The bone morphogenetic protein pathway in colorectal cancer progression

Voorneveld, P.W.

Chapter 10

Conclusion, Discussion & Future Perspectives

Introduction

We set out the get a better understanding of the role of Bone Morphogenetic Protein (BMP) signalling in normal intestine and in carcinogenesis. The BMP pathway is known to be a major player in the development of colorectal cancer (CRC). CRC is one of the leading causes of cancer-related deaths in the western world. Although survival and recurrence of CRC have improved, 5-year survival is low at only 65% (https://seer.cancer.gov – US data). Improving our understanding of the molecular pathways involved in CRC will potentially allow earlier detection, better prediction and personalized therapy. To briefly summarise the research we have done, we started by investigating the function of BMP in the normal intestine. We then went on to study the role of BMP signalling in carcinogenesis, mainly the role of non canonical BMP signalling in the development of metastasis. We ended with a focus on patients by explaining how we can improve estimation of prognosis using expression levels of several BMP components and how targeting the BMP pathway can be used for personalized treatment of patients.

The basics: BMP pathway is important in maintaining intestinal homeostasis

In normal intestinal epithelium Bone Morphogenetic Protein signalling is active in a decreasing gradient from top to bottom along the vertical crypt-villus axis. BMP signalling counteracts Wnt signalling which has an expression pattern exactly opposite that of BMP.[1] Stem cells reside at the bottom of the crypts where Wnt signalling is active, responsible for maintaining stemness. Stem cells give rise to transit amplifying cells that are highly proliferative under the influence of Wnt. These cells move upward and differentiate as a result of BMP signalling activation. Eventually the cells will undergo apoptosis, also an effect driven by BMP, and are shed into the intestinal lumen.

The importance of an intact BMP signalling pathway in the intestine was first recognized when germline mutations in key BMP signalling components (SMAD4 and BMPR1A) were found in Juvenile Polyposis and Hereditary Mixed Polyposis.[2] See **chapter 1** for more information on the BMP signalling cascade and its components. In two hallmark studies, mouse models in which the BMP signalling pathway was inactivated by deleting BMPR1A or by overproduction of the BMP inhibitor Noggin, resulted in the development of polyps.[3,4] Both methods resulted in inactivation of BMP signalling mimicking the germline mutations found in hereditary colorectal cancer syndromes. In **chapter 2** we achieved the opposite by using an inducible mouse model in which BMP signalling could be constitutively activated in both the crypt and villi, but not Paneth cells. Our goal was to further understand the role of BMP signalling in the intestinal epithelium. Overactivation of BMP signalling in both stem cells and differentiated cells led to downregulation of Wnt signalling in the stem cell compartment together with a major disruption of the crypt-villi architecture. Interestingly, no trace of this disruption was seen after 6 months. We do not fully understand the mechanism behind this phenomenon. Immediately after induction, leading to overactivation of BMP, loss of the stem cell marker OLFM4 was seen. Five days after induction, OLFM4 suddenly reappears. We have several theories to explain the repopulation, but nothing conclusive. Recombination after induction is not established in a 100% of the cells. In our model the efficiency was very high, so we think it is unlikely that a relative small portion of the stem cells would be able to repopulate the entire intestine in such a short time period. A more likely explanation is that

some cells dedifferentiate or that Paneth cells behave as quiescent stem cells, but this needs to be further investigated.

We are currently investigating if this model of BMP activation could be used to impede cancer formation by combining this mouse model with carcinogenesis models. The drastic morphological changes and remarkable recovery of the epithelium resembled a sort of 'reset' that could be useful in intervening in cancer development.

Different paths: Non canonical SMAD4 independent BMP signalling

In the previous paragraph we describe the importance of intact BMP signaling on the crypt-villi architecture and that disruption of the BMP cascade could result in carcinogenesis. The most common disruption of the BMP pathway is loss of SMAD4, which occurs in in 40-60% of sporadic CRCs and is associated with a poor patient survival.[5] The consequences of loss of SMAD4 were initially ascribed to the loss of canonical SMAD4 dependent BMP and TGFβ signalling. It is known that BMP signalling in the presence of SMAD4 acts as a tumour suppressor.[6] However, TGFβ can switch from being a tumor suppressor to become a tumor promoter, driving invasion and metastasis.[7] In **chapter 3** we show that the BMP signalling pathway changes into a pro-metastatic pathway upon loss of SMAD4. In the absence of SMAD4, BMP stimulation induces epithelial-to-mesenchymal transition and enhancing invasion and metastasis. We analyzed non canonical SMAD4 independent BMP signalling using a kinome array and found that it activates a complex network of kinases; p38MAPK, ERK, JNK, PI3K, AKT, GSK and Rho/ROCK/LIMK. These are targets of pathways involved in inflammation, mitosis, cell survival, proliferation, stemness and cytoskeleton rearrangement. Using a panel of inhibitors, we observed that inhibiting the Rho/ROCK pathway resulted in reduction of the pro-metastic effects of non-canonical BMP signalling. At the end of this chapter we will discuss the ROCK pathway as a possible target of treatment. We will now describe our effort to relate BMP activity to another signalling pathway that is important in crypt-villi homestasis and in carcinogenesis, namely Wnt signalling.

Battle at the front: the ß-catenin paradox

In **chapter 1** we mentioned the beta-catenin paradox. This term describes the occurrence of active Wnt signalling at the invasive front and low Wnt signalling activity in the center of the tumour despite a similar mutational status of APC and CTNNB1 (beta-catenin).[8] Apparently, Wnt signalling activity is regulated differently throughout the tumour. At the invasive front specific cell type features attributed to Wnt signalling, like proliferation and migration, are beneficial for growth and infiltration of the surrounding tissue. BMP signalling is known to interact with Wnt.[9] We hypothesized that BMP signalling is involved in the regulation of Wnt signalling activity at the invasive front.

In **chapter 4** we observed a relation between SMAD4 loss, p53 expression and Wnt signalling activity in human colon cancer tissue specimens at the invasive front. SMAD4 loss and/or aberrant p53 expression is associated with high beta-catenin expression. P53 was also chosen in this screening because it has been published that p53 mutations can increase Wnt signalling. In subsequent *in vitro* experiments we found that BMP signalling can only inhibit Wnt signalling when p53 Wild-type and SMAD4 expression is present. Interestingly, in the absence of SMAD4, BMP signalling results in Wnt signalling activation. We can combine this with the results in **chapter 3** describing that non canonical SMAD4 independent BMP signalling drives proliferation, invasion and eventually metastases and conclude that non canonical BMP signalling could in part explain the high Wnt signalling activity at the invasive front. Our next question was what role the stromal cells (the cells surrounding the tumour) play and how they contribute to cancer progression and invasion?

Stroma: not so innocent?

In **chapter 5** we hypothesized that the stroma could be a source of BMP ligands which activate the non canonical BMP signalling pathway at the invasive front. This hypothesis was fueled by publications that show that large amounts of stroma surrounding a tumour negatively influences patient survival and that this is especially true in SMAD4 negative cancers. [10-12]

Stroma consists of inflammatory cells, endothelial cells, extracellular matrix, but mostly of fibroblasts. For this reason, we focused on the effects of fibroblasts on SMAD4 negative cancers, well aware of the fact that other cell types could be of importance as well. Future studies will be an opportunity to take these cell types into account.

Using *in vitro* invasion assays we found that SMAD4 negative cancer cells migrate faster towards fibroblasts than SMAD4 positive cancer cells. From this we concluded that fibroblasts produce something that specifically influences SMAD4 negative cells. We found that when in contact with medium from SMAD4 negative cancer cells (which contain unknown excreted products of the cancer cells), fibroblasts express more BMP2. BMP2 is a BMP ligand capable of activating the BMP signalling pathway. In **chapter 3** we describe that BMP activation in SMAD4 negative cancer cells results in an increase in migration, invasion and metastasis. Our theory is that, when in contact with SMAD4 negative cancer cells, fibroblasts in the stroma start to producing more BMP2, which then activates the non-canonical BMP pathway of the cancer cells, driving invasion and eventually metastases.

Crystal ball: The use of BMP signalling components as prognostic markers

A different part of our research focused on finding prognostic markers. Estimation of prognosis plays an important role in decisions about treatment, and this is currently almost entirely dependent on histopathological staging. In the search for simple molecular markers KRAS and p53 mutations and loss SMAD4 have been the most intensively investigated as possible prognostic factors. Several studies have reported the prognostic value of SMAD4 expression using immunohistochemistry, but others did not conclude this.[13-15] To establish the prognostic value of SMAD4 expression we conducted a meta-analyses described in **chapter 6**. In this study we conclude that immunohistochemical analysis of SMAD4 expression is a useful prognostic marker in colorectal cancer, but that International standardization of SMAD4 scoring methodology is required for reproducibility and comparison.

As the selection of suitable markers is currently limited we attempted to add

phospho-SMADs to the arsenal. In **chapter 7** we present a study where we evaluated the prognostic value of nuclear expression of pSMAD1,5,8 and pSMAD2,3. Nuclear expression of these molecules can be used to monitor the activity of the BMP and the TGF-β pathways respectively. Reduced phospho-SMAD expression (both molecules combined and separate) was not associated with a worse survival making them unfit as prognostic markers.
Chapter 3 shows that the presence of BMP Receptors in SMAD4 negative colorectal cancers is associated with a worse survival. In these cancers non canonical BMP signalling is most likely active further negatively influencing patient survival. Those with low BMP receptor expression have a prognosis similar to patients with SMAD4 positive CRC. In the future SMAD4 could be refined as a prognostic marker by analysis of BMP receptor expression. However, these results are preliminary because of the low number of patients included and the absence of adjustment for confounders. In **chapter 5** we added stromal expression of BMP2 to SMAD4 expression of cancer cells. We observed that high expression levels of BMP2 in the stroma is associated with a poorer prognosis in SMAD4 negative CRCs. This further supports our theory that fibroblasts produce BMP2 which then activate non-canonical BMP signalling in SMAD4 negative cancers resulting in invasion and metastasis, which negatively impacts patient survival. It also shows that markers expressed in the stroma can help predicting patients' outcome. In conclusion, if used appropriately, BMP signalling component expression can be an asset in predicting patient survival.

A little sidestep: Pancreatic cancer

Pancreatic cancer has a 5-year survival of only 4%. At the time of diagnosis, tumour cells have usually invaded surrounding organs or metastasized, resulting in 80–85% being inoperable. Also, a poor response to chemotherapy is seen.[16]

Mutations in SMAD4 leading to inactivation occur relatively late in the adenoma-carcinoma sequence and are observed in more than 50% of pancreatic cancers.[17, 18] Inactivation of SMAD4 is associated with a poorer prognosis indicating the importance of intact BMP signalling in the pancreas[19-21], but not much was known about the other components of the BMP signalling pathway and about the BMP

functionality. In **chapter 8** we reveal that in 53.7% of the cases BMPR1A expression is reduced, and that this is associated with a poorer survival (*p=0.008*). This is only true in SMAD4 positive cancers. Subsequent reduction of BMPR1A expression in SMAD4-negative cancer does not reduce the canonical BMP signalling activity further and probably has little additional impact. By manipulating BMPR1A expression in vitro, we have also shown that BMPR1A has tumour-suppressive effects, which are dependent on the SMAD4 status. The next step would be investigating BMP as a possible treatment target in pancreatic cancer.

BMP as targeted treatment: aiming for the impossible?

As described in the earlier paragraphs of this chapter, the BMP pathway is considered to be tumour suppressive. A tumour suppressive pathway makes an interesting treatment target. Theoretically, such a pathway can be (over)activated to reduce tumour progression, especially if the pathway activity is reduced. BMP signalling can be activated using BMP ligands. There is a whole selection of ligands either enhancing or inhibiting BMP signalling. Recombinant human BMPs are already used in orthopaedic and oral surgery to improve bone formation.[22, 23] After initial praise and FDA approval sparked by the perception of complete safety, more and more side-effect have been reported now that the use of BMPs has been widely implemented. Among the side effects reported are swelling of the injection site, inflammation, osteolysis, bladder retention and wound healing problems.[24] The fact that these side effects are reported after using a single injection must also be taken into account. It cannot be predicted what long systemic exposure might invoke.

In **chapter 3** we presented that BMP activation in the absence of SMAD4 results in the activation of the non-canonical arm of the BMP pathway, inducing migration, invasion and metastases formation. The results in **chapter 5** show us that this already occurs in patients through the production of BMP2 in stromal cells. Activating BMP in cancers in which 100% of the cancer cells are SMAD4 positive might have a positive effect, but in SMAD4 negative cancer cells it is likely to have deleterious effects. This dichotomy makes it difficult to implement BMPs in cancer

treatment. One could argue that BMP needs to be activated in SMAD4 positive cancers and inhibited in SMAD4 negative cancers, but how can you be certain that all cancer cells have an equal level SMAD4 expression. Another option is ROCK inhibition. Non-canonical BMP signalling results in the activation of the Rho/ROCK pathway. ROCK is involved in cytoskeletal rearrangement and cell motility and could be a feasible target for SMAD4 negative cancers and at least not have any tumour progressive effects on SMAD4 positive cancer cells. Fasudil, a ROCK inhibitor, has already been safely used in patients with pulmonary hypertension and cerebral vasospasm.[25, 26] However, further safety assessment is imperative considering the fact that ROCK inhibition in mouse embryos resulted in developmental defects.[27] We found that in a mouse model in which we injected SMAD4 negative cancer cells in the spleen, treatment with a ROCK inhibitor resulted in less metastases formation.

Statins: an old drug with new tricks?

For now, we have moved away from direct activation or inhibition of the BMP pathway, and instead focussed on statins. In our previous studies, we conducted *in vitro* and *in vivo* experiments and found that statins activate BMP signalling and inhibit proliferation and induce apoptosis in SMAD4 positive colon cancer cells. Statins are widely used for cardiovascular risk management and the side effects are well known. A large number of studies have been performed relating statin use to a lower incidence in colon cancer; in other words, using statin as chemoprevention.[28] Very few studies investigated the use of statins as cancer therapy. Due to mixed outcomes of these studies there is no consensus whether or not statins actually work against colon cancer.

In **chapter 9** we present a large observational study that shows that statin use initiated or continued after colon cancer diagnosis is associated with a significantly reduced risk of death. Looking back at our initial *in vitro* data, which shows that statin inhibit tumour growth through BMP activation, it could be argued that statins would only have an effect in SMAD4 positive cancers. We found that in our cohort the survival benefit of statin use was stronger in SMAD4 positive cancers

compared to SMAD4 negative cancers. In the future, BMP signalling functionality may serve as a predictive biomarker to select patients for adjuvant statin therapy. However, randomized clinical trials are needed to confirm the effects of statins on colon cancer survival and BMP signalling as a predictive biomarker.

To conclude, with this thesis we hope to have increased understanding of the involvement of BMP signalling in carcinogenesis, more specifically metastasis formation and the tumour-stroma interaction. Furthermore, we have attempted to use BMP signalling components as prognostic makers and to use BMPs as a targeted treatment in colon cancer. We are currently still working on these subjects which will hopefully generate more results in the future.

References

1. Hardwick JC, Kodach LL, Offerhaus GJ, et al. Bone morphogenetic protein signalling in colorectal cancer. Nat Rev Cancer 2008;8:806-12.

2. Howe JR, Bair JL, Sayed MG, et al. Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. Nat Genet 2001;28:184-7.

3. Haramis AP, Begthel H, van den Born M, et al. De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. Science 2004;303:1684-6.

4. He XC, Zhang J, Tong WG, et al. BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. Nat Genet 2004;36:1117-21.

5. Alazzouzi H, Alhopuro P, Salovaara R, et al. SMAD4 as a prognostic marker in colorectal cancer. Clin Cancer Res 2005;11:2606-11.

6. Hardwick JC, Van Den Brink GR, Bleuming SA, et al. Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon. Gastroenterology 2004;126:111-21.

7. Massague J. TGF-beta signal transduction. Annu Rev Biochem 1998;67:753-91.

8. Fodde R, Brabletz T. Wnt/beta-catenin signaling in cancer stemness and malignant behavior. Curr Opin Cell Biol 2007;19:150-8.

9. Radtke F, Clevers H. Self-renewal and cancer of the gut: two sides of a coin. Science 2005;307:1904-9.

10. Mesker WE, Junggeburt JM, Szuhai K, et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol 2007;29:387-98.

11. Mesker WE, Liefers GJ, Junggeburt JM, et al. Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I-II colon cancer patients. Cell Oncol 2009;31:169-78.

12. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 2004;4:839-49.

13. Carethers JM, Hawn MT, Greenson JK, et al. Prognostic significance of allelic lost at chromosome 18q21 for stage II colorectal cancer. Gastroenterology 1998;114:1188-95.

14. Jen J, Kim H, Piantadosi S, et al. Allelic loss of chromosome 18q and prognosis in colorectal cancer. N Engl J Med 1994;331:213-21.

15. Walther A, Johnstone E, Swanton C, et al. Genetic prognostic and predictive markers in colorectal cancer. Nat Rev Cancer 2009;9:489-99.

16. Vincent A, Herman J, Schulick R, et al. Pancreatic cancer. Lancet 2011;378:607-20.

17. Hahn SA, Schutte M, Hoque AT, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 1996;271:350-3.

18. Rozenblum E, Schutte M, Goggins M, et al. Tumor-suppressive pathways in pancreatic carcinoma. Cancer Res 1997;57:1731-4.

19. Tascilar M, Offerhaus GJ, Altink R, et al. Immunohistochemical labeling for the Dpc4 gene

product is a specific marker for adenocarcinoma in biopsy specimens of the pancreas and bile duct. Am J Clin Pathol 2001;116:831-7.

20. Tascilar M, Skinner HG, Rosty C, et al. The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma. Clin Cancer Res 2001;7:4115-21.

21. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. Cancer Res 2000;60:2002-6.

22. Campana V, Milano G, Pagano E, et al. Bone substitutes in orthopaedic surgery: from basic science to clinical practice. J Mater Sci Mater Med 2014;25:2445-61.

23. Wikesjo UM, Sorensen RG, Wozney JM. Augmentation of alveolar bone and dental implant osseointegration: clinical implications of studies with rhBMP-2. J Bone Joint Surg Am 2001;83-A Suppl 1:S136-45.

24. James AW, LaChaud G, Shen J, et al. A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2. Tissue Eng Part B Rev 2016;22:284-97.

25. Fujita H, Fukumoto Y, Saji K, et al. Acute vasodilator effects of inhaled fasudil, a specific Rho-kinase inhibitor, in patients with pulmonary arterial hypertension. Heart Vessels 2010;25:144-9.

26. Shibuya M, Suzuki Y, Sugita K, et al. Effect of AT877 on cerebral vasospasm after aneurysmal subarachnoid hemorrhage. Results of a prospective placebo-controlled double-blind trial. J Neurosurg 1992;76:571-7.

27. Wei L, Roberts W, Wang L, et al. Rho kinases play an obligatory role in vertebrate embryonic organogenesis. Development 2001;128:2953-62.

28. Poynter JN, Gruber SB, Higgins PD, et al. Statins and the risk of colorectal cancer. N Engl J Med 2005;352:2184-92.