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**Author:** Naber, Hildegonda Petronella Henriëtte

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## **Chapter 8**

### **Summary and discussion**

Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) and Bone Morphogenetic Proteins (BMPs) are involved in a multitude of biological processes. Dysregulation of their signaling pathways is often observed in cancer. TGF- $\beta$  has a dual role in cancer; in early stages it inhibits cell growth and promotes apoptosis, whereas in later stages it promotes invasion and metastasis. . Given its pro-tumorigenic role, many efforts have been made in developing TGF- $\beta$  signaling inhibitors and some of them are currently in clinical trials (reviewed in [1]). However, given its growth inhibitory role, these inhibitors should be used with care. Therefore, these therapies might benefit from the use of additional inhibitors targeting downstream effectors of TGF- $\beta$  that are involved in the pro-tumorigenic action of TGF- $\beta$ .

To study invasion in more detail *in vitro*, a 3 dimensional model was developed, as described in **Chapter 3**. In this model, breast cancer cells, aggregated into spheroids, were allowed to invade into a collagen type I matrix. Collagen I is often found in breast cancer tissue [2] and is present to the so-called dense tissue in mammography, which is a poor prognosis factor for breast cancer [3]. An advantage of using spheroids in the invasion assay is that they have an organization similar to a tumour with an inner hypoxic core and more metabolically active cells at the outside [4]. In this way, cells organize themselves in structures which resemble more their parental tissue. In a model set up by the group of Bissell, mammary epithelial cells form acinar structures. Using that model one can easily distinguish normal mammary cells from malignant breast cancer cells, as malignant cells fail to organize themselves into acinar structures [5]. 3D models also better recapitulate resistance to chemotherapeutic drugs than cells in monolayers [6,7]. Our spheroid model complements these models by focusing on invasion. In our model, TGF- $\beta$  potently induces invasion. Using a series of cell lines representing different stages of cancer development, it was demonstrated that the invasiveness of the cell lines correlated with their malignancy. Thus, the spheroid invasion model allows to study TGF- $\beta$ -induced breast cancer cell invasion.

Further characterization of the TGF- $\beta$ -dependent invasive properties of these cells in **Chapter 4** revealed that invasion of the metastatic cell line was dependent on Smad3

and Smad4. This is in concordance with previous studies *in vivo* which demonstrated the importance of the Smad pathway by overexpression of a Smad binding defective mutant of ALK5 [8] or knockdown of Smad4 [9,10]. Also Smad3 has been shown to be involved in breast cancer metastasis, although this effect was mainly due to effects on angiogenesis [11].

Both Smad3 and 4 were necessary for induction of MMP2 and MMP9. This is in agreement with a previous study using the Smad-binding defective mutant of ALK5 [8]. Since MMPs especially MMP9, are involved in angiogenesis [12], Smad3 may promote both invasion and angiogenesis through the induction of MMPs. On the other hand, Smad2 has been shown to have anti-metastatic effects [11]. The role of Smad2 in our invasion model remains to be established.

Although Smads were shown to be crucial for TGF- $\beta$ -induced invasion, we can not rule out that non-Smad pathways also contribute to invasion. Various studies have shown that RAS and TGF- $\beta$  collaborate in promoting malignant progression of breast cancer [13,14,15]. TGF- $\beta$  is able to activate RAS through phosphorylation of ShcA [16]. Downstream effectors of RAS include the Raf-MEK-ERK, PI3K-PKB and Ral pathways [17]. The PI3K-PKB pathway has been shown to primarily counteract TGF- $\beta$ -induced growth arrest [18], although the PKB- $\beta$  isoform was able to enhance invasion in a mouse model of breast cancer [19]. The Raf-MEK-ERK pathway appears to be the mediator of an invasive phenotype downstream of Ras [18,14]. The role of the small GTPase Ral in the interplay between Ras and TGF- $\beta$  in invasion has not yet been investigated, although Ral has been implicated in migration of breast cancer cells and leukocytes [20,21]. In addition to the above-mentioned pathways, H-RAS is able to induce p38 activity [22]. In MCF10A cells, TGF- $\beta$  and H-RAS cooperate to induce MMP2 expression through activation of p38 and ATF2 [23]. However, it remains to be established if this also occurs in the MCF10A metastatic derivative (M-IV) in our invasion system.

We established a crucial role for MMPs in our invasion model. MMP1, which is able to cleave type I collagen, was dispensable for TGF- $\beta$ -induced invasion, whereas MMP2 and MMP9 proved to be necessary for invasion. Although these latter MMPs are not able to

cleave collagen I, their pro-invasive capacities may depend on cleavage of adhesion molecules, such as E-cadherin [24]. In addition, MMP2 and MMP9 are able to cleave latent growth factors. For example, MMP9 is able to cleave latent TGF- $\beta$ , thus functioning in a feed-forward loop [12]. The individual roles of MMP2 and MMP9 in invasion remains to be established.

MMP2 and MMP9 appear attractive targets for therapeutic intervention of breast cancer invasion. Given the multifunctionality of MMPs, they may also have anti-tumorigenic effects. The latter appears in particular an issue for targeting of MMP9 [25]. Tumors in MMP9 null mice were found to be more aggressive, indicating that MMP9 inhibits certain aspects of tumor progression [26]. On the other hand, knockdown of MMP9 reduced metastasis of TGF- $\beta$ RI-expressing cells [27]. MMP2 appears to be a better target, since overexpression in breast cancer cells augments orthotopic tumor growth and metastasis in mice [28]. In humans, MMP2 is one of the predictors for lung metastasis [29]. The development of specific MMP inhibitors remains a challenge, since the enzymatic pocket of these proteins are highly homologous.

The pro-invasive action of TGF- $\beta$  is thought to be dependent on the ability of TGF- $\beta$  to induce EMT, resulting in a more motile mesenchymal phenotype. Therefore, the role of key regulators in EMT in TGF- $\beta$ -induced invasion, Snail and Slug, was studied in **Chapter 6**. Overexpression of the transcription factors Slug and Snail enhanced invasion, both in our *in vitro* spheroid invasion assay as well as in a *in vivo* zebrafish model, thus linking EMT to invasion. In addition, Slug was necessary for TGF- $\beta$ -induced invasion *in vitro*. A role of Snail in TGF- $\beta$ -induced invasion awaits further clarification.

Furthermore, we observed that either Slug or Snail overexpression enhanced single cell invasion. This type of movement resembles the amoeboid type of movement which occurs in the presence of MMP-inhibitors, without pericellular proteolysis [30,31]. Interestingly, this type of movement has been linked to TGF- $\beta$ -signaling *in vivo* [32]. Amoeboid movement is promoted by Rho-ROCK signaling [30]. Rho GTPases are induced by Snail and Slug [33,34] and the Rho signaling axis is involved TGF- $\beta$ -induced EMT and actin fiber rearrangements [35,36]. Therefore, it is tempting to speculate that the induction of Rho by

Slug or Snail contributes to their effects on migration. These data described in chapter 6 further emphasize the role of these EMT mediators in metastasis.

BMP-7 has been shown to negatively affect TGF- $\beta$ -induced EMT [37] and BMP-7 inhibited bone metastasis of breast cancer cells [38]. Therefore, we investigated the effect of BMP-7 on TGF- $\beta$ -induced invasion in **Chapter 5**. Indeed, BMP-7 inhibited TGF- $\beta$ -induced invasion, whereas BMP-6, its closest homolog, was not able to do so. Although BMP-6 and BMP-7 share 73 % amino acid homology, differences between these two BMPs have been observed previously. For example, BMP-6 is more potent than BMP-7 in inducing bone formation [39]. However, in our cells BMP-6 and BMP-7 activated the BMP pathway to the same extent. If activation of non-Smad signaling pathways is differentially induced by these BMPs is still unresolved.

Interestingly, BMP-7 did not affect the growth inhibitory effect of TGF- $\beta$  in these metastatic cells. Thus BMP-7 only inhibits pro-oncogenic actions of TGF- $\beta$ . Furthermore, BMP-7 only inhibited TGF- $\beta$ -induced invasion in a metastatic cell line, but not in its pre-malignant precursor cell line, further suggesting that BMP-7 only inhibits oncogenic TGF- $\beta$ -signaling. Indeed, treatment with BMP-7 reduced bone metastasis in breast cancer [38], thus reinforcing the rationale that this cytokine might be useful in cancer therapy.

In chapter 5, it is described that BMP-7 inhibited the expression of integrin  $\alpha_v\beta_3$ , a critical factor for TGF- $\beta$ -induced invasion. Overexpression of integrin  $\beta_3$  could rescue TGF- $\beta$ -induced invasion from inhibition by BMP-7, thereby demonstrating the importance of this inhibition. Integrin  $\alpha_v\beta_3$  is able to interact with TGF- $\beta$ RII and to enhance the activity of this receptor and resulting in increased TGF- $\beta$ -induced EMT [40]. Furthermore, this integrin is able to interact with MMP2 and MMP9 to enhance invasion [41,42]. Interestingly, a complex between  $\alpha_v\beta_3$ , MMP2 and Bone Sialic Protein, another TGF- $\beta$  target gene [43], promotes invasion. Given the importance of these MMPs in invasion, it is likely that this action of integrin  $\alpha_v\beta_3$  also contributes to its pro-invasive effect.

Integrin  $\alpha_v\beta_3$  appears to be an attractive target for therapeutic intervention in breast cancer invasion. The integrin complex is highly expressed in bone-metastasized breast cancer cells [44]. Overexpression of integrin  $\alpha_v\beta_3$  promoted bone metastasis of breast cancer

cells [45], whereas inhibition of  $\alpha_v\beta_3$  diminishes osteotropism of breast cancer cells [46]. Also, integrin  $\beta_3$  identified cancer stem cells in mouse models of breast cancer [47]. Therefore, several drugs targeting integrin  $\alpha_v\beta_3$  have been developed. The integrin  $\alpha_v\beta_3/\alpha_v\beta_5$  inhibitor Cilengitide enhanced the tumoricidal effect of radioimmunotherapy in a mouse xenograft model [48], whereas Cilengitide alone decreased progression of bone metastasis [49]. Also CNTO 95, a humanized antibody directed against integrin  $\alpha_v\beta_5$  and  $\alpha_v\beta_3$  reduced the invasion and metastasis capacity of human breast cancer cells in pre-clinical breast cancer models [50].

Although we discovered which genes are involved in the inhibition of TGF- $\beta$  by BMP-7, the signaling pathway involved is not clarified. A recent report demonstrated that BMP-7 could inhibit Smad3 translocation to the nucleus by inducing SnoN in kidney cells [51]. However, in M-IV cells BMP-7 did not affect Smad3 transcriptional activity (Chapter 5). Since induction of integrin  $\beta_3$  by TGF- $\beta$  has been reported to be dependent on p38 and Src in lung fibroblasts [52], BMP-7 could exert its effects through inhibition of non-Smad pathways. Further studies are awaited to shed more light into the mechanisms by which BMP7, but not BMP6, inhibit TGF- $\beta$  induced invasion.

The BMP signaling pathway is far less well characterized than the TGF- $\beta$  pathway. To identify novel kinases involved in the BMP signaling pathway, we performed an overexpression protein kinase screen. In **Chapter 7** we described the identification of both known and novel players in BMP signaling. Further validation revealed RPSKA4 as a novel positive regulator of BMP signaling. RPS6KA4 acts downstream of p38 to phosphorylate CREB [53]. Phosphorylated CREB is known to enhance BMP-Smad signaling [54]. Thus, RPS6KA4 may promote BMP signaling by increasing the amount of phosphorylated CREB. Further study is needed to characterize this mechanism and to show how this action affects the role of BMPs in cancer.

We also identified CDK8 as a negative regulator of BMP signaling. Recently, it was shown that CDK8 phosphorylated Smad1 at residues in the linker region. This creates docking sites for Smurf1, resulting in degradation of Smad1. However, also the transcriptional co-activator YAP is able to bind to these phosphorylated residues, resulting in enhanced

signaling [55]. Given the negative effect of CDK8 on BMP signaling, Smurf1 probably out-competes the YAP present in the cells.

In this thesis, a novel model was described to study TGF- $\beta$ -induced invasion of breast cancer cells *in vitro*. Key players in this process were identified, including MMP2, integrin  $\alpha_v\beta_3$ , Slug and ZEB, which hold therapeutic promise. The inhibitory effect of BMP-7 on TGF- $\beta$ -induced invasion also provides a rationale for using this cytokine in breast cancer therapy. Further studies are needed to confirm these findings.

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