Chapter 6

Summary and Discussion

Since the discovery of TGF-β nearly three decades ago [1, 2, 3, 4] tremendous scientific effort has led to a sophisticated understanding of the multifunctional actions of this pleiotropic cytokine. TGF-β regulates a myriad of processes in normal tissues and in cancer pathogenesis a characteristic is the frequent loss of TGF-β-induced growth arrest and abnormal secretion of this cytokine (reviewed in [5, 6, 7, 8, 9]). The pathogenic branch of TGF-β signaling is an attractive target for therapeutical intervention. However, in order to specifically direct therapy to this arm of TGF-β signaling a deeper understanding of its cellular actions and intercellular signal transduction pathways in specific contextual settings is crucial. Several studies are presented in this thesis, which are aimed at unraveling the mechanisms by which TGF-β is involved in the pathogenesis of breast cancer, breast cancer bone metastasis and in renal fibrosis.

The key findings presented in this thesis are:

1. The intracellular TGF-β effector proteins, Smad2 and Smad3, play differential roles in breast cancer bone metastasis. Smad2 mediates anti-angiogenic properties whereas Smad3 contributes with pro-angiogenic effects in vitro and in human breast cancer bone metastasis (chapter 2).

2. HMGA2 was identified as a Smad4-dependent TGF-β target gene in breast cancer cells. This factor is required for TGF-β-induced EMT of epithelial cells. Ectopic expression of HMGA2 results in a down-regulation of E-cadherin, which is mediated via enhanced expression of its transcriptional repressors Snail1, Snail2/Slug and Twist (chapter 3).

3. TGF-β induces EMT, growth arrest and initiates specific gene responses in epithelial cells. The actions of TGF-β can be inhibited by administration of the ALK5 and TβRII antagonist GW788388 (chapter 5).

4. Expression of constitutively active ALK2, a BMP-7 type I receptor, in osteotrophic breast cancer cells impairs tumor-induced osteolysis in an intra-bone tumor growth model (chapter 4).
5. Constitutive activation of the BMP-7 receptor, ALK2, in breast cancer cells led to the findings that the anti-metastatic actions of BMP-7, in breast cancer bone metastasis can occur via direct effects on the tumor cells. This mediates paracrine signaling in cells of the bone and tumor-associated microenvironment. We speculate that enhanced BMP signaling can counteract TGF-β in cancer cells hereby disturbing the vicious cycle of bone metastasis (chapter 4).

6. When the TGF-β inhibitor GW788388 was orally administered to diabetic db/db mice with advanced nephropathy, GW788388 significantly reduced renal fibrosis and decreased expression of key mediators of fibrosis in kidneys (chapter 5).

The significance of these observations and how we can relate them to the field of TGF-β research and potentially lead to clinical applications will be discussed in the following. A simplified schematic overview of our findings is given in figure 6.1.

6.1 Modulation of the TGF-β signaling pathway

TGF-β may exert dual roles in carcinogenesis. Acting at first as a tumor suppressor, in premalignant stages of tumorigenesis, via inhibition of proliferation and induction of apoptosis. At later stages of disease, TGF-β can shift to being a tumor promoter (reviewed in [5, 6, 7]). Frequently in cancer there is a loss of the TGF-β cytostatic responses and the switch to a tumor promoting role of TGF-β. Concomitantly, high dose secretion of active TGF-β is often observed in carcinomas. The net result being tumor progression and possibly metastasis (reviewed in [5, 8, 9]). The molecular determinants which mediate this switch remain poorly understood [10]. However, dissecting the individual steps of the metastatic cascade has led to pivotal findings and the mechanistic of breast cancer metastasis are gradually being unveiled.

Smad2, Smad3, HMGA2 downstream mediators of TGF-β signaling

We studied several aspects of the pro-tumorigenic functions of TGF-β in breast cancer cells and in breast cancer metastasis models in vivo. It was previously shown that knockdown of Smad4 inhibits the formation and progression of bone metastasis of MDA-MB-231 cells [11, 12]. However, Smad4 is a central regulator of both TGF-β and BMP signaling. In chapter 2 we describe a comparative analysis of the two direct downstream mediators of TGF-β signaling Smad2 and Smad3. The function of these signaling molecules were analyzed in the highly invasive and osteotropic breast cancer cells the MDA-MB-231. Smad2 or Smad3 expression was genetically knocked down by lentiviral transduction of microRNAi (miRNAi).

When analyzing the effect of eliminating these TGF-β effector molecules we found that key metastatic TGF-β target genes [13] were critically dependent on Smad3 and not Smad2 (see figure 6.1). These genes include IL-11, PAI-1 and CTGF. That Smad3 is
6.1 Modulation of the TGF-β signaling pathway

We modified TGF-β signaling and downstream responses through several mechanisms in NMuMG and MDA-MB-231 breast cancer cells. By lentiviral transduced microRNAi we eliminated the expression of either Smad2 or Smad3. Smad2 and Smad3 were shown to play specific roles in MDA-MB-231 breast cancer cells. Smad3 was necessary for TGF-β inducible gene responses of IL-11, CTGF, PAI-1 and VEGF-A. In contrast, loss of Smad2 enhanced VEGF mRNA expression (left side of figure). We studied the function of HMGA2 a direct target gene of TGF-β and found HMGA2 to be dependent on Smad3 and Smad4 in MDA-MB-231 cells and on Smad2, Smad3, and Smad4 in NMuMG cells. Enhanced HMGA2 expression significantly induced the expression of the E-cadherin repressors Snail1, Snail2 and Twist. Furthermore, expression of Id2 was repressed when HMGA2 was overexpressed. Thus, ectopic expression of HMGA2 induce EMT of NMuMG cells independent of TGF-β and loss of HMGA2 inhibits TGF-β mediated EMT (middle part of figure). Continuous activation of the BMP-7 receptor caALK2 was shown to counteract TGF-β signaling in MDA-BO2 cells as observed in transcriptional reporter assays (right side of figure).

the major regulator of TGF-β target genes is in line with several other previous reports [10, 14, 15, 16, 17]. Also, specific knockout of Smad2 or Smad3 in hepatocytes identified Smad3 as the main transcriptional inducer or repressor downstream of TGF-β [10].

We further analyzed the effect of knockdown on the migratory properties of breast cancer cells. MDA-MB-231 cells readily migrate in response to TGF-β and this can be inhibited by administration of an ALK5 kinase inhibitor (chapter 2, [18]). We found that depletion of either Smad2 or Smad3 was sufficient to inhibit TGF-β-induced migration of MDA-MB-231 cells.
To specifically determine the actions of TGF-β in metastasis we used the Smad2 and Smad3 knockdown cells in a breast cancer metastasis model where cells are inoculated into the left heart ventricle and metastasize to bone [12]. Knockdown of Smad2 resulted in enhanced progression of metastasis compared to control and Smad3 knockdown tumor-bearing animals. In contrast, Smad3 knockdown cells displayed delayed growth kinetics in the early phases of the experiment. These findings were directly correlated with differential roles of Smad2 and Smad3 in tumor-induced angiogenesis (see figure 6.1 and 6.2). VEGF secretion was significantly enhanced in MDA-MB-231 cells depleted for Smad2. In Smad3 knockdown cells, VEGF-A expression and secretion were no longer induced by TGF-β.

To further examine this, we visualized and quantified the newly formed capillary networks in bone tumor metastasis by immunohistochemical CD31 staining. Strikingly, Smad2 knockdown metastasis showed a four fold increase in CD31 staining and the micro vascular density was significantly enhanced compared to Smad3 knockdown and control metastasis. Thus, Smad2 may inhibit tumor-induced angiogenesis by negatively regulating tumor-cell secretion of VEGF into the bone microenvironment. Smad3, on the contrary, induce angiogenesis by stimulating production of angiogenic factors (like VEGF) and chemotactic factors, which mediate the recruitment of inflammatory cells (see figure 6.2).

In agreement with our findings, Smad2 was found to mediate secretion of factors with anti-angiogenic properties, whereas Smad3 induced the secretion of pro-angiogenic factors in tubular epithelial cells [19].

A study by Ju et al. [10] clearly demonstrated differential roles of Smad2 or Smad3 in conditional knockout (KO) hepatocytes. Smad2KO cells displayed enhanced migratory potentials and spontaneously underwent mesenchymal transition suggesting that Smad2 is required to maintain a stable epithelial morphology [10]. In contrast, TGF-β was unable to induce EMT in Smad3KO cells. Moreover, Smad3 was found to be an essential mediator of apoptosis and TGF-β-induced G1 cell cycle arrest whereas in Smad2KO cells cyclinD1 and c-Myc were up-regulated [10]. Thus, Smad2 and Smad3 exert distinct roles in TGF-β-mediated cell cycle regulation in hepatocytes. In our breast cancer model we found that Smad2 knockdown cells proliferated slightly slower in vitro than control and Smad3 knockdown cells. However, we cannot exclude effects on apoptosis or survival since proliferation was analyzed in a cell viability assay. It would be interesting to examine the proliferative capacities of Smad2 miR RNAi cells versus control and Smad3 miR RNAi cells in our bone metastases sections with immunohistochemical markers for apoptosis and proliferation.

When analyzing TGF-β-induced responses in hepatocytes all genes examined were shown to be dependent on Smad3 [10]. Similarly, we found that most TGF-β-responsive genes were regulated by Smad3 not Smad2. Many of these genes were also dys-regulated in MDA-MB-231 Smad4 knockdown cells [11, 12]. However, even though many pro-metastatic genes lost their responsiveness to TGF-β in both Smad3 and Smad4 knockdown cells loss of Smad4 gave rise to a much less severe metastatic phenotype in vivo. Metastasis-free survival was significantly enhanced when Smad4 was depleted and the
Figure 6.2: Smad2 and Smad3 play distinct roles in breast cancer bone metastasis. Model of the opposing roles of Smad2 and Smad3 in osteotropic MDA-MB-231 cells. Knock down of Smad3 (left panel) results in loss of TGF-β-inducible gene responses of critical metastatic inducers such as IL-11, CTGF, PAI-1, VEGF-A and HMGA2. When Smad3 knockdown cells were used in a bone metastatic model prolonged latency in metastatic growth was observed. In contrast, when Smad2 was eliminated (right panel) enhanced expression and secretion of VEGF was observed. This translated into enhanced metastatic potential of these cells in vivo and significantly enhanced tumor-induced angiogenesis. Together, these observations support a role for Smad2 as a tumor suppressor and Smad3 as a tumor promoter in breast cancer cells.

frequency of bone metastasis was reduced by 75% [11, 12]. In animal injected with Smad3 knockdown cells we observed a clear latency in tumor progression in the early phase of metastatic progression compared to control and Smad2 inoculated animals. Since Smad4 is a common transcription factor shared by both TGF-β and BMP these observations could suggest that abrogation of both signaling pathways result in a more efficient blockade of metastasis compared to only eliminating Smad3.

The molecular determinants that mediate the functional switch, which transform TGF-β from being a tumor suppressor to a tumor promoter remain poorly understood. We hypothesize that loss of Smad2 (genetic or epigenetic) during tumorigenesis may increase the metastatic potential, whereas loss of Smad3 could decrease the metastatic behavior of breast tumor cells (chapter 2, [10]). This is in line with findings in patients with stage II breast cancer where loss of P-Smad2 staining is correlated with shorter overall survival [20]. Thus, it is possible that the functional switch may be determined by changes of the relative balance of Smad2 (anti-metastatic signaling) and Smad3 (pro-metastatic signaling) in malignant cells (see figure 6.2) hereby affecting their distinct properties on the microenvironnt [10]. It would therefore be of great interest to perform a more detailed genetic analysis of the exact roles of Smad2 and Smad3 both in response to TGF-β and in non-induced conditions and to analyze the impact of knock-
down or either Smad2 or Smad3 on the tumor-associated stroma in bone metastasis in further detail.

Thus, our findings suggest that Smad2 and Smad3 play distinct roles in mammary cancer metastasis. In hepatocytes, Smad2 knockout cells spontaneously acquired mesenchymal features with a pro-migratory phenotype [10]. Smad3, on the other hand, was necessary for TGF-β-induced EMT and migration [10]. Also, keratinocyte-specific knockout of Smad2 displayed clear mesenchymal characteristics [21]. Together, these studies suggest that Smad2 is necessary to maintain a stable epithelial phenotype and that the two TGF-β R-Smads have distinct functions in several cellular systems. The breast cancer model used in our study is not suited for studying aspects of EMT as MDA-MB-231 cells display partial hypermethylation of the E-cadherin promoter [22]. Moreover, we employ intracardiac inoculation of the cancer cells and hereby circumvent the critical steps of EMT.

EMT facilitates cell movement and the generation of new tissues in embryogenesis. In a cancer setting, gain of mesenchymal characteristics allow tumor cells to disseminate and intravasate and hereby escape from the primary tumor as extensively described in chapter 1 (reviewed in [5, 23, 6, 24]). The downstream mediators of TGF-β Smad2, Smad3 and Smad4 have all been extensively studied in normal epithelial cell models. Homozygous deletion of Smad2 triggered complete EMT in skin tumors [21]. In contrast, over-expression of activated Smad2/3 was shown to increase cell motility in a squamous skin tumorigenesis models [25]. In line with these findings, Valcourt et al. showed that ectopic expression of either Smad2 and Smad3, in combination with Smad4 could induce EMT in NMuMG cells [26]. In these cells, loss of Smad3 or Smad4 by RNAi completely abrogated TGF-β-induced EMT [17, 12]. Moreover, overexpression of the antagonist of TGF-β signaling, Smad7, in NMuMG cells blocked TGF-β-mediated EMT [26].

The discovery of HMGA2 as being a direct target gene of TGF-β and necessary for TGF-β-mediated EMT of mammary epithelial cells is described in chapter 3 (see figure 6.3). We found that knockdown of Smad4, in NMuMG cells blocked TGF-β-induced up-regulation of Hmga2. In MDA-MB-231 cells, knockdown of either Smad3 or Smad4 could block TGF-β-mediated HMGA2 mRNA induction. When HMGA2 was over-expressed this alone could drive the EMT response through direct up-regulation of the E-cadherin repressors, i.e. Snail1, Snail2 and Twist. In addition, ID2, another key player in EMT, which expression is reduced in response to TGF-β [27], was down-regulated in HMGA2 over-expressing cells. Furthermore, NMuMG cells depleted for HMGA2 by RNAi no longer undergo EMT in response to TGF-β. Together these findings establish a strong role for HMGA2 as a mediator of EMT, which is directly induced by TGF-β [28].

In correlation with these observations, it was shown that HMGA2 is highly up-regulated during embryogenesis in cells which have not yet undergone overt differentiation [29, 30]. In normal tissues, the expression of HMGA2 is lost and various tumor types abundantly re-express HMGA2, which is correlated with enhanced malignancy or metastatic potential [29, 31]. Together, these findings suggests that HMGA2 indeed could be a key pro-tumorigenic mediator downstream of TGF-β.

We persistently tried to study the role of HMGA2 in the MDA-MB-231 breast can-
6.1 Modulation of the TGF-β signaling pathway

Figure 6.3: HMGA2 induce the expression of E-cadherin repressors resulting in EMT.
TGF-β up-regulate HMGA2 via Smad-dependent mechanisms. Ectopic HMGA2 expression significantly induced the mRNA expression of Snail1, Snail2 and Twist which functions as critical E-cadherin repressors. In addition, HMGA2 could repress the expression of Id2. Thus, ectopic expression of HMGA2 induce EMT of NMuMG cells independent of TGF-β as observed on morphological and EMT marker level.

cancer bone metastasis model. This was done by knock down of HMGA2 expression by shRNAi and analyzing the effect in various tumorigenic assays. HMGA2 knockdown cells displayed reduced capacity to invade and migrate in matrigel coated transwell chambers (Thuault unpublished observations). No differences were observed in other in vitro models such as anchorage-independent growth or proliferation assays. When HMGA2 knockdown cells were used in our in vivo metastasis model we did not observe any differences in metastasis formation and progression between control cells and cells silenced for HMGA2. Despite technical difficulties in conducting these experiments we hypothesize that HMGA2 is important in a primary tumor setting where EMT is needed for migration and invasion to distant sites. This is in line with findings in squamous carcinomas where HMGA2 is observed at the invasive front in carcinoma tissue where tumor cells migrate into the connective tissue [31]. However, in later stages of the metastatic cascade HMGA2 may not be necessary for the establishment and progression of breast cancer bone metastasis.

We studied the knock down of HMGA2 in osteotropic MDA-MB-231 cells a highly metastatic cell line, which displays complete loss of E-cadherin [22]. This metastasis mode circumvents critical steps of EMT since the main pro-tumorigenic function of HMGA2 is the induction of the E-cadherin repressors this may explain the outcome of our preliminary in vivo study using cells in which there is no E-cadherin present to be repressed. HMGA2 may therefore be irrelevant in the later stages of tumorigenesis in MDA-MB-231 cells. Olmeda et al. showed that knock down of the E-cadherin repressor SNAIL in MDA-MB-231 could inhibit orthotopic growth in vivo [32]. This might have been a superior model for studying the effect of HMGA2 depletion or overexpression. Alternatively we could have used immortalized human mammary epithelial cells as described by Onder et al. who recently used this cell line to examine the function of E-cadherin in breast cancer [33]. Otherwise, the murine epithelial breast cancer line 4T1 which express E-cadherin and give rise bone-tropic metastasis could have been a better
suited model for these studies [34].

**Counteracting TGF-β signaling by continuous activation of ALK2**

Metastatic cancer affects millions of people worldwide yet the majority of patients with cancer metastasis remain incurable [6, 35]. Cancer research have largely been focussed on the cells from which a given cancer originates in a primary tumor setting. However, tumor growth depends on interactions between multiple inter-dependent cell types [36]. How the microenvironment and tumor-infiltrated stromal cells influence tumorigenesis and the role played by TGF-β herein are currently topics of intense investigation.

In patients with breast cancer, loss of BMP-7 mRNA expression in primary tumor is associated with the formation of bone metastasis [37]. In prostate cancer, the expression of BMP-7 was shown to be down-regulated compared to normal prostate tissue [38]. Moreover, in a panel of cancer cell lines the expression of BMP-7 negatively correlated with tumor cell aggressiveness [37, 38]. BMP-7 can counteract TGF-β signaling and reverse TGF-β-induced EMT. This is mediated through re-expression of the key EMT marker E-cadherin [39, 37, 40]. In MDA-MB-231 breast cancer cells, BMP-7 was shown to counteract TGF-β at the transcriptional level and reverse the TGF-β-induced up-regulation of vimentin [37]. When BMP-7 was ectopically expressed in MDA-BO2 cells or human recombinant BMP-7 systemic administered to MDA-BO2 tumor-bearing mice a significant inhibition in the number of osteolytic metastasis and progression of these lesions was observed [37].

In chapter 5, we describe the identification of the functional BMP-7 receptor, ALK2, in MDA-BO2 cells. Furthermore, we explore whether ectopic expression caALK2, a genetically constitutively activated receptor, can mediate similar responses as over-expression of BMP-7 ligand in vitro and in vivo. Ectopic expression of caALK2 resulted in continuous activation of downstream BMP signaling, thus mimicking a state of continuous BMP-7 signaling. When analyzing classical downstream target genes of BMPs, we found sustained up-regulation of ID2, SMAD6, and SMAD7. Moreover, TGF-β signaling was significantly abrogated at transcriptional level (see figure 6.1). Hence, these observations are directly in line with our findings in cells ectopically expressing the BMP-7 ligand [37].

Whether the inhibitory actions of BMP-7 on bone metastasis and intra-bone tumor growth are caused by direct cell autonomous actions on the cancer cells or through direct inhibitory signals on the tumor-associated stroma has remained elusive. With over-expression of caALK2 we mimic a situation where BMP signaling is active only in the tumor cells not in the entire microenvironment as is the case for BMP-7 over-expressing cells (see figure 6.4). The metastatic behavior of BMP-7 versus caALK2 over-expressing cells was studied in intra-bone tumor growth and metastasis models.

In the intra-bone tumor growth model the effects of either caALK2 or BMP-7 over-expression on tumor-induced bone remodeling was analyzed by quantifying changes in bone mass and bone architecture by μ-CT. We found that the formation of overt osteolytic lesions was significantly inhibited in mice injected with either caALK2 of BMP-7
Figure 6.4: Over-expression of BMP-7 or caALK2 inhibits osteolytic breast cancer metastasis. A tumor cell-induced vicious cycle of osteolysis and tumor growth in the bone is dependent on active TGF-β signaling. Tumor cells secrete pro-osteolytic factors which stimulate osteoclastogenesis and osteoclastic activation hereby inducing bone resorption. Bone encapsulated growth factors such as TGF-β is released into the microenvironment and these act back on the cancer cells to stimulate further growth and osteolysis. We overexpressed BMP-7 (top insert) or caALK2 (lower insert) in osteotropic breast cancer cells. BMP-7 secreted from MDA-BO2 cells over-expressing the ligand act both on the tumor cells and cells in the bone microenvironment. Cells over-expressing caALK2 on the other hand solely activate BMP-7 signaling in the tumor cells. Both cell lines displayed significantly reduced tumor-induced osteolysis and metastatic progression. High up-regulation of Smad7, a well described inhibitor of TGF-β and BMP signaling was also observed in these cell lines. We hypothesize that this induction of Smad7 or alternatively ID proteins could play an important inhibitory role in breast cancer bone metastasis and intra-bone tumor growth.
sis [47]. Furthermore, adenoviral administration of Smad7 to mice bearing jygMC(A) murine mammary tumors or ectopic expression of Smad7 in these cells [48] significantly inhibited metastasis and increased metastasis-free survival [48].

Thus, Smad7 plays a crucial role in metastatic progression of various cancers. Stable overexpression of Smad7 inhibited the up-regulation of TGF-β-inducible genes such as IL-11, CXCR4, PTHrP and Osteopontin in melanoma cells [47]. We analyzed the expression levels of IL-11, PTHrP and CTGF in cell over-expressing caALK2 or BMP-7 MDA-BO2 compared to control cells and found no changes in expression patterns. In order to determine the contribution of either Smad7 or ID2 in our model it would be of great interest to genetically deplete these proteins from caALK2 and BMP-7 cells and analyze their changed metastatic behavior in vivo.

Continuous activation of ALK3, a receptor for BMP-2 and BMP-4 [49], was recently shown to mediate invasion and metastasis of MDA-MB-231 breast cancer cells. This pro-metastatic function of caALK3 could be blocked by over-expression of a dominant negative ALK3 [50]. We show that in MDA-BO2 cells BMP-7 signals via ALK2 and over-expression of continuous active ALK2 receptor gives rise to a less metastatic phenotype in vivo. Thus, active BMP-7 signaling in tumor cells distinctively induce anti-metastatic properties in these human breast cancer cells. Together, these observations highlight the importance of discriminating between different BMPs when characterizing their functions.

Taken together, these studies demonstrate that BMP-7 can block tumor progression in bone via directly inhibiting tumor cell-induced osteolysis possibly by antagonizing TGF-β signaling in the breast cancer cells (see figure 6.4). To determine the mechanism in detail a more thorough gene analysis would have to be performed to analyze the regulation of down-stream effectors in these cells.

6.2 Clinical applications and therapeutic opportunities

The dichotomous role of TGF-β in diseases represents a great therapeutic challenge. Targeted strategies must be directed towards the pathogenic functions of the signaling pathway and protect the normal homeostatic role of TGF-β such as its potent anti-inflammatory actions and the anti-mitogenic effects of TGF-β on primary tumors (reviewed in [7, 51]). Moreover, to properly stratify patient subpopulations, which may benefit from anti-TGF-β treatment regimens, new and robust diagnostic tools must be developed [52].

Abrogating TGF-β signaling by means of synthetic inhibitors have been attempted by several pharmaceutical companies and research groups, as introduced in chapter 1. In chapter 5 we describe the characterization of a novel TGF-β receptor antagonist GW788388 in epithelial cells of various origins [53, 54]. We show for the first time that GW788388 targets the ATP binding domain of both the TGF-β type I and the type II receptors. When analyzing downstream TGF-β signaling we found that GW788388
efficiently blocked TGF-β-mediated EMT. Furthermore, the compound inhibited TGF-β-induced growth arrest in NMuMG cells. Thus, down-stream mechanisms of TGF-β signaling can efficiently be inhibited by low doses of GW788388 [53]. These findings are summarized in figure 8 of chapter 5.

In a mouse model of diabetic nephropathy, the db/db model, we found that daily administration of GW788388 (2 mg/kg/day for 5 consecutive weeks) significantly attenuated glomerulopathy in mouse kidneys [53]. This was correlated with reduced mRNA expression of critical factors in ECM remodeling induced by TGF-β namely PAI-1, collagen I, collagen III and Fibronectin in GW788388 treated animals versus controls. All together, these data provide a strong foundation for using TGF-β receptor kinase inhibitors for treating advanced renal diseases. However, the use of such drugs for renoprotection may require chronic treatment and the impact of blocking of TGF-β signaling in perspective of its immunosuppressive and tumor suppressor functions could have adverse side effects [53, 55, 51].

In continuation of these studies, we initiated an curative drug administration regimen in mice induced with metastatic breast cancer. GW788388 was given daily (at 3 and 15 mg/kg) mixed with powdered rodent chow. However, due to technical difficulties and the publications of similar studies [18, 56, 57] during the course of these experiments this project was discontinued and our attention was focused to other research areas. Other groups elegantly showed that administration of small kinase inhibitors to ALK5 can inhibit metastasis formation in mice both in a preventive setting [18] and with curative treatment regimens [56, 57].

With genetic interference of the expression of downstream TGF-β signaling mediators, Smad2 or Smad3, we found that Smad2 can act as a negative regulator of breast cancer metastasis. In contrast, downstream TGF-β signaling was dependent on Smad3, which was shown to have a pro-metastatic function in metastatic breast cancer (chapter 2). Current therapeutic strategies aim at targeting TGF-β signaling through receptor inactivation [51]. This leads to a complete halt of downstream Smad2 and Smad3 signaling. Despite efficacious results using these approaches in animal models (reviewed in [51]) our findings (chapter 2) together with those of others [10, 21] suggests that selectively targeting Smad3 may lead to more effective anti-metastatic and anti-fibrotic therapy [10].

HMGA2 was identified as a critical Smad-dependent TGF-β target gene which directly induce EMT and is crucial for TGF-β-mediated EMT [28]. Our preliminary findings, in a breast cancer metastatic model, suggests that HMGA2 may be more important in a primary tumor setting compared to the progression of metastasis at a secondary site (unpublished observations). Thus, targeting HMGA2 in the primary tumor could lead to preservation of the epithelial morphology and hereby inhibit the acquisition of a migratory phenotype which facilitates metastatic progression. Since HMGA2 is lost in normal cells and highly re-expressed in various cancerous tissues it presents as an excellent target for anti-cancer therapy.

The let-7 family members of miRNAi’s are characterized as tumor suppressors and clearly define an epithelial gene signature. Interestingly, the loss of miRNA let-7 and
enhanced expression of HMGA2 were recently suggested as superior prognostic markers with more clinical relevance than the classical markers E-Cadherin, vimentin and Snail1 in cancer [58].

We compared the intra-bone tumor growth of BMP-7 overexpressing breast cancer cells with cells ectopically expressing a continuous active ALK2 receptor (chapter 4). We found that BMP-7 can act as a negative regulator of TGF-β signaling likely by up-regulating the expression of Smad7 and by reversing TGF-β-induced EMT [37]. Thus, BMP-7 inhibits local tumor progression in the bone through tumor cell-autonomous actions. These findings suggests, that systemic or local administration of BMP-7 could be used to treat patients with breast or prostate cancer metastasis (reviewed in [37, 41, 59]).

Due to the pleiotropic nature of TGF-β the application of TGF-β receptor inhibitors or drugs which target this signaling pathway present unique challenges that must be considered in drug development programmes. Targeted drug delivery using liposome-based therapy can specifically deliver a drug to the site of interests. Systemic administration Bisphosphonate-coupled liposome encapsulated ALK5 inhibitors, to patients with bone metastasis, would allow specific delivery of biological relevant doses of compounds to sites of extensive bone remodeling and hereby prevent systemic toxicity and severe immune side-effects.

Also, novel studies examining combinatorial therapies targeting both HIF-1α and TGF-β signaling are currently being explored [60]. Ectopic expression of DN-TβRII and silenced expression of HIF-1α significantly enhanced survival of mice induced with cancer metastasis. Thus, targeting both TGF-β and HIF-1α showed additive inhibitory effects on tumor metastasis [60]. Whether combined therapy of human recombinant BMP-7 and an ALK5 inhibitor synergistically could provide a more potent anti-metastatic therapy would also be of great interest to examine in our osteotropic breast cancer models. In addition, Dr. Theresa Guise and colleagues are further exploring whether Halofuginone can block metastatic progression in mice models. This compound has been described to interfere with TGF-β signaling through down-regulation of TβRII expression and up-regulation of Smad7 [61]. In renal fibrosis, halofuginone was shown to prevent ECM deposition in db/db mice by inhibiting TGF-β signaling [62]. Together, these examples highlight the potential of targeting the TGF-β pathway in the treatment against cancer and fibrosis.

6.3 Perspectives

By understanding the stochastic nature of the metastatic cascade and the molecular mechanisms that facilitate metastasis we have begun to understand not only how metastasis proceeds but also why they occur in the first place [63, 64]. Understanding the role of the tumor-associated stroma and the cells herein on tumorigenesis is one field which holds great promise for future research and novel therapeutic strategies [36]. The pioneering work of Dr. Mina Bissell and many others have resulted in the design of complex 3D multi-cellular models in vitro which allow simulation of in vivo settings.
One model for tumorigenesis suggests that cancer develops through augmented cell-autonomy and rare cellular variants survive a darwinian selection process for enhanced metastatic abilities [65]. Another model proposes that metastatic traits are acquired through exposure of epithelial cancer cells to paracrine signals received from mesenchymal cell types in the tumor-associated stroma (reviewed in [66, 36, 67, 68, 69]). In support of this theory, Karnoub et al. recently showed that MSCs dramatically promote metastasis of breast cancer cells when these are co-injected orthotopically. When tumor cells were isolated and re-injected without MSCs they no longer possessed enhanced metastatic properties compared to controls [68]. Thus, supporting the hypothesis that acquisition of an invasive metastatic morphology is reversible and maintenance of this phenotype depends on continuous contact with stromal cells [68]. Furthermore, this suggests that the metastatic characteristics are expressed transiently in a minority of cells which respond to paracrine signals from the stromal compartment. Locating and identifying these key genes which mediate invasiveness should therefore be done by procedures rather than bulk analysis of the primary tumor [68] and focus should be aimed at analyzing the responsiveness of primary tumor cells to stromal signals and the effect of the cancer cells on the tumor stroma and cells herein [36, 69].

The observations described in this thesis are based on the current models available for studying metastatic bone disease [42, 41, 70]. Few human breast cancer cell lines give rise to bone metastasis and most of our studies are based on an osteotropic subclone of the MDA-MB-231 cell line. A cancer line originally isolated from a pleural effusion of a breast cancer patient suffering from widespread metastasis many years after removal of her primary tumors [65, 71]. That most research is based on this one model is a drawback and it is therefore of high relevance to identify new in vivo models for breast cancer bone metastasis. Furthermore, is it of great importance to correlate our finding in mouse models with observations in human material to produce clinical relevant data.

The discovery of miRNAs and their potential tumor suppressor and tumor promoting properties holds great promises for future drug design strategies [72] along with the use of miRNAs as prognostic biomarkers [73]. Also, the emerging field of tumor-initiating cancer stem cells (CSCs) and the pathways that regulate self-renewal and survival of breast CSCs may give rise to a promising novel cancer therapies. Moreover, the EMT program was recently shown to support the differentiation of mammary epithelial cells with self-renewal properties similar stem cell [74]. The impact of EMT on tumor-initiating CSCs and on miRNAs and how these observations can be translated into therapy will be interesting to follow in the near future.

On the prognostic side, the FDA recently approved an in vitro diagnostic test “the Mamma-print” which can predict the likelihood of distant metastatic recurrence in breast cancer based on a 70 gene signature [75, 76]. The application of expression profiling of primary human cancers is a promising approach to identify mechanisms responsible for tumorigenesis. Well-defined EMT and tumor models can complement the human cancer studies and the synergism between expression profiles could identify promising key target genes and pathways involved in late-stage tumorigenesis. This would support the identification of molecular markers and the development for anti-cancer therapies and
eventually stratify patient group which could benefit from such treatment regimens.
References


6.3 References


6.3 References


