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The bone morphogenetic protein pathway in colorectal cancer progression

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Chapter 1

General introduction

Cancer cell progression is the result of multiple genetic and epigenetic changes that give cells a survival advantage over their neighbouring cells. The cumulative acquirement of alterations can eventually select highly proliferative cells that are prone to metastasize and are chemo resistant. Morphogenetic pathways play an important role in the regulation of tissue homeostasis by controlling processes like differentiation, proliferation and apoptosis and are often affected by the genetic and epigenetic changes occurring in cancer cells.[1]

Morphogens act by forming a concentration gradient through tissue thereby creating different levels based on the distance from its source.[2] Morphogenetic pathways of the target cells are activated based on concentration thresholds. This allows complex morphological organization at a tissue level controlling the phenotype of individual cells within that tissue.

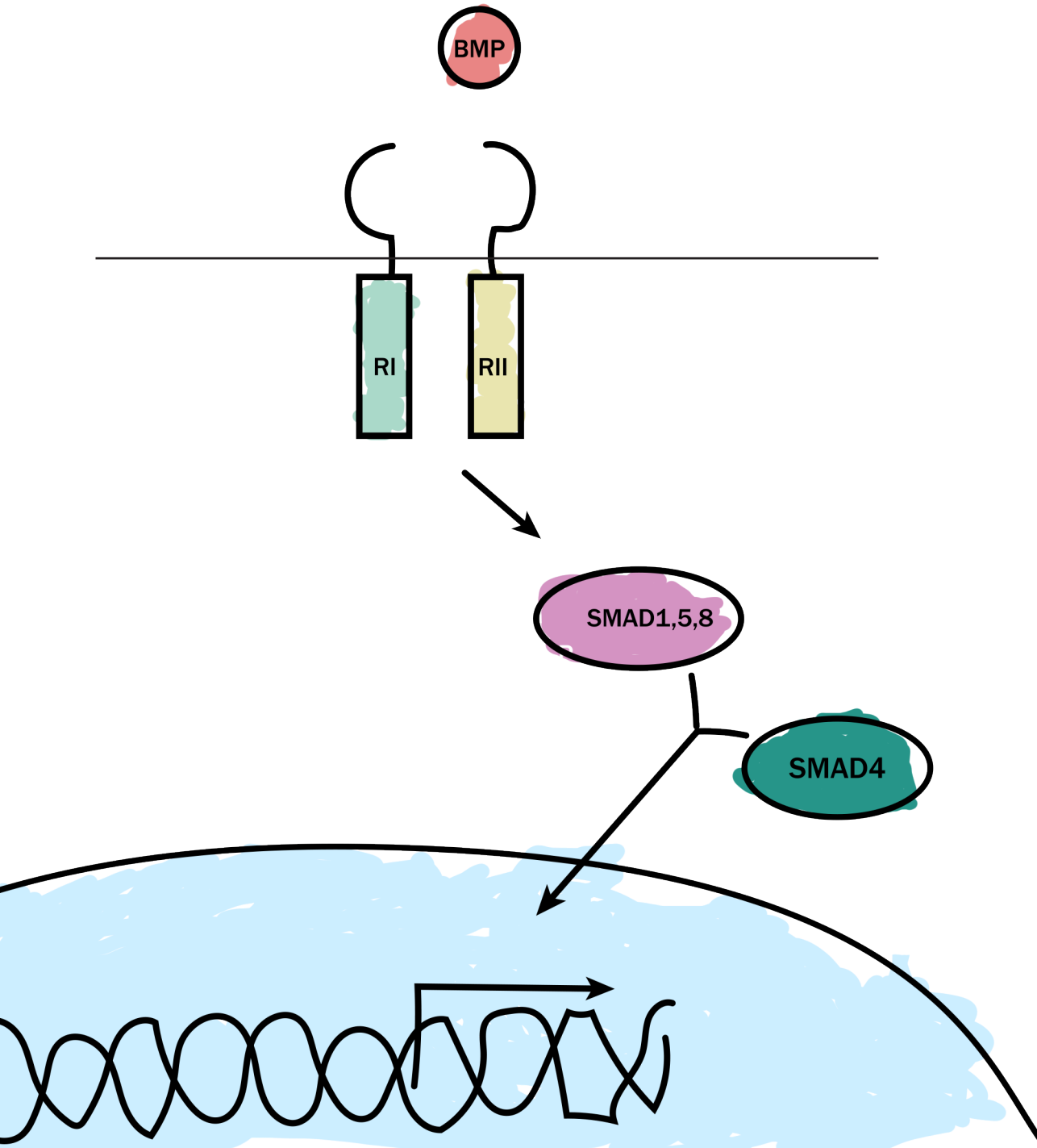
One important morphogenetic pathway is the Bone Morphogenetic Protein (BMP) signalling pathway, which was originally discovered in bone because of its ability to induce the formation of bone and cartilage.[3] It is now known that the BMP pathway is a key morphogenetic pathway involved in tissue organization throughout the body.[4]

In this thesis, we deepen our knowledge of the tumour suppressor effects of BMP, mainly in colorectal cancer (CRC), but also explore its previously unknown tumour promoting capabilities. These new findings shed a completely different light on the role of BMP signalling in CRC development and could have clinical implications. They create new possibilities to use the BMP signalling pathway in prediction of disease development and also question the use of BMPs in cancer treatment as they can have deleterious effects by not only inhibiting but also enhancing tumour growth.

BMP signalling cascade

BMPs are part of the transforming growth factor β (TGFβ) superfamily of morphogenetic proteins. Ligands bind to a complex of transmembrane serine threonine kinase receptors type 1 and 2, resulting in phosphorylation and activation of the BMP receptor type 2 (BMPR2). The activated BMPR2 activates BMP receptor type 1 (BMPR1), which phosphorylates receptor-associated SMAD1,5 or 8 that subsequently complexes with SMAD4 and translocates to the nucleus to regulate gene transcription.[5] TGFβ signals in a similar manner but

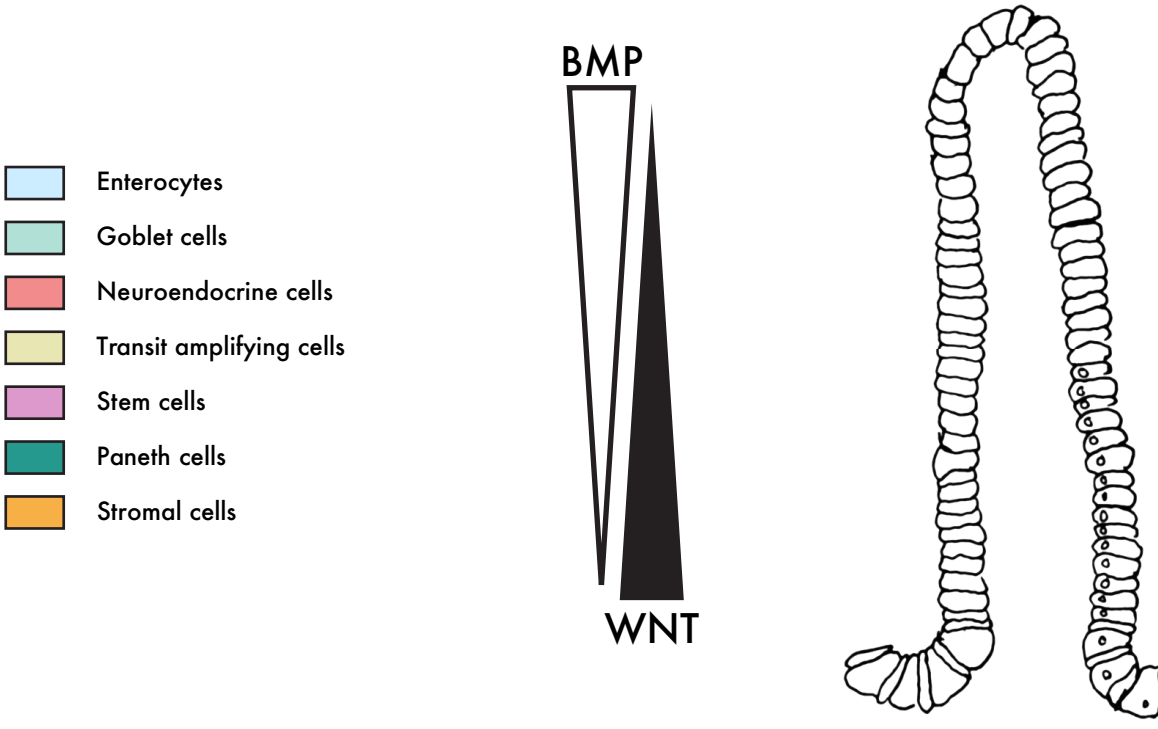
through distinct receptors and receptor associated SMADs, but both TGFβ and BMP signal through the common mediator SMAD4 to transduce a signal to the nucleus. This is the canonical SMAD dependent signalling route, but TGFβ and BMP can also signal independently from SMADs, a matter that will be discussed further on this introduction.



Role of BMP signalling in maintaining intestinal homeostasis

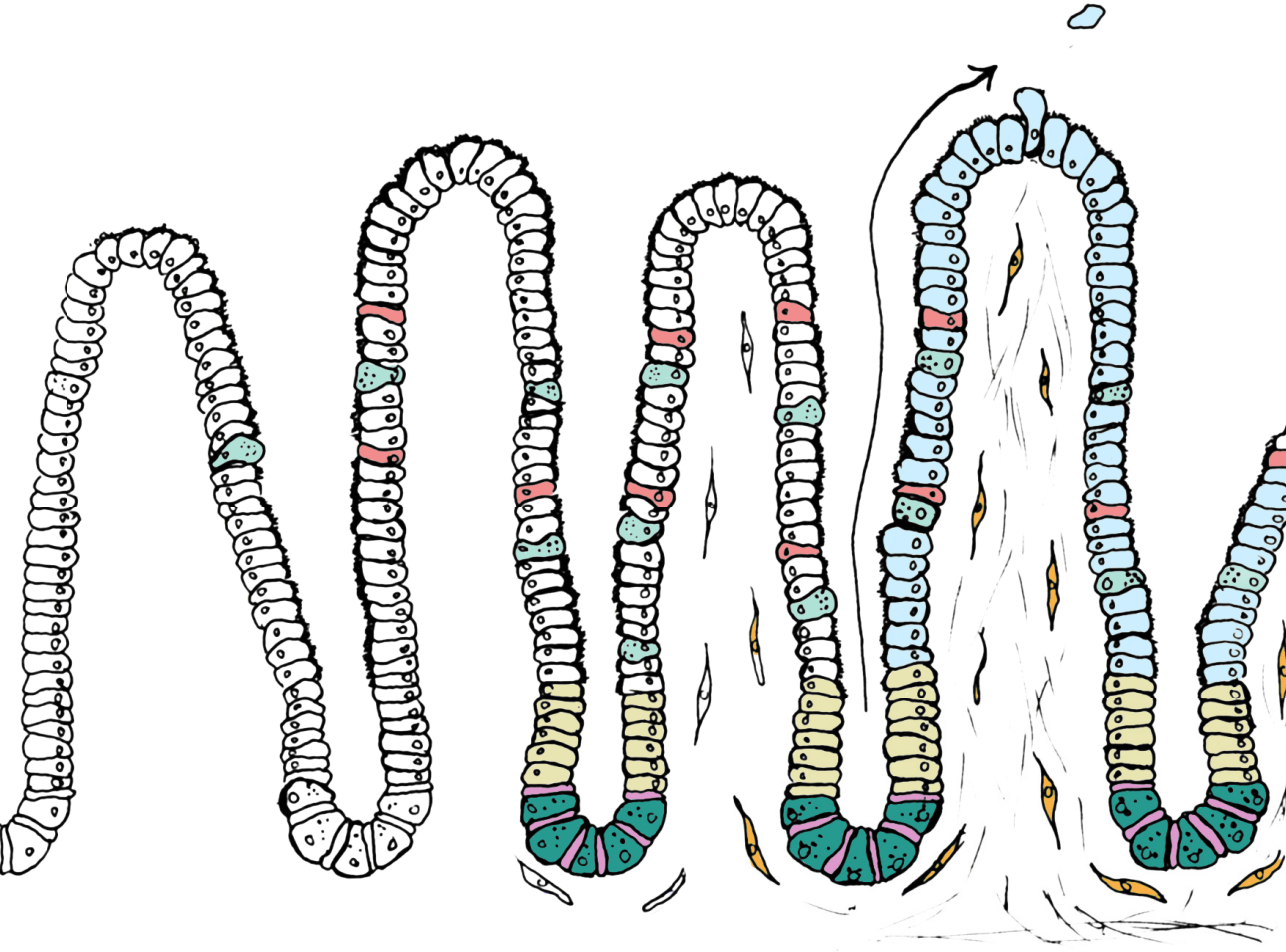
A massive surface area is squeezed into a small tube that we call intestine. This results in a sort of wrinkled epithelial cell monolayer. In the small intestine there are crypts and villi, while the colon only consists of crypts. Stem cells reside at the bottom of crypts giving rise to cells in the transit amplifying zone. Transit amplifying cells are highly proliferative cells that go through a couple of cell cycles while being pushed upwards. These cells differentiate and continue to move towards the villus tip, or to the top of the crypt for colonic cells. Eventually they are shed into the intestinal lumen.

BMP signalling is mostly active at the upper most part of the crypt-villus axis inducing differentiation and apoptosis. Wnt signalling, another morphogenetic pathway, is active in the stem cell compartment, inducing stemness and proliferation, and counteracts BMP signalling. A proper balance between Wnt and BMP signalling pathways along the crypt-villus axis is necessary for maintaining intestinal homeostasis. In **chapter 2** we show the consequences of BMP signalling activation in the stem cell compartment.



Significance of BMP signalling in colorectal cancer

The importance of morphogenetic signalling pathways in the development of cancers was recognized through the identification of Germline mutations in hereditary cancer syndromes. In the case of the BMP pathway SMAD4 and BMPR1a germline mutations were first found to be associated with Juvenile Polyposis (JP) accounting for approximately 50% of the JP cases.[6, 7] Later it was found that BMP signalling also plays a major role in sporadic CRC. 40-60% of the sporadic CRCs have lost protein expression of SMAD4, a late-stage event that is associated with the development of metastases[8], chemo resistance[9, 10] and a poor patient prognosis[11]. Methylation of the promoter region resulting in transcriptional silencing of *BMP2*, a tumour suppressor gene, occurs in a subgroup CRCs with the CpG island methylator phenotype (CIMP).[12] The expression of *BMPR2* can be impaired in Microsatellite Instable (MSI) cancer through mutations in the long polyadenine tract of the 3'UTR.[13] More recently, Genomic Wide Association Studies have identified genetic variants of BMP signalling components independently predisposing CRC. The CRC susceptibility single



nucleotide polymorphisms (SNPS) were found that are close to BMP pathway loci *GREM1*, *BMP2* and *BMP4*.[14, 15] All these findings underline the significance of a proper functioning BMP signalling pathway in the intestinal homeostasis.

Non canonical BMP signalling

The accepted view of the BMP and TGF signalling pathways is that of tumour suppressors and barriers to tumour progression and metastasis[16]. The consequences of loss of SMAD4 were therefore initially ascribed to the loss of BMP and TGFβ signalling. In many cancers TGFβ switches from being a tumour suppressor to become a tumour promoter, driving invasion and metastasis[5]. A possible explanation for the switch could be loss of SMAD4, thereby activating non canonical TGFβ signalling[8] [9]. These studies do not take into account the effects of BMP signalling in the absence of SMAD4.

In **chapter 3** we describe that the BMP pathway can switch from being a tumour suppressor to become a tumour promoter, driving invasion and metastasis**.**

BMP and WNT; a happy couple or arch enemies?

Earlier it was mentioned that Wnt signalling induces stemness, drives proliferation and counteracts BMP signalling. Wnt signalling activity can be inferred by the nuclear presence of β-catenin. When Wnt signalling is not active, β-catenin is phosphorylated by Glycogen Synthase Kinase-3b (GSK-3b) in the APC (Adenomatous Polyposis Coli)/AXIN/GSK-3b-complex. Afterwards phosphorylated β-catenin is targeted for ubiquitin mediated proteosomal degradation. Upon Wnt signalling activation, this process is prevented which results in high levels of β-catenin. β-catenin then translocates to the nucleus which results in transcription of genes favouring cell growth and proliferation. Mutations in *APC* or β-catenin (*CTNNB1) also prevent* β-catenin degradation. *APC* mutations occur in 70% of CRC and *CTNNB1 mutations in 15% of the cases. Interestingly, APC/CTNNB1* mutations are identical throughout a clonal tumour, but immunohistochemical analysis reveal a different, heterogenous expression pattern. Nuclear β-catenin tends to be high at something that we call the ‘ invasive front’ and low in the centre of the tumour. The invasive front is the part of tumour that is in contact with the surrounding tissue, which mostly consists of stroma. This indicates that the ‘constitutively active’ Wnt signalling caused

by *APC/CTNNB1* mutations is actually still modulated by tumour cell intrinsic and/or extrinsic factors (Fodde and Brabletz, 2007)[17]. This effect is known as the β-catenin paradox. We don't know what causes this phenomenon, but it is probably the result of interaction between tumour cell intrinsic and extrinsic factors. BMP and Wnt signalling are known to interact in normal intestinal cell homeostasis and both pathways are important in cancer cell progression, whether it is through activation or inhibition. **Chapter 4** attempts to shed some light on the BMP-Wnt interaction at a cellular level and at the invasive front, also taking into consideration the BMP non-canonical pathway.

Stroma and the invasive front

Cancer cells at the invasive front are in contact with stromal cells. Tumour stroma consists of fibroblasts, inflammatory cells and endothelial cells. It has become clear that the stroma plays an important role in the progression of cancer cells. Tumour tissue produces growth factors, which can activate surrounding fibroblasts, inflammatory cells and endothelial cells. In turn, stromal cells produce proteases, growth factors and extracellular matrix components that can promote angiogenesis and malignant tumour growth.[18, 19] Just the amount of stromal cells surrounding a tumour negatively affects patient survival. This is especially true in SMAD4 negative colon cancers.[20] In **chapter 5** we hypothesized that stroma might act on SMAD4 negative cancer cells through activation of non-canonical BMP signalling.

Prognostic/predictive markers

Although colorectal cancer patient survival has improved significantly over the last decade it remains one of the leading causes of cancer-related death in the western world. Headway has been made regarding early stage detection with many countries currently implementing CRC endoscopy screening programs. Another frontier in cancer research and opportunity for further improving patient prognosis is personalized prognosis estimation and personalized treatment based on molecular profiling of individual tumours. Estimation of the prognosis is currently almost entirely dependent on histopathological staging mostly using the TNM classification (originally devised between 1943 and 1952) or a system based on the Dukes staging system (1932)[21].

Several protein and genetic markers have been suggested in an attempt to optimize prognosis predictions and treatment response. So far, despite promising results, none are standardized in colorectal cancer evaluation.[22] Among the many suggested molecular markers, BMP signalling components are well represented. Most promising is loss of SMAD4 protein expression measured using immunohistochemistry showing an association with a poor prognosis, the development of metastases and a poorer response to 5-FU treatment. Of all the molecular markers investigated in CRC SMAD4 is the only one consistently showing a relation with patient survival. SMAD4 is located on chromosome 18q which is deleted in up to 70% of the colorectal cancers dependent on the detection method, an event that was already connected to tumour aggressiveness in 1994.[23] Originally it was thought that the poor prognosis associated with deletion of the long arm of chromosome 18 was the result of the loss of a gene called deleted in colorectal carcinoma (DCC), but studies investigating the independent prognostic value of DCC loss did not demonstrate a clear link with prognosis.[24] It has now become clear that the poor prognosis associated with 18q loss is the result of SMAD4 loss.

In **chapter 6** we review the prognostic value of SMAD4 using a meta-analysis. **Chapter 7** describes our attempt to use pSMADs as prognostic markers in CRC.

BMP signalling in pancreatic cancer

SMAD4 mutations occur in pancreatic cancer, just as they do in CRC. More than 50% of pancreatic cancers have a SMAD4 mutation and, just as in CRC, this is associated with a poorer prognosis.[25-27] SMAD4 restoration in SMAD4 depleted pancreatic cancer cell lines leads to a reduction of growth.[28]

As stated before, SMAD4 is the central component of both the BMP and TGF-β pathways. There is already some evidence for the importance of TGF-β signalling in pancreatic cancer, but the role of BMP signalling is much less clear. A small percentage (4-7%) of pancreatic cancers have TGF-β receptor II mutations and TGF-β signalling can be tumour suppressive in normal epithelial cells and tumour promoting in the later stages of cancer with different functional effects dependent on the SMAD4 status.[29]

In **chapter 8** we investigate the effects of BMP signalling on pancreatic cancer cell lines and relate the expression of BMP signalling components to patient survival.

Statins as a way to alter BMP signalling and treat colorectal cancer patients

Recombinant human BMPs are used as a treatment in orthopaedic and oral surgery to promote bone formation. Using BMPs in cancer treatment could be problematic because of the dichotomous effects we have observed *in vitro* and *in vivo*. There are no trials that investigate the effects of BMP on cancer patient survival and there are not enough users of BMPs to perform a retrospective study. Statins, however, are widely used for cardiovascular risk management and have the ability to activate BMP signalling. *In vitro* and *in vivo* studies indicate that statins inhibit proliferation and induce apoptosis in colorectal cancer cells[30, 31] Interestingly, in these in vitro and in vivo studies, statins are only effective in colorectal cancer cells with intact BMP signalling pathways[31]. In **chapter 9** we evaluated whether statins are effective as adjuvant therapy in colon cancer and related this to BMP signalling pathway functionality (canonical vs non-canonical).

To conclude, this thesis is an attempt to increase our knowledge of the mechanisms involved in carcinogenesis, metastases formation and tumour-stroma interaction; with a particular focus on the BMP signalling pathway.

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