequences of BMP signaling on patient outcome. In ductal breast cancer patients, BMP2 expression did not affect the overall or relapse-free survival [75], whereas overexpression of BMP receptor IB associated with poor prognosis in estrogen receptor (ER)-positive breast cancer [76]. In 170 breast cancer patients with IDC, Schwalbe and co-workers observed that expression of BMP7 protein was highly associated with ER and progesterone receptor (PR) levels. Using a tissue microarray (TMA), we confirmed indeed that expression of BMP7 in 520 breast cancer patients was associated with ER + PR levels (manuscript in preparation). To follow up on these findings, we are currently investigating whether treatment with the hormones estrogen and progesterone regulates BMP7 gene expression in breast cancer as well (manuscript in preparation). Furthermore, our TMA data did not show an association between BMP7 expression in primary tumor and (disease free) survival or metastatic pattern. This seeming discrepancy with the data that BMP7 mRNA was associated with formation of bone metastasis (**chapter 6**), may be explained by the findings from Alarmo and co-workers who showed that there is no significant correlation between BMP7 copy number, BMP7 mRNA and BMP7 protein levels [77]. This is substantiated by the fact that invasive lobular carcinoma (ILC) of the breast expressed elevated levels of BMP7 protein when compared to IDC, even though an increase in *BMP7* gene copy number was found in IDC, but not in ILC [77]. The observation that regulating the recruitment of histone deacetylase HDAC2 to the BMP7 promoter region acts as a regulatory mechanism to control BMP7 expression [78], suggests that the lack of association between BMP7 copy number and BMP7 mRNA may be due to epigenetic regulation. Moreover, the lack of correlation between BMP7 mRNA and protein expression levels could be a result of the stability of mRNA or protein. The 3'UTR of BMP7 mRNA is very long, which is a characteristic that has been linked to mRNA stability [79]. Subsequently, translation could occur from very few copies of mRNA. The involvement of microRNAs — non-coding small RNAs that primarily function as gene regulators [80] — on regulating BMP7 mRNA has not been addressed to date, but the presence of such microRNAs may greatly influence BMP7 mRNA stability. In addition, BMPs are synthesized as pre-proteins, which are modified to mature BMPs by pro-domain cleavage, and are subsequently secreted. Pro-domains are known to affect the stability of the BMP protein [81]. Taken together, it is likely that both mRNA and protein processing are involved in the regulation of BMP7 expression levels.

Although the finding from Alarmo and co-workers that BMP7 expression was lost from most of the local recurrences as compared with the corresponding primary tumor [82] is in agreement with our data, the observation that BMP7 protein levels in primary breast cancer are associated with the formation of bone metastases [82], is not. In contrast to Schwalbe and our findings, Alarmo and co-workers did not find an association between BMP7 and ER or

PR [82]. They explained this discrepancy by differences in tumor materials and classification of immunohistochemical staining results. Surprisingly, only 37% of primary IDC and 57% of primary ILC were classified as being positive for BMP7, while in an earlier study the same authors showed that the majority (71.4%) of 91 breast cancer patients (IDC+ILC) showed strong BMP7 staining, whereas moderate staining was observed in the rest of the cases [77]. Even though they performed an additional antigen retrieval step, one would not expect that BMP7 staining was detected exclusively in the cytoplasm, when an earlier study showed exclusive nuclear staining in primary breast cancer cells using the same antibody (sc-9305; D.Bobinac, personal communication).

Using tissue micro arrays containing malignant primary skin melanomas [83] or primary renal carcinomas [84], BMP7 was associated with poor and good prognosis, respectively. This apparent contradiction underlines once more the heterogeneous effects that specific BMPs can exert on different cell types.

BMP7, Cellular Function and Signaling

BMPs have a well-known role in osteogenesis by inducing differentiation of cells from the osteoblast lineage and enhancing osteoblast activity [85,86]. For example, BMP7 has the ability to enhance osteoblast activity, partly by inducing VEGF expression [87]. Therefore, BMPs have also been implicated in the sclerotic phenotype of bone metastases. Noggin is a BMP antagonist and inhibits BMP2, -4, -6, -7 signaling by binding BMP ligands [88]. Indeed, overexpression of Noggin in osteosclerotic prostate cancer cell lines significantly decreased the osteoblastic response *in vivo*, but tumor burden was not affected [89,90]. In contrast, overexpression of Noggin in the osteolytic prostate cancer cell line PC-3 significantly inhibited the expansion of the lesion *in vivo* [91]. In this and other studies, particularly BMP2 and BMP6 stimulated migration and invasion of prostate cancer cells, while BMP4 and BMP7 had no or almost no effect [89,91,92]. Direct experimental evidence that a specific BMP could affect the osteoblastic response was provided when neutralizing BMP6 antibodies inhibited, besides intra-bone tumor growth, the blastic response [92]. Taken together, the inhibitory effects of Noggin on cancer progression are most probably mediated by antagonizing the pro-tumorigenic effects of BMP2 and BMP6, rather than antagonizing BMP4 or BMP7.

Many different BMP7-induced biological effects are known, but they are mostly cell type-dependent as well as dose-dependent. In prostate cancer, addition of BMP7 to *in vitro* cultured cells has resulted, so far, in at least three different kinds of responses, affecting 1) proliferation, 2) apoptosis, and 3) cellular plasticity (Fig. 2*A*)[93-97].

Although under normal culture conditions — 10% fetal calf serum (FCS) in culture medium — BMP7 did not affect proliferation of PC-3 cells, BMP7 could inhibit the proliferation of the LNCaP, PC-3 and DU 145 cells under culture conditions of 1% FCS [94]. The observed G1-arrest was most likely caused by BMP7 induced up-regulation of the cyclin-dependent kinase inhibi-

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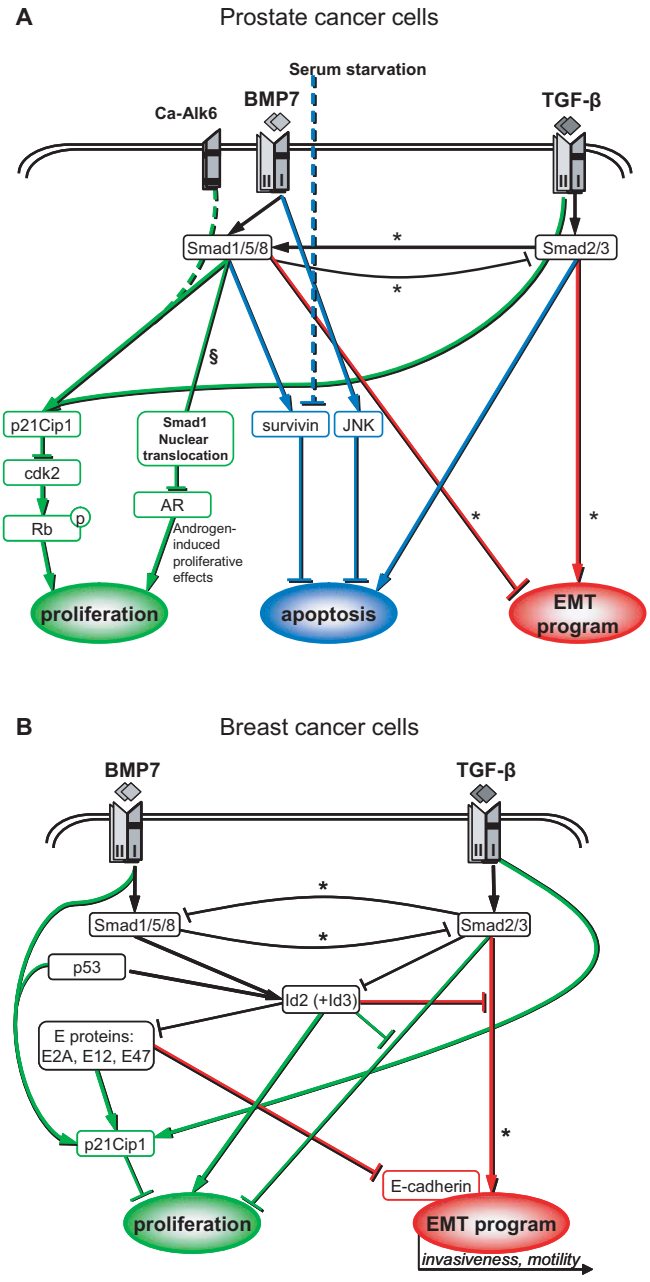

Figure 2 **BMP7-induced intracellular signaling cascades described in prostate (A) and breast (B) cancer cells, regulating cellular processes, such as proliferation, apoptosis and epithelial plasticity.** In addition, a very limited scheme for intracellular TGF-ß signaling is depicted . *, signaling pathway described in this thesis; §, described for BMP signaling; based on data from [93-96](A) and [115,116](B).

tor (CDKI) p21CIP1/WAF1 [94], as was also demonstrated for BMP2 and BMP4 in LNCaP cells [98]. Interestingly, BMP7 induced a concentration-dependent biphasic effect on the proliferation of LNCaP in the absence of exogenous androgen. While higher concentrations of BMP7 inhibited proliferation of LNCaP cells, lower concentrations of BMP7 stimulated the proliferation in the absence of androgen [94]. At yet another FCS concentration (0.5% FCS), the anti-proliferative effect of BMP7 was only detected in the cell line representing a benign prostatic epithelial hyperplasia (BPH-1), but not in the invasive PC-3 and DU 145 cells [97].

In keeping with the findings that BMP signaling can exert anti-proliferative effects, Kim and co-workers demonstrated that transfection of a dominant negative type II BMP receptor (BMPRII) into PC-3M cells (a PC-3 subclone), resulted in a growth rate 10 times higher than that of control cells in the murine tumor model [99]. In addition, the introduction of the constitutively active ALK6 (BMPRIB) in PC-3 cells resulted in the inhibition of cell proliferation *in vitro* as well as inhibition of tumor growth after subcutaneous injection [94].

In the second type of response, BMP7 protected prostate cancer cells from stress-induced apoptosis. Yang and co-workers have shown that BMP7 inhibited serum starved-induced apoptosis in LNCaP and C4-2B cells [96]. In the C4-2B cells, this was mediated via both Smad5-dependent upregulation of Survivin and Smad-independent activation of the JNK pathway.

Finally, the third type of described responses to BMP7 is related to cell plasticity. It has long been recognized that TGF-ß can induce morphologic conversion, invasiveness, and migration in epithelial cells, collectively referred to as an EMT [64]. BMP7, however, was shown to induce the opposite process, the mesenchymal-to-epithelial transition (MET) in renal epithelial cells [100,101]. In **chapter 7**, we demonstrate that BMP7 induce MET in prostate cancer cells, as demonstrated by induced E-cadherin promoter activity and E-cadherin protein expression, and concomitant inhibition of vimentin expression. Therefore, we hypothesize that BMP7 expressed in normal epithelial cells induces E-cadherin expression and contributes to maintenance of an epithelial state, whereas loss of BMP7 during cancer progression may result in EMT and metastasis (Fig. 1). In prostate cancer cells, the effects of BMP7 could be further potentiated by addition of TGF-ß (Fig. 3). The synergistic actions of BMP7 and TGF-ß were not detected in any other epithelial cell line studied so far and may be cell-specific for reasons that remain to be explained.

In contrast, Yang and co-workers showed that BMP7, but not TGF-ß, stimulated EMT in PC-3 cells [97]. However, in addition to our *in vitro*, *in vivo*, and clinical data, a protective role for BMP7 is substantiated by data obtained in numerous studies that showed that 1) loss of BMP7 increases the invasive and migratory properties of prostate cancer cells [102], 2) BMP7 is downregulated in recurrent prostate cancer [74], and 3) that BMP receptors are frequently lost during prostate cancer progression [99,103]. Interestingly, the accessory type III receptor endoglin was shown to decrease prostate cancer cell migration and invasion through activation of ALK2–Smad1 pathway [104]. This is in total agreement with earlier data, which showed

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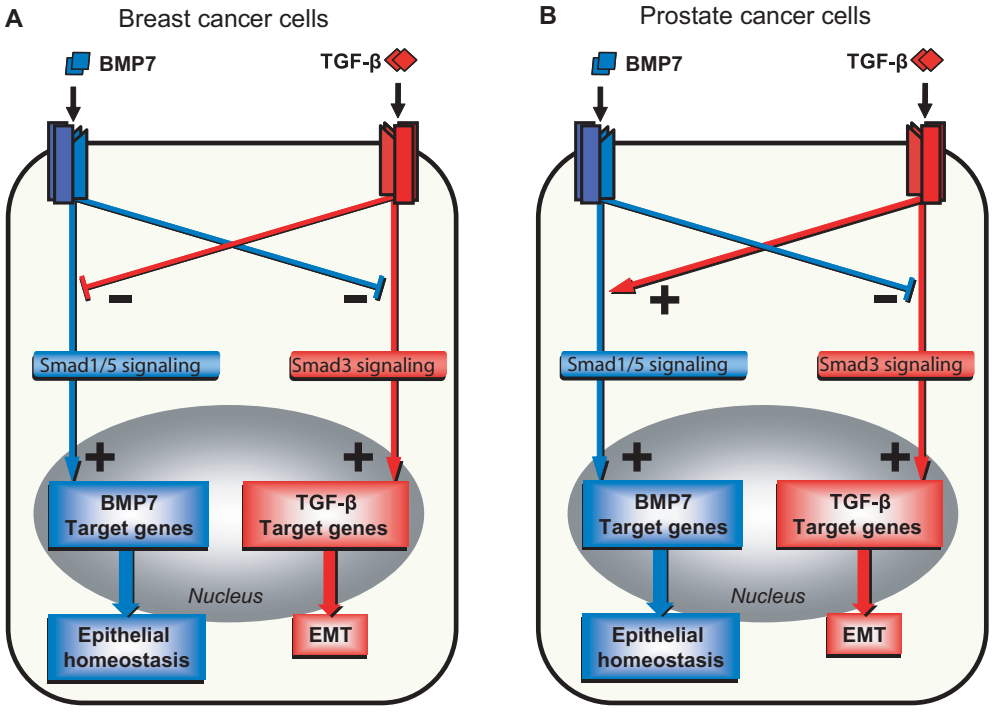

Figure 3 **Interactions between TGF-ß and BMP7 signaling.** The biological distinct effects of TGF-ß and BMP7 can be explained at the level of intracellular signaling. *A,* in breast cancer cells, BMP7 can inhibit Smad-dependent TGF-ß-signaling, and vice versa, TGF-ß can inhibit Smad-dependent BMP7-signaling. *B.* In prostate cancer cells, TGF-ß has an stimulatory rather than an inhibitory effect on BMP7-signaling. Consequently, BMP7 alone or in combination with TGF-ß, results in epithelial maintenance. However, when BMP7 expression is lost, TGF-ß may still trigger the TGF-ß-induced EMT and acquisition of a metastatic phenotype. So, whereas in breast cancer prevailed TGF-ß-signaling might already result in induction of an EMT, in prostate cancer, loss of BMP7 is a prerequisite for EMT to occur.

that endoglin inhibits TGF-ß1-induced ALK5–Smad3 signaling and promotes BMP7–Smad1/Smad5 signaling [105,106]. Therefore, we suggest that the inhibitory effects of BMP7 on prostate cancer cell migration and invasion are most likely mediated via its type I receptor ALK2 and is Smad-dependent.

Similarly, expression of BMP receptors was reduced, and BMP signaling was diminished in colorectal cancer, indicating that loss of BMP signaling also correlates tightly with progression of adenomas to colorectal cancer occuring relatively early during cancer progression [107,108].

Compared to prostate cancer, even less is known about the BMP effects in breast cancer cells. A dominant negative truncated BMPRII inhibited proliferation of T47D breast cancer cells suggesting a stimulatory role for BMPs [109]. In contrast, a dominant negative mutated

BMPRII — inhibiting BMP–Smad signaling, but not p38^{MAPK} signaling — stimulated proliferation of NMuMG breast cancer cells suggesting a stimulatory role for BMPs via p38^{MAPK} [110]. In breast cancer, BMP7 has not yet been studied in many functional studies. BMP2, however, is one the the most investigated BMPs in breast cancer. BMP2 can inhibit proliferation [111,112], inhibit hypoxia-induced apoptosis [75], stimulate migration of cancer cells, and induce tumor formation *in vivo* after subcutaneous implantation in nude mice [113]. In **chapter 6**, we describe that BMP7 does not affect proliferation, induces MET, and inhibits TGF-ß induced EMT in MDA-231 breast cancer cells. In line with its inhibitory role in cancer progression, we show that stable overexpression of BMP7 in MDA-231 breast cancer cells inhibits *de novo* formation and progression of osteolytic bone metastases.

In mammary (and other) epithelial cells, members of TGF-ß superfamily strongly regulate the expression of Inhibitor of DNA binding (Id) proteins in normal and cancerous cells in a Smad-dependent manner. Id proteins antagonize basic helix-loop-helix transcription factors by inhibiting them from DNA binding and transactivation of their target genes, thereby playing critical roles in animal development and cancer [114]. Kowanetz and co-workers have shown that Id2 and Id3 showed long-term repression by TGF-ß and sustained induction by BMP7 in NMe mammary, lens and other epithelial cells (Fig. 2*B*) [115,116]. Moreover, overexpression of Id2, and to a lesser extent Id3, blocked TGF-ß-induced EMT in these cells. Furthermore, BMP7 could counteract TGF-ß-induced EMT by overriding the downregulation of Id2 and Id3 by TGF-ß in lens epithelial cells. Surprisingly, Id2 knockdown in these cells not only enhanced TGF-ß-induced EMT, but also permitted EMT by BMP7. Thus, during tumor progression, misregulation of epithelial Id2 and Id3 expression could possibly allow even BMP pathways to cause EMT and enhance tumor invasiveness. Accordingly, Id2 was found to play an important role in the preservation and maintenance of the differentiated and non-invasive phenotype of breast cancer cells [117]. However, the role of Id proteins in our models has not yet been addressed. If regulation of Id proteins is also critically involved in biological responses in other carcinomas, such as prostate, the absence or very low basal level of Id2 and Id3 proteins in the PC-3 cells used by Yang might explain that BMP7 was capable to induce EMT [97].

Recently, it was demonstrated that BMP7 is a novel target of the p53 family in breast cancer [116]. In fact, Id2 is also regulated by p53, and whereas a functional BMP7 or an active p53 pathway was sufficient to maintain basal levels of Id2, loss of both pathways abrogated Id2 expression. Subsequently, loss of Id2 resulted in inhibition of cell proliferation [116]. Interestingly, p53 is the most frequently mutated gene found in human cancers, including breast cancer. For this reason, it is tempting to speculate that loss of both p53 and BMP7, which is the case for MDA-231 cells, may be a prerequisite for EMT in cancer cells that have already escaped from the anti-proliferative effects exerted by restrained Id2 signaling.

Although much remains to be understood about the complexity of signaling from BMPs in cancer, there is emerging evidence from a variety of tumor systems that the observed

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effects are cell specific and could be either pro- or anti-tumorigenic. Consequently, results derived from the study of one cell system frequently could not be applied to other systems of the same cancer type. While the BMPs themselves may hold some of the answers, BMP receptors are probably also determining factors in distinguishing between the pro- or anti-tumorigenic effects seen in cancer cells. There is a good possibility that further understanding of the expression pattern of the type I and type II receptors and their relationship to the development and progression of cancer may provide much more information on this phenomenon. In addition, the physiological response of many different types of cancer cells might also very well be explained by the basal level and regulation of sensitive gene targets, such as *Id2* and *Id3*.

It is important to note that analogous to its protective role in breast and prostate cancer progression (**chapter 6** + **7**), we have recently demonstrated that decreased BMP7 expression also contributes to progression of uveal melanoma [118].

BMP7 Treatment in Experimental Models

It has never been specifically addressed whether systemic treatment with (any) BMP might influence the progression of (breast and prostate) cancer. In line with our earlier observations that BMP7 inhibits tumorigenicity in breast cancer, we describe in **chapter 6** that daily intra venous administration of BMP7 (100 µg/kg/d) significantly inhibited orthotopic and intra-bone growth of MDA-231-B/Luc+cells in nude mice. In addition, we describe in **chapter 7** that BMP7 treatment also inhibits PC-3M-Pro4/Luc+ prostate cancer bone metastatic growth. However, prostate cancer growth was not inhibited after orthotopic implantation. Clearly, the tumor microenvironment is an important determinant of the therapeutic response to BMP7 in prostate cancer. In fact, it cannot be excluded that the successful treatment may also be explained by direct effects of BMP7 on tumor stroma. This question may, for example, be adequately addressed in an experiment in which cancer cells are transfected with a constitutively active form of ALK2, excluding the direct BMP7 effects on the stroma.

Interestingly, the effects of BMP7 can be further potentiated in prostate cancer cells by an environment rich in TGF-ß, such as in bone, because the combination of BMP7 and TGF-ß increased E-cadherin and decreased vimentin expression to greater extent than BMP7 alone (Fig. 1-3). In prostate cancer, these synergistic actions of BMP7 and TGF-ß may explain the successful experimental treatment of skeletal metastases and intra-bone growth, but not of orthotopic growth. For the full beneficial effects of BMP7 in prostate (cancer) cells, paradoxically, the presence TGF-ß may be needed for BMP7 to fully function as a growth factor that maintains the epithelial homeostasis. Taken together, the tumor microenvironment is an important determinant of the therapeutic response to BMP7 in prostate cancer.

In the bone matrix, TGF-ß is highly concentrated in an inactive form that can be released and activated by osteoclastic resorption [119]. Upon activation, TGF-ß may act as a paracrine

growth factor for neighboring cancer cells that may have colonized the BM [120]. In this context, our observation that BMP7 could counteract TGF-ß signaling in both breast and prostate cancer cells (Fig. 1-3), may be very important in explaining the successful treatment of osteolytic bone metastases from breast and prostate cancer (Fig. 4). The important role of TGF-ß in the progression of bone metastasis was established in experiments that showed that interference with TGF-ß signaling by either small-molecule inhibitors of ALK5 (TßRI) kinase [121,122], overexpression of the inhibitory Smad7 [123], or introduction of a dominant negative TßRII [58] could reduce osteolytic tumor burden. Nevertheless, we did not provide direct evidence that BMP7 inhibits TGF-ß signaling *in vivo* as it does *in vitro*. We are in the process

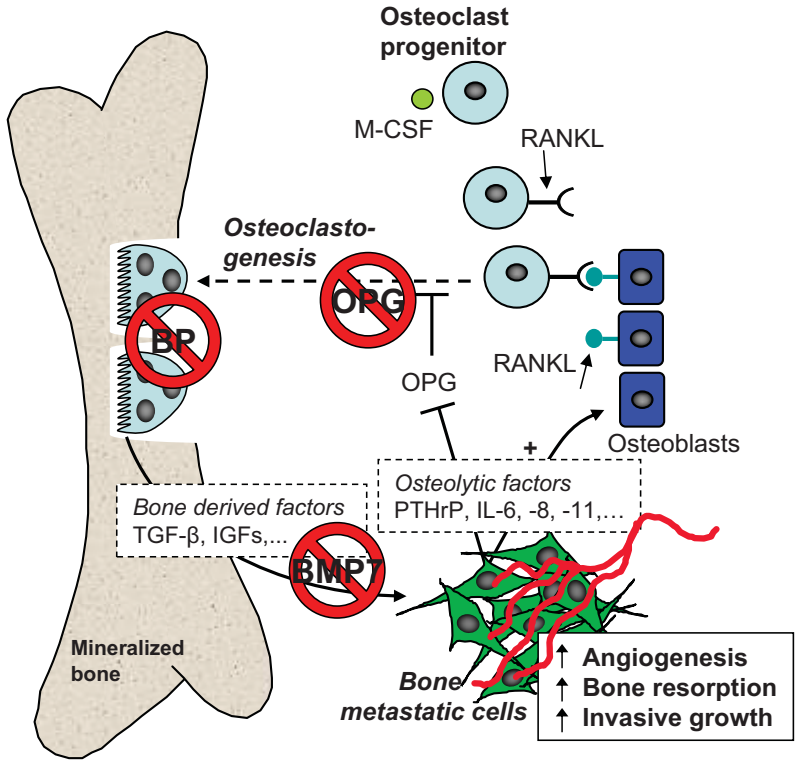

Figure 4 **Potential mechanisms to inhibit bone metastatic growth**. Osteoclastic bone resorption results in release of growth factors, such as TGF-ß, from the bone matrix that may stimulate (cancer) cell growth. Therefore, inhibition of bone resorption provide a potential mechanism for treatment and prevention of bone metastasis. Either treatment with OPG, inhibiting the formation of osteoclasts, or treatment with a bisphosphonate (BP), reducing the number of active osteoclasts, may not only inhibit osteolysis but also the development and growth of bone metastases. It is important to note that in both blastic and lytic lesions the osteoclastic bone resorption is increased and plays an important role in the pathophysiology of the bone metastasis. BMP7, either endogenously expressed or given as a therapeutic agent, can inhibit TGF-ß signaling in cancer cells. Therefore, BMP7 may also represent a novel therapeutic molecule that may also halt or slow down the progression and development of bone metastases.

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of addressing this using an *in vivo* model in which cancer cell line lines stably expressing the TGF-ß-specific reporter construct CAGA-luciferase are monitored (manuscript in preparation). Using this enhancement of our *in vivo* models, the effects of various compounds, such as BMP7, on TGF-ß signaling in cancer cells will be elegantly monitored.

In conclusion, we provide a role for BMP7 expression in the maintenance of epithelial cell behavior in the breast and prostate. We propose a model wherein BMP7 can induce the expression of E-cadherin, repress vimentin synthesis, and prevent EMT in normal epithelial cells. In later stages of tumorigenesis, cancer cells lose BMP7 expression or become resistant to the BMP7 secreted by surrounding normal epithelial cells. Consequently, malignant cells may lose their expression of E-cadherin under the influence of TGF-ß, resulting in EMT and subsequent metastatic spread. In prostate cancer, TGF-ß may even contribute to the epithelial homeostasis as long as BMP7 remains present as well. This model re-emphasizes the double-edged-sword properties and the complexity of members of the TGF-ß superfamily in cancer biology. It also provides a rationale for further investigation of BMP7 in the prevention of osteolytic bone metastases from breast and prostate cancer. Overall, we provide novel and possibly clinically relevant perspectives for the therapeutic use of BMP7, but also raise numerous questions regarding the molecular mechanisms involved, and its role at different stages of cancer that exemplify the promises but also the versatility of the members of the TGF-ß superfamily.

BMP7, Future Perspectives

Since we position BMP7 as a promising novel therapeutic molecule for repression of systemic breast and prostate cancer progression, it is of utmost interest to unravel the molecular and biological roles of BMP7 in cancer progression and bone metastasis formation that have not yet been addressed. Given the correlation between BMP7 and androgens — BMP7 expression is associated with androgen receptor status, and stimulated by androgens — it is of great interest to study the effects of BMP7 treatment on androgen-positive cell lines in experimental models as well. In addition, the effect of BMP7 on the initial phases of metastasis and on osteosclerotic lesions also remains to be investigated.

In our animal models, orthotopic breast and prostate cancer growth does not lead to distant metastases. Hence, studying the role of BMP7 in inhibiting EMT in the process of metastasis requires investigation in animal models in which tumor cells metastasize from the primary site to distant sites, such as bone. For this, very few models are available that recapitulate the events of the entire metastatic cascade attributable to prostate or breast cancer. A relevant model that fulfills this requirement, and shows enhanced metastasis with increased TGF-ß signaling uses a genetically engineered expression of the proto-oncogene Her2/Neu (erBB2), a receptor tyrosine kinase under the control of the mouse mammary tumor virus in mouse mammary gland [124]. In this system, mammary tumors spontaneously

developed and metastasized to lung. Overexpression of either active TGF-ß [125] or a constitutively active form of ALK5 [126] increased metastasis formation, whereas impairment of the TGF-ß pathway in mammary cells with a dominant negative form of the TßRII [126], or administering a soluble form of TßRII [125], decreased the frequency of metastasis to the lungs. Analysis of endogenous expression of BMP7 and EMT markers, as well as testing the effects of a BMP7 treatment could answer questions about BMP7 antagonism of TGF-ß-induced EMT and the early steps of metastasis. However, bone metastases do not develop in this model.

Particularly, transgenic mice that spontaneously develop primary breast or prostate tumors may very well be used to further elucidate the role of BMP7 in development and progression of these carcinomas [128]. For example, in these transgenic mice the BMP7 gene could be knocked-out using the Cre/loxP system in a prostate or breast specific manner.

In another model, inoculation of the 4T1 murine breast cancer cells into the mammary gland results in formation of a primary tumor that disseminates to lung, adrenal glands, and bone. Using this model, Muraoka and co-workers showed that inhibition of TGF-ß signaling with a soluble type II receptor significantly reduced metastases [127]. Unfortunately, EMT or bone metastases have not been studied in this model.

Most patients with prostate cancer metastases manifest with radiologically osteoblastic lesions. However, enhanced osteoclastic bone resorption is also clearly evident and contributes to the pathophysiology of bone metastasis in this setting. In fact, the bone resorption marker N-telopeptide is increased in prostate cancer patients with bone metastases and is a stronger predictor of death than prostate specific antigen [46]. Furthermore, BPs also improve skeletal morbidity in patients with osteoblastic disease [129]. Although our animal model lacks a strong osteoblastic component that is often evident in humans with prostate cancer bone metastases, important information can be gleaned regarding the role of BMP7 in osteolytic prostate (and breast) cancer bone metastases. Nonetheless, the effect of BMP7 on bone metastases with a sclerotic phenotype has not yet been established, and should, therefore, also be evaluated in osteoblastic models such as those induced by the breast cancer cell line ZR-75-1 [130], and prostate cancer cell lines LuCap 23.1 [131] or C4-2B [132].

Cancer Stem Cells and the Bone Metastatic Process

It is becoming increasingly clear that tumor cell populations arising in breast and prostate are organized much like normal tissues, in which self-renewing stem cell populations are responsible for spawning the bulk of the cells [133]. The bulk populations (>>95%) of tumor cells behave much like the transit-amplifying cells found in a normal tissue [134,135]. While the cancer stem cells are tumorigenic, in that they can seed a new tumor mass when implanted into the host, the non-stem cells (i.e., the transit-amplifying cells) in the tumor mass are unable to do so, because they are endowed with only a limited replicative potential. Strikingly, TGF-ß and BMPs affect stem cell renewal and induction of differentiation into spe-

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cialized-epithelial structures (reviewed in [136-139]). These observations may have important implications for the process of metastasis. If the non-stem cells in a primary tumor mass are truly non-tumorigenic, then these cells may well succeed in leaving the primary tumor and lodging in distant sites, but they will be unable to colonize these sites because of their limited proliferative potential. Accordingly, the ability to create macroscopic metastases may be limited to the relatively small number of tumor stem cells that escape from the primary tumor. These MICs may possess the ability to replicate to an unlimited extent and are therefore ideally suited to found new metastatic colonies that eventually expand to a life-threatening size. Such dynamics may well contribute to metastatic inefficiency — the failure of the vast majority of DTCs to colonize the tissue sites in which they have landed [140-143].

In addition to the TGF-ß and BMP pathways, the Notch, hedgehog and Wnt pathway are critically involved in normal and cancer stem cell proliferation (reviewed in [134]). Interestingly, multiple interactions between these pathways and different BMPs have been established. BMP7 could inhibit Notch signaling in normal prostate gland development [144], and BMP signaling can inhibit intestinal stem cell-renewal through suppression of Wnt/ß-catenin signaling [145]. For instance, we have found that BMP7 is able to inhibit Wnt signaling in PC-3M-Pro4 prostate cancer cells (unpublished data). Furthermore, recent data suggest that BMP(4)-induced Smad-signaling can inhibit the tumorigenic potential of human brain-tumor initiating cells, mediated via a significant reduction in the stem-like, tumor-initiating precursors [146]. Accumulating evidence suggests that cancer stem cells with tumor-initiating potential exist in human breast [147] and prostate cancer [141]. Although speculative at present, some of the observed effects of BMP7 described in this thesis may be mediated, at least in part, by osteotropic cancer cells with tumor-initiating (and metastatic) potential.

Further research is certainly warranted to address the role of BMP7 and other members of the TGF-ß superfamily in these pathways that are crucially important in developmental processes as well as in cancer progression. Furthermore, identification and characterization of MICs may provide new strategies for the treatment of patients with (as yet) incurable metastatic bone disease.

Summary and Conclusion

In this thesis, several processes have been unraveled that are critically important in the formation of metastases, particularly to bone. The presence of PAS+ networks in lymph node metastases from breast cancer is a prognostic factor for the formation of distant metastases. In addition, low BMP7 mRNA expression levels in primary breast cancer may be used as a prognostic indicator for the formation of bone metastases. After validation, these markers may be used to predict which patients are at high risk for distant metastases and become the rationale to offer patients a more adequate treatment, e.g., prophylactic BP treatment for patients that have low BMP7 levels.

Furthermore, we propose a model in which DTCs from osteotropic cancers are spread throughout the body, but with a modestly higher propensity for bone. Only DTCs with stem-cell like characteristics, i.e., MICs, which end up in a suitable environment can ultimately develop into a metastasis. In the bone environment, MICs can stay dormant for many years until a BMU comes into close proximity. When this happens the BMU may induce high local concentrations of growth factors such as TGF-ß, which can stimulate the outgrowth of a micrometastatic deposit to an overt bone metastasis. So, only MICs that are in the close proximity of a BMU may be activated to form a bone metastasis. Also, therapies that may reduce the actual number of BMUs, like OPG and BP, may inhibit the formation of bone metastases (Fig. 4). This may also be true for treatments that antagonize the pro-tumorigenic effects of TGF-ß. In addition, BMP7 may not only inhibit TGF-ß-induced EMT, but also induce epithelial homeostasis in normal breast and prostate epithelium, representing a putative novel therapeutic molecule for breast and prostate cancer.

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