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The bone morphogenetic protein pathway in colorectal cancer progression

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The Bone Morphogenetic Protein pathway in Colorectal Cancer progression

Philip W. Voorneveld

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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.

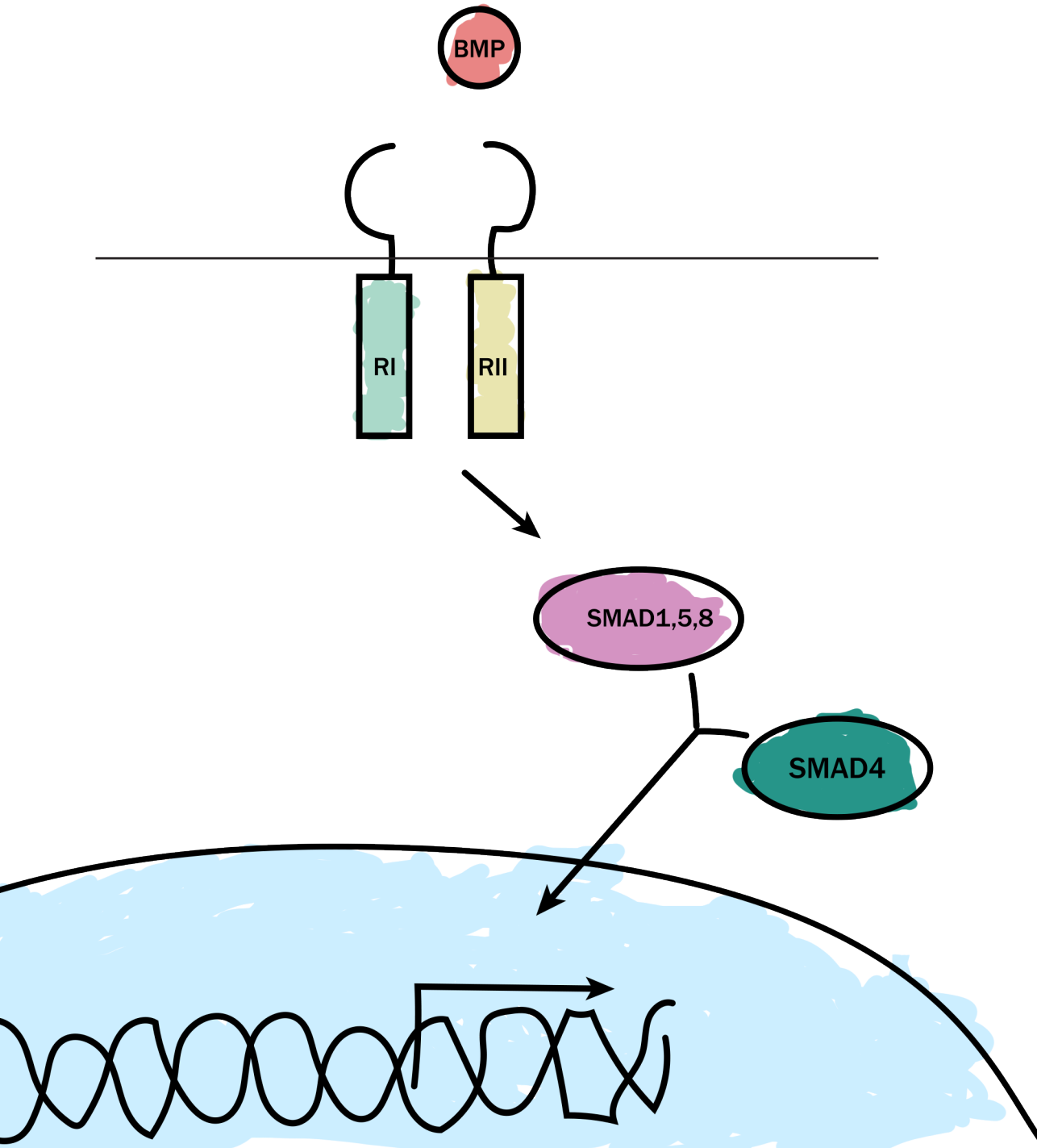
One important morphogenetic pathway is the Bone Morphogenetic Protein (BMP) signalling pathway, which was originally discovered in bone because of its ability to induce the formation of bone and cartilage.[3] It is now known that the BMP pathway is a key morphogenetic pathway involved in tissue organization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

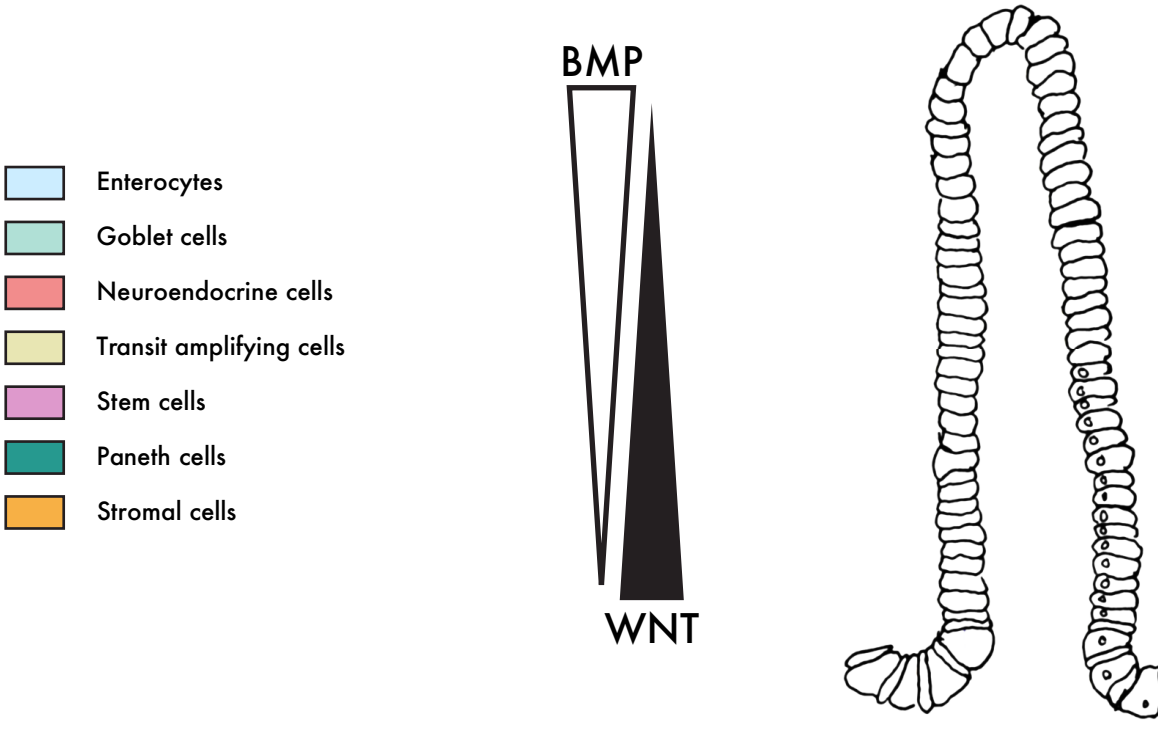
through distinct receptors and receptor associated SMADs, but both TGFβ and BMP signal through the common mediator SMAD4 to transduce a signal to the nucleus. This is the canonical SMAD dependent signalling route, but TGFβ and BMP can also signal independently from SMADs, a matter that will be discussed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



Role of BMP signalling in maintaining intestinal homeostasis

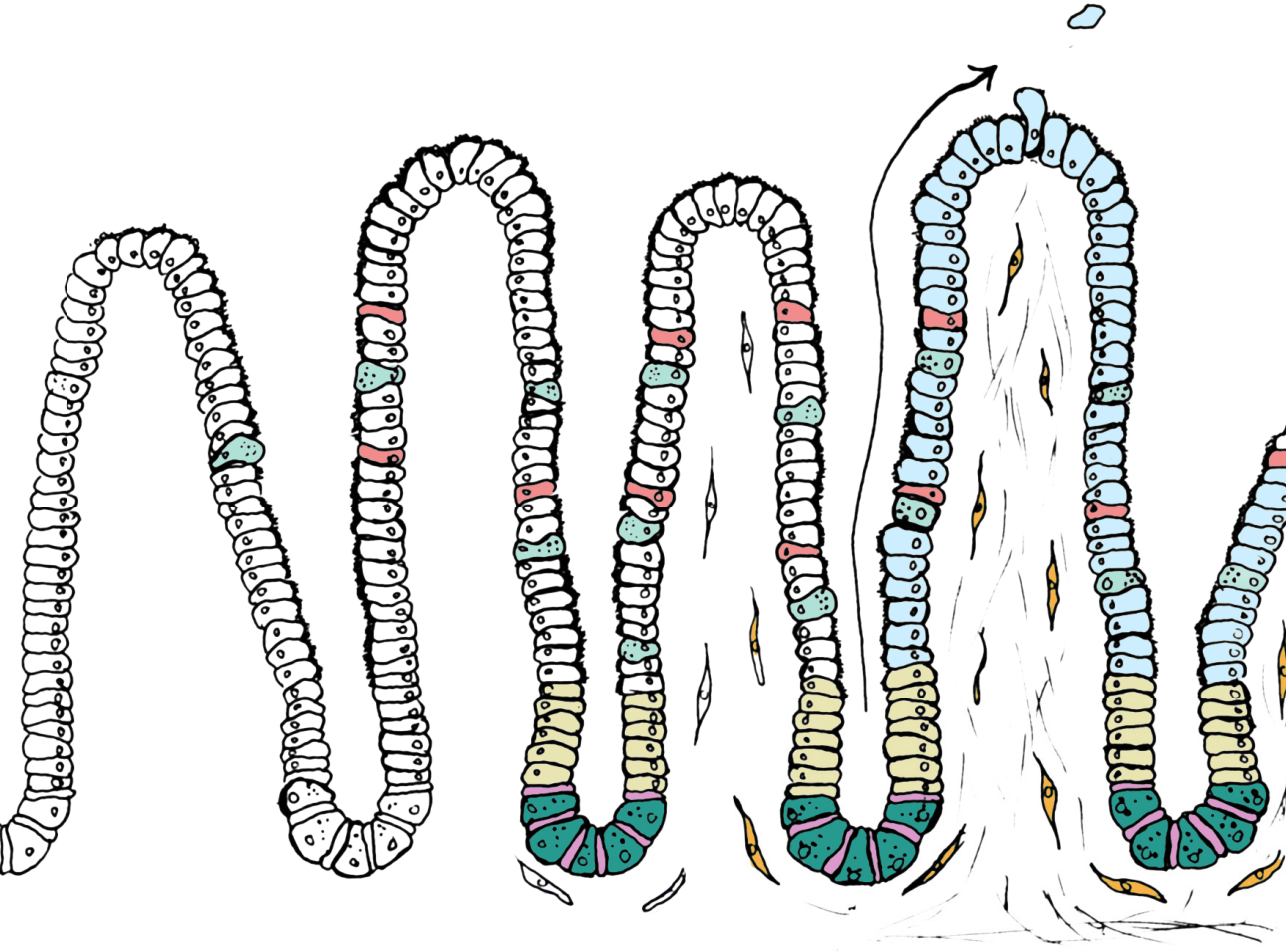
A massive surface area is squeezed into a small tube that we call intestine. This results in a sort of wrinkled epithelial cell monolayer. In the small intestine there are crypts and villi, while the colon only consists of crypts. Stem cells reside at the bottom of crypts giving rise to cells in the 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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Chapter 2

Conditional activation of BMP signalling leads to loss of the stem cell compartment

Manuscript in preparation

Philip Voorneveld, Liudmila Kodach, Rutger Jacobs, Sanne Rozekrans,
Gijs van den Brink, James Hardwick

Aim

Bone Morphogenetic Protein (BMP) signalling is essential for normal intestinal epithelial homeostasis. In transgenic mice inactivation of BMP signalling results in the development of polyps. This approach only alters the existing BMP activity at the top of the villi and does not affect BMP levels in the stem cell compartment, where BMP signalling is virtually non-existent. To study the importance of the BMP axial gradient we used an inducible mouse model in which BMP signalling can be constitutively activated in both the crypt and villi.

Methods

Mice containing an inducible constitutively active human BMPR1A sequence were crossed with CypCre. Eight-week-old mice were injected intraperitoneally with β-naftoflavone for 5 consecutive days. Cre-negative mice iwere used as controls. The mice were sacrificed at different time points.. Immunohistochemistry was performed for recombination (GFP), proliferation (BrdU & Ki-67), apoptosis (Caspase3), differentiation (CAII, Villin & Alcain Blue), BMP signalling (pSMAD1,5,8) and Wnt signalling (β-catenin). In situ hybridization was performed for intestinal stem cell marker OLFM4.

Results

Conditional induction of BMP activity results in a major disruption of the small intestinal crypt-villus architecture at days 3 and 5 and a 5% loss of body weight. Morphological changes include crypt fissioning, crypt enlargement, loss of villi and the appearance of cystic like structures. Paneth cells are mispositioned and shifted upwards, nuclear β-catenin is lost at the base of the crypt and expression of OLFM4 is completely absent. Subsequently the mice recover to regain a normal small intestinal phenotype. Recovery is preceded by the re-expression of OLFM4 day 5 after induction.

Conclusion

We conclude that the axial gradient of BMP activity in the crypt-villi is important for maintaining intestinal epithelial homeostasis. Low BMP activity at the bottom of the crypt is imperative for the preservation of the stem cell compartment.

Introduction

In normal intestinal epithelium Bone Morphogenetic Protein (BMP) signalling is active in a decreasing gradient from top to bottom along the vertical crypt-villus axis; it is active at the top of the villi and inactive at the bottom of the crypt. BMP signalling counteracts Wnt signalling which is active in an opposite increasing gradient; inactive at the top of the villi and active at the bottom.[1] Both pathways are thought to be essential for normal intestinal homeostastis. Wnt signalling induces stemness at the bottom of the crypt and leads to proliferation in the transit amplifying zone. Further up the villi, cells differentiate under the influence of BMP signalling, and eventually go into apoptosis at the top of the villi.

The importance of BMP signalling is signified by Juvenile Polyposis and Hereditary Mixed Polyposis; two intestinal polyp diseases both resulting from germline mutations in BMP signaling components.[2]

In two mouse models, namely BMPR1A floxed mouse and Noggin (BMP inhibitor) mouse, BMP signalling was inactivated resulted in the development of polyps.[3, 4] Inactivation of BMP signalling also led to upregulation of Wnt signalling. In these mouse models the existing BMP signalling at the top of the villi is inhibited. The level of BMP signalling in the crypt, which is very low, is unaffected. This approach does not affect BMP levels in the stem cell compartment, where BMP signalling is virtually non-existent. To study the importance of the BMP axial gradient we used an inducible mouse model in which BMP signalling can be constitutively activated in both the crypt and villi.

Normally, BMP ligands bind to a complex of transmembrane serine threonine kinase receptors consisting of type I (BMPR1A or BMPR1B) and type II (BMPR2) receptors. On binding of the BMPs to the receptor complex, the type I receptor (BMPR1A or BMPR1B) is phosphorylated by BMPR2, which leads to downstream phosphorylation and activation of the BMP-specific SMADs, SMAD1, 5 and 8. Phosphorylated SMAD1, 5 or 8 then complexes with the co-SMAD, SMAD4. SMAD4 is crucial for the modulation of gene transcription as it facilitates the translocation of pSMAD1/5/8 to the nucleus.

After recombination a constitutively active version of BMPR1A is transcribed resulting in constant phosphorylation and activation of pSMAD1,5,8 and eventually BMP signalling.

Methods

Mouse model

We used mice containing an inducible constitutively active human BMPR1A (caBMPR1A) sequence and crossed these with CypCre mice.

The caBMPRIA mouse was generated by Kamiya et al. [5] A modified chicken beta-actin promoter (CAG) was used to drive ubiquitous expression of a floxed lacZ gene with a nuclear localization signal and a triple polyadenylation sequence. The floxed cassette is followed by a constitutively active human BMPR1A sequence with the amino acid substitution of aspartic acid for glutamine at position 227 (Q227D), an IRES sequence, and the EGFP coding sequence. Expression of the constitutively active BMPR1A and EGFP are cre-dependent.

CypCre leads to recombination in both villus and crypt (except for paneth cells) and mostly in the proximal part of the small bowel.

Experimental set-up

Eight-week-old mice were injected intraperitoneally (ip) with 80ug/kg β-naftoflavone three times on one day. Cre-negative caBMPR1A mice injected with β-naftoflavone were used as controls. The mice were sacrificed 1 day, 3 days, 5 days, 1 month, 3 months and 6 months after induction (Figure1A). Bromodeoxyuridine (BrdU) (7.5mg/0.5ml NaCl) was injected ip one hour prior to sacrificing the animals.

Immunohistochemistry

The analysis was performed on the proximal parts of the small intestine where Cypcre is mostly recombined. Tissue was fixed overnight in 10% formalin, embedded in paraffin, and sectioned. Sections were deparaffinized using xylene and rehydrated in ethanol. Endogenous peroxidases were blocked using Methanol with 0.3% H_2O_2 was used to block endogenous peroxidases. Antigen retrieval was done by boiling in either sodium citrate solution (pH 6.0) or sodium EDTA (pH 9.0) for 20 min. After that slides were blocked for 30 min in TENG-T (10 mM Tris, 5 mM EDTA, 0.15 mM NaCl, 0.25% gelatin, 0.05% Tween 20) or 10% goat serum. Subsequently, slides were incubated with a primary antibody in PBS with 1% bovine serum albumin (BSA) and 0.1% Triton X-100 for 1 hour or overnight.

After PBS washing PowerVision secondary antibody (Immunologic) was used for 1 hr. DAB (diaminobenzidine) by Sigma was used according to manufacturer's instructions.

Antibody	Manufacturer	Antigen Retrieval	Block	Dilution	Incubation time
Anti-GFP	Rockland	Citrate	10% goat serum	1:400	Overnight
Anti-BRDU	Roche	Citrate	No blocking	1:500	1 hour
Anti-Ki67	Monosan	Citrate	TENG-T	1:200	Overnight
Anti-Caspase3	Cell Signaling	Citrate	No blocking	1:200	Overnight
Anti-CAII	Santa Cruz	Citrate	TENG-T	1:200	Overnight
Anti-Villin	Santa Cruz	Citrate	No blocking	1:100	Overnight
Anti-pSMAD1,5,8	Cell Signaling	Citrate	TENG-T	1:200	Overnight
Anti-beta-Catenin	BD	Tris/EDTA	No blocking	1:400	Overnight

In situ Hybridization

In situ probes were made by mRNA amplification. Amplicons were cloned into T-Easy Vector (Promega), according to instructions. Subsequently, dig-labeled probes were made using dig-labeled dUTP (Roche) and transcribed with T7 or Sp6 RNA polymerase according to manufacturer's instructions. Formalin-fixed paraffin-embedded sections were used. Sections were deparaffinized and rehydrated in H_2O, incubated for 10 min in 1 M HCl, digested with Proteinase K for 20 min, refixed in 4% paraformaldehyde for 10 min, and acetylated with acetic anhydride. 2% Blocking Powder, 0.05% Chaps, 50% formamide, 5× saline sodium citrate, 5 mM EDTA, 100 μg/ml heparin, and 100 μg/ml yeast RNA was applied to the slides afterwards. Subsequently, slides were incubated for 72 hr at 68°C complementary RNA probe. Washing was done using a buffer containing 50% formamide and 2× SSC. Slides were then blocked with 0.5% Blocking Powder in TBS-T, and incubated overnight with sheep anti-dig alkaline-phosphatase-conjugated Fab fragments (Roche). Staining was done using NBT/BCIP substrate

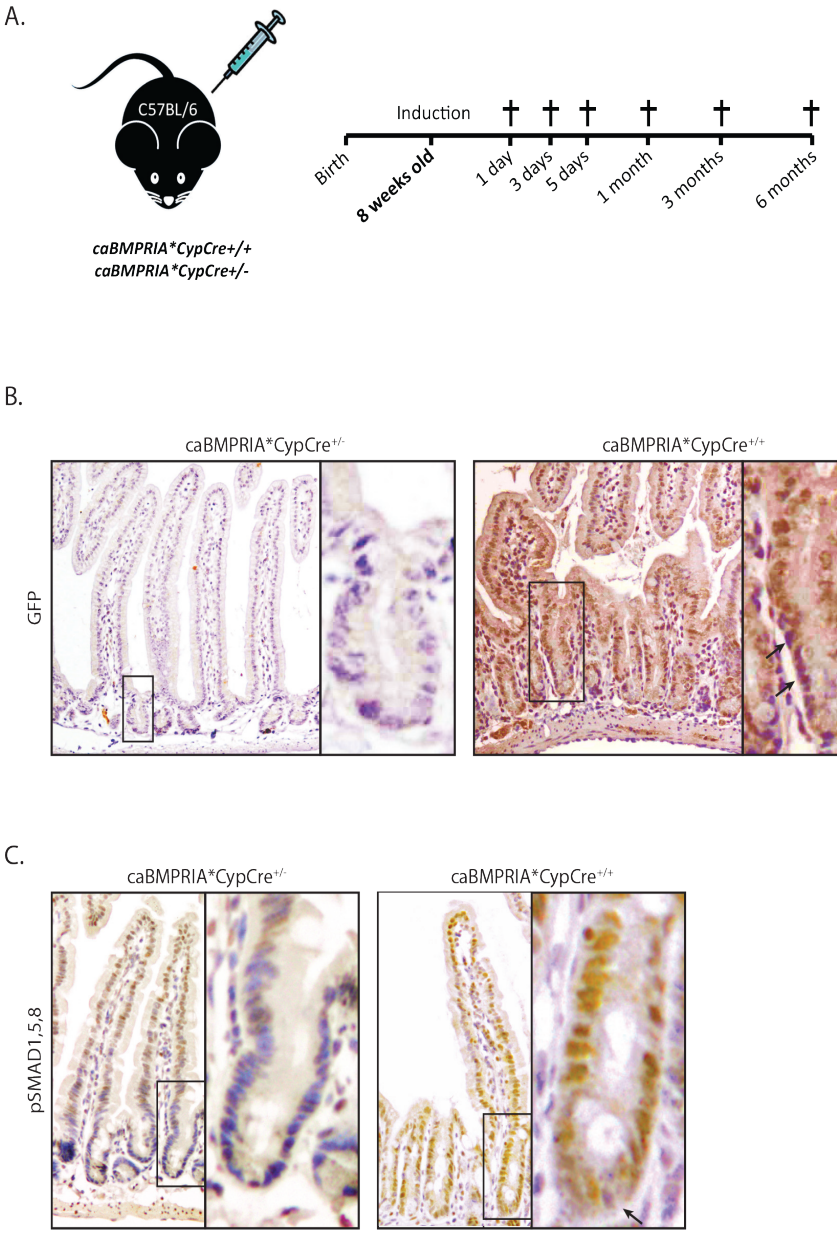


Figure 1. Activation of BMP signalling in the crypt-villus of the duodenum after induction

(Sigma) for 2 hours.

Results

Induction of caBMPR1A construct leads to activation of BMP signalling in stem cell compartment.

The caBMPRIA construct contains a GFP reporter; recombination could be visualized using GFP immunohistochemistry (Figure 1B). Injection of caBMPR1A*CypCre $^{+/-}$ mice with β-naftoflavone does not lead to GFP expression in the intestinal epithelial, while caBMPR1A*CypCre $^{+/+}$ mice injected with β-naftoflavone show intestinal epithelial expression in both the crypts and vili. Almost all cells are positive for GFP, meaning that is a high percentage of recombination.

To demonstrate that β-naftoflavone injection of caBMPR1A*CypCre $^{+/+}$ mice leads to BMP signalling activation in the stem cell compartment, we used immonuhistochemical staining of pSMAD1,5,8. Figure 1C confirms that this is indeed true, as nuclear pSMAD1,5,8 expression can be observed throughout the whole crypt and vili, wheras the control mouse shows the normal gradient expression pattern of BMP signalling.

Activation of BMP signalling disrupts crypt-villus architecture

We noticed that caBMPR1A*CypCre $^{+/+}$ lost weight in the first week after induction, to almost completely recover after two weeks (Figure 2). Looking at the hematoxylin-eosin sections of the proximal small bowel we observed major changes in crypt-villi architecture (figure 3). One day after induction the crypts are slightly elongated, but the size of the villi is still similar to the control mice. Three days after induction the crypt are elongated more clearly, crypt fissioning occurs, there is an increase in stromal cells and the villi are shorter. After five days the intestinal epithelium is changed unrecognizable with

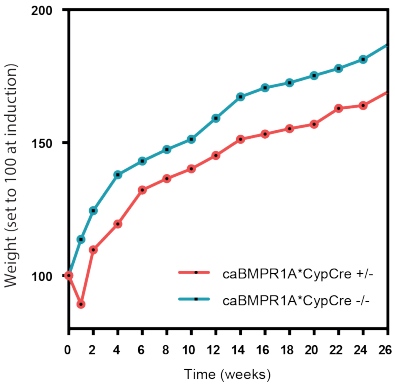


Figure 2. Weight after induction

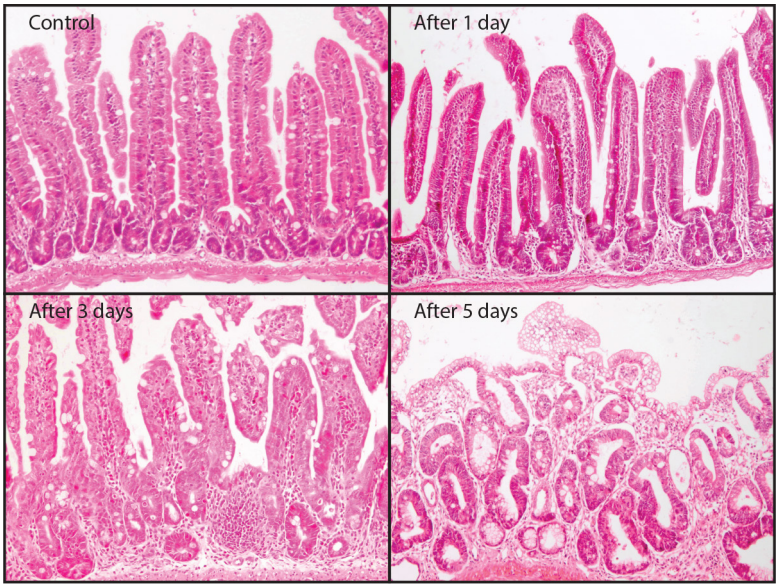


Figure 3. Activation of BMP signalling disrupts crypt-villus homeostasis

the appearance of cystic like structures. Interstingly, the mice sacrificed 6 months after induction had completely normal crypt-villus architecture similar to their controls.

There is an increase in Ki-67 and BrdU positive cells three days after induction (Figure 4). This is only relative as the BrdU index (percentage of BrdU positive cells) is unchanged. This means that when the crypt length increases more cells stay in a proliferative state. At day five we observed that in part of the former crypts most cells are Ki-67 positive, while in other crypts almost no proliferation occurs.

We hypothesized that activation of BMP signalling in the crypts would lead to inhibition of Wnt signalling as these signalling pathways counteract each other. Epithelial cells in the bottom of the crypt normally have ß-catenin positive nuclei, which means that there is active Wnt signalling in those cells. In the crypts of caBMPR1A*CypCre $^{+/+}$ mice there are no b-catenin positive nuclei three days after induction (Figure 5A).

Next, we investigated different cell lineages of the intestinal epithelium. The number of cells positive for chromogranin A (neuroendocrine cells) is equal

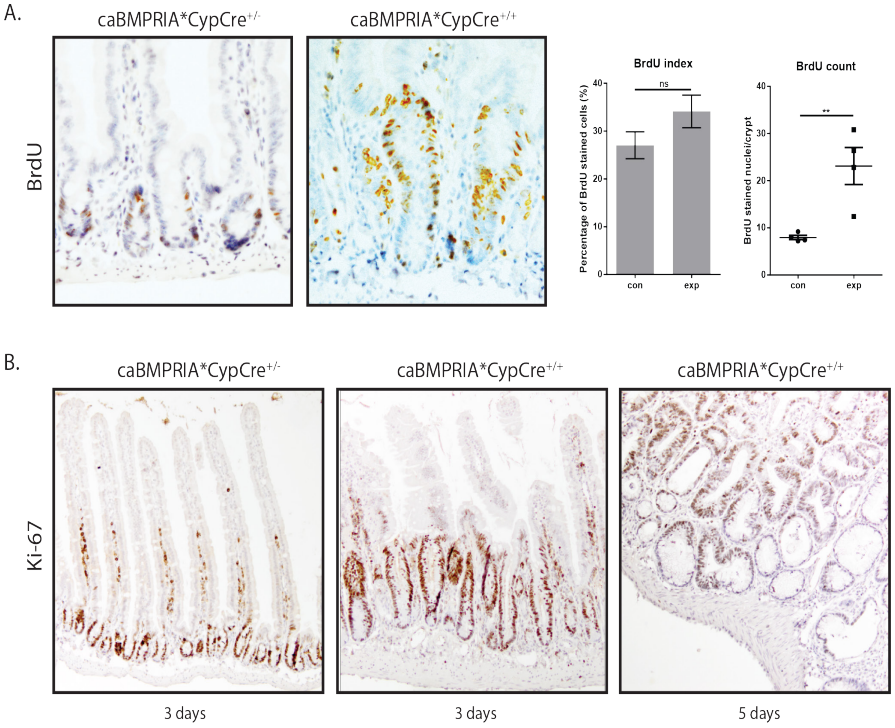


Figure 4. Proliferation

in the caBMPR1A*CypCre $^{+/+}$ versus caBMPR1A*CypCre $^{+/-}$ mice (Figure 5B). The number of lysozyme cells (Paneth cells) is also equal, but most of them are mispositioned. Paneth cells normally reside in the stem cell compartment interspersed between the stem cells, but in the caBMPR1A*CypCre $^{+/+}$ mice there are also found in the villi (figure 5C).

In an attempt to understand why the mice regain their weight after initial weight loss and why their crypt-villus architecture is transformed back to normal, we visualized OLFM4 expression using in situ hybridization. OLFM4 is a marker expressed only in stem cells as can be seen at day 0 in figure 6. However, one day after induction OLFM4 expression is completely absent. OLFM4 regains expression five days after induction.

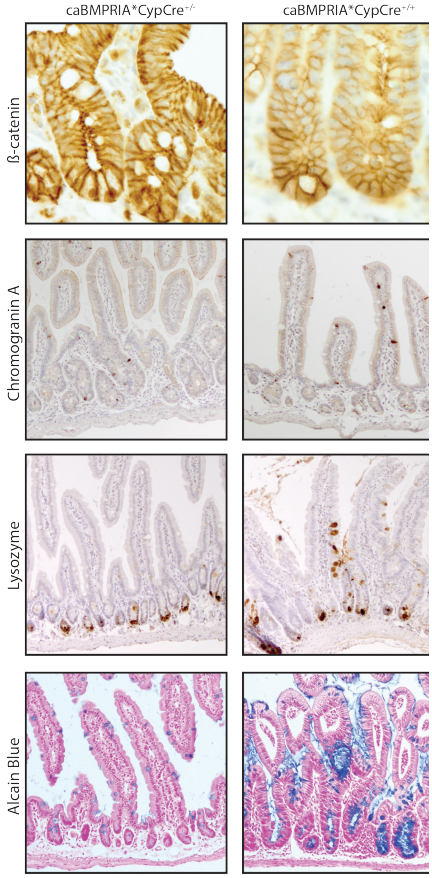


Figure 5. Differentation markers

Discussion

Previous studies have already underlined the importance of intact BMP signalling in the intestine. Inhibition of BMP signalling in rodent models leads to the formation of polyps[3, 4] and loss of BMP signalling is seen in a portion of CRC cancers.[6-11] We found that intestinal crypt-villus homeostasis can not only be disrupted by abolishing BMP signalling, but also by enhancing it.

In our *in vivo* model BMP signalling is constitutively active in both the crypt and the villus resulting in a major disruption of the crypt-villus architecture. At first the crypts only elongate, but after three days crypt fissioning occurs and after five days cyst like structures appear with a portion of the former crypts highly proliferative and parts with no proliferation. After 6 months the architecture appears normal again.

In the normal intestine BMP signalling is not active in crypts; there Wnt signalling is active inducing stemness and proliferation. Nuclear expression of beta-catenin is an indication of active Wnt signalling and can be found at the bottom of crypts in normal intestinal epithelium.[12] We found that beta-catenin is downregulated at the bottom of the crypt of the small intestine in caBMPR1A*CypCre $^{+/+}$ mice compared to caBMPR1A*CypCre $^{+/-}$ mice where BMP signalling is not activated in the crypts. This is in accordance with previous in vitro and in vivo studies that have shown that BMP inhibition leads to Wnt activation and that BMP induction can inhibit Wnt signalling.[13]

In a mouse model where Wnt signalling is abolished by deleting the Wnt/tcf-4 target gene Myc a somewhat similar phenotype occurs; initially a decrease in proliferation is seen, expression of the stem cell marker CD44 is reduced and crypt fissioning occurs. Later, the intestinal epithelium is regenerated, probably by cells that are not recombined and maintain Myc expression.[14]
In a study by Heijmans[15] et al loss of stemness is seen after induction of ER stress by activating the unfolded protein respons (UPR). Here, also a remarkable repopulation is seen shortly after induction. The authors argue that it is unlikely the result of incomplete recombination because of the high efficiency they achieved, which is also the case in our model. Their suggestion is that it could be the result of dedifferentiation or that Paneth cells, not being recombined, could behave as quiescent stem cells.
In future studies we need to investigate the pathways involved in BMP induced differentiation of stemness. Also the complete regeneration of the epithelium after loss of stemness in the crypts requires further analyses.

In a future study we plan to combine this model with a CRC model. BMP activation can possibly intervene in carcinogenesis. Relapse after chemotherapy could be the result of remaining cancer stem cells (CSC). It is know that CSCs are less sensitive to chemotherapy than differentiated cancer cells. Lombardo et al treated CSC with BMP4 and observed differentiation.[16] When combining BMP4 with chemotherapy (5-FU and oxaliplatin) a better reduction in tumour growth in a xenograft model was seen. Activating BMP signalling can possibly push CSCs towards differentiation making them more susceptible for chemotherapy. Our study shows that BMP signalling can be used to differentiate intestinal stem cells.

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Chapter 3

Loss of SMAD4 Alters BMP Signaling to Promote Colorectal Cancer Cell Metastasis via Activation of Rho and ROCK

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Aims

SMAD4 is frequently lost from colorectal cancers (CRC), which is associated with the development of metastases and a poor prognosis. SMAD4 loss is believed to alter transforming growth factor (TGF)β signaling to promote tumor progression. However, SMAD4 is also a central component of the bone morphogenetic protein (BMP) signaling pathway, implicated in CRC pathogenesis by human genetic studies. We investigated the effects of alterations in BMP signaling on the invasive and metastatic abilities of CRC cells and changes in members in this pathway in human tumor samples.

Methods

We activated BMP signaling in SMAD4-positive and -negative CRC cells (HCT116, HT-29, SW480, and LS174T); SMAD4 was stably expressed or knocked down using lentiviral vectors. We investigated the effects on markers of epithelial–mesenchymal transition (EMT) and on cell migration, invasion, and formation of invadopodia. We performed kinase activity assays to characterize SMAD4-independent BMP signaling and used an inhibitor screen to identify pathways that regulate CRC cell migration. We investigated the effects of the ROCK inhibitor Y-27632 in immunocompromised (CD-1 Nu) mice with orthotopic metastatic tumors. Immunohistochemistry was used to detect BMPR1a, BMPR1b, BMPR2, and SMAD4 in human colorectal tumors; these were related to patient survival times.

Results

Activation of BMP signaling in SMAD4-negative cells altered protein and mRNA levels of markers of EMT and increased cell migration, invasion, and formation of invadopodia. Knockdown of the BMP receptor in SMAD4-negative cells reduced their invasive activity in vitro. SMAD4-independent BMP signaling activated Rho signaling via ROCK and LIMK. Pharmacologic inhibition of ROCK reduced metastasis of colorectal xenograft tumors in mice.

Loss of SMAD4 from colorectal tumors has been associated with reduced survival time; we found that this association is dependent on the expression of BMP receptors but not TGFβ receptors.

Conclusion

Loss of SMAD4 from colorectal cancer cells causes BMP signaling to switch from tumor suppressive to metastasis promoting. Concurrent loss of SMAD4 and normal expression of BMP receptors in colorectal tumors was associated with reduced survival times of patients. Reagents that interfere with SMAD4-independent BMP signaling, such as ROCK inhibitors, might be developed as therapeutics for CRC.

Introduction

Colorectal cancer (CRC) remains a frequently fatal disease due to the high incidence of metastases for which current therapy is largely unsuccessful. This makes the understanding of the process of metastasis critical to improve the prognosis.

Loss of SMAD4 is seen in 30-40% of CRCs[1], occurs late in the adenoma-to-carcinoma sequence[2] and is associated with liver metastases, a poor prognosis and a poor response to chemotherapy[3, 4]. SMAD4 is the central critical signal transduction element of the Transforming Growth Factor (TGF)-β signaling pathway. The consequences of loss of SMAD4 were therefore initially ascribed to the loss of canonical TGFβ signaling. However, in many cancers TGFβ switches from being a tumor suppressor to become a tumor promoter, driving invasion and metastasis[5]

The nature of the switch, that determines whether TGFβ acts as a tumor suppressor or as a tumor promoter, has been the subject of intense research[6, 7]. Mutant p53 has been shown to be one potential explanation [8], while others have shown that SMAD4 mutations bring about this switch [9].

To date most research into the role of SMAD4 in CRC has focused exclusively on its role as part of the TGFβ pathway. However, SMAD4 is also the central component of the Bone Morphogenetic Protein (BMP) signaling pathway which we and others have shown plays an important role in cancer development[1, 10, 11]. There is even perhaps stronger evidence for a critical role for the BMP pathway than for the TGFβ pathway in CRC with germ line mutations in BMP receptor 1A (BMPR1A) in Juvenile Polyposis but not in TGFβ receptors[12] and from Genome Wide Association Studies in CRC multiple associations exclusively within the BMP pathway (BMP2, BMP4, BMPR1A and Gremlin1) but none exclusively within the TGFβ pathway, while two others are involved in both (SMAD4 and SMAD7)[13, 14]. BMP signaling is classically regarded as tumor-suppressive in CRC inducing cell differentiation and apoptosis of colonic epithelial cells and negatively regulating WNT signaling[10, 15]. However, while some studies show that activation of BMP signaling results in growth suppression and induction of differentiation in cancer cell lines[10, 16] others have found that BMP activation leads to pro-metastatic effects.[17]

In this study we set out to investigate the role of BMP signaling in the processes of Epithelial–Mesenchymal Transition (EMT), invasion and metastasis in CRC with particular focus on the role of loss of SMAD4 and SMAD4-independent signaling (also known as non-canonical or non-SMAD BMP signaling).

Methods

Cell culture

SW480, HT29, DLD1 and HCT116 colon cancer cell lines were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland) with 4.5g/l glucose and with 580mg/l L-glutamine. This was supplemented with penicillin (50U/ml) and streptomycin (50µg/ml) and with 10% fetal calf serum (FCS) (Gibco) unless stated otherwise. HCT116 SMAD4-/- cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA). HCT116 SMAD4-/- cells were cultured in DMEM medium supplemented with 0.4 mg/mL G418 and 0.1 mg/mL hygromycin B. All in vitro experiments were performed on cells growing exponentially.

Activation or inhibition of BMP signaling

Activation: Treatment with BMP-2 ligand (100ng/ml) (BD biosciences) dissolved in DMEM supplemented with 1% FCS compared to the same volume of PBS dissolved in DMEM supplemented with 1% FCS or transfection of a pcDNA4/TO plasmid (Invitrogen) expressing BMPR2 compared to transfection of an empty vector pcDNA4/TO. Inhibition: recombinant human Noggin (500ng/ml) (BD biosciences) or the small molecule LDN-193189 (5nM) (Selleckchem) in culture medium. The controls were PBS and DMSO respectively, added in the same volume to the culture medium.

Transfection, luciferase reporter assay and siRNA knockdown

Cells were transiently transfected with either a pcDNA4/TO*BMPR2 expressing plasmid, a pcDNA4/TO*TGFβR2 expressing plasmid or a pcDNA4/TO control vector (Invitrogen, Breda, the Netherlands), or. The efficiency of the transfection was evaluated by co-transfection with a pmaxGFP control vector (from Amaxa

GmbH, Cologne, Germany) and the measurement the measurement of GFP-positive cells, which was at least 70%. The activity of canonical BMP and TGFβ signaling was measured using co-transfection of BRE-Luc vector or CAGA-Luc vector respectively and a CMV promoter-driven Renilla luciferase vector (Promega, Leiden, The Netherlands). Transfections were performed using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection luciferase activity was assayed using a Dual-Glo Luciferase Assay System (Promega) on a Luminometer (Berthold Technologies, Bad Wildbad, Germany). Each Firefly luciferase value was corrected for its co-transfected Renilla Luciferase value.

For siRNA knockdown of BMP receptors, cells were transfected with either siRNA against one of the BMP receptors (BMPR1A, BMPR1B or BMPR2) or scrambled siRNA (all Ambion, Invitrogen) using Lipofectamine 2000 (Invitrogen).

BMPR1A Ambion Silencer Select Pre-designed siRNA ID: s280
BMPR1B Ambion Silencer Select Pre-designed siRNA ID: s2041
BMPR2 Ambion Silencer Select Pre-designed siRNA ID: s2045

Stable shRNA-mediated knockdown of SMAD4

Lentiviral constructs expressing shRNAs targeting SMAD4 (TRCN0000040028) and a non-targeting control construct (SHC002) were obtained from the Sigma MISSION shRNA library (Sigma-Aldrich, St. Louis, MO). Production of lentiviruses by transfection into 293T cells has been described earlier [1]. Cells were transduced in the presence of 8 μg/ml polybrene (Sigma-Aldrich). Cells were selected using puromycin.

Roundness

Phase-contrast images of HCT116 and HCT116 SMAD4-/- cells were obtained using ZEISS Axiovert 200m (Sliedrecht, the Netherlands). Images were analyzed using ImageJ software and roundness was calculated using the following principle. Roundness (R) is based on a circularity ratio between the surface area of the cell and a perfect circle that has the same perimeter as the cell. The roundness can be calculated using two variables, namely surface area (A) and the perimeter (P). The given formulas for a perfect circle with R=1 are:

$A = \pi r^2$

and

$P = \pi r$ *or* $r = P/2\pi$

then substituting the radius in A gives:

$A = \pi (P/2\pi)^2$ →

$A = \pi P^2/(4\pi^2)$ →

$A = P^2/4\pi$

If the R is a ratio between the surface area of the test shape and the surface area of a perfect circle then

$R = A_{cell}/A_{circle}$

With the previous area of a perfect circle filled into the equation it gives:

$R = A_{cell}/(P^2/4\pi)$ or

$R = 4\pi (A/P^2)$

In this formula, a perfect circle is equal to 1 and a line approaches 0. The roundness of a 100 cells per condition was measured in each experiment.

Wound healing assay

Cells were plated in 6-well plates (Corning Incorporated, Corning, NY, USA) and grown in a monolayer until 70-80% confluent. At this point a scratch was made using the tip of a micropipette. Bright field images were obtained at 0hrs and after 24hrs and cell migration was calculated using ImageJ software (rsbweb.nih.gov/ij/) as the ratio of the wound closure relative to the initial wound.

Invasion assay

Cells were labeled with 5 μM CellTracker Green CMFDA (Invitrogen) after which they were detached using trypsin with 2 mM ethylenediaminetetraacetic

acid (EDTA). 10^5 cells/insert were transferred to 8 µM pore size HTS FluoroBlok Cell Culture Inserts (BD Falcon, Breda, the Netherlands) in medium containing 1% FCS. The inserts were coated with 100 µl of 1:1 mix of serum free-medium and Matrigel (BD Biosciences, Breda, the Netherlands). Inserts were placed in 24-well plates (Corning Incorporated) in which 600µl of 10% FCS-containing media without phenol red were present. Fluorescence of the invaded cells at the bottom well of the transwell was measured every 2 hours using the BioTek Flx800 (BioTek, Winooski, VT, USA). Data was corrected for background fluorescence and migration start points were set to zero.

Liver metastases model

Liver metastasis were generated by injecting 0.5 *10^6 HT29 cells expressing luciferase dissolved in 50 µl of medium:matrigel (1:1) into the spleens of 6 week old immunocompromised CD1 nude mice (males). The spleen was not removed after injection. The mice were treated by intraperitoneal injection (i.p.) with 2.5mg/kg Y-27632 (R&D systems) dissolved in PBS (n=9) or with PBS (n=9) every other day. Weekly scans bioluminescence scans were performed and after seven weeks the mice were euthanized. The model is described in detail in the supplementary methods.

Simultaneous correlative scanning electron and high-NA fluorescence microscopy

The following section contains a summary of the microscopy set-up. For a complete microscope set-up and explanation of tissue and staining methods we refer to two papers by our collaborating groups[18, 19].

All imaging experiments were done on a fluorescence microscope integrated in a commercial Scanning Electron Microscope (SEM) (Quanta™ 200 FEG microscope (FEI, Eindhoven, The Netherlands)) as developed at Delft University of Technology and DELMIC BV (Delft, The Netherlands). The fluorescence microscope was equipped with a 100x 1.4 NA objective lens (Nikon CFI Plan Apochromat VC) mounted below the sample holder in the SEM chamber, and excitation and emission filters for Alexa-fluor-488.

Seeding and transfection

ITO-coated microscope slides (thickness #1, 22x22mm with 8–12 Ωsq^{-1} or

22x40mm with 70– 100 Ωsq^{-1}; SPI Supplies, West Chester, PA, U.S.A.) were disinfected with 70% ethanol, placed in culture dishes with the conductive side upwards and incubated with culture medium. The cells were seeded onto the ITO coated glass slides. When cell reached a confluence of 60% they were transfected with either pcDNA4/TO or BMPR2 and cultured for 48hrs at 37°C 5%CO_2. Cells were then washed twice with Phosphate Buffered Saline (PBS) and fixed for 10 minutes with a mixture of 2.5% paraformaldehyde and 1.25% glutaraldehyde in 0.1M PBS, pH 7.4. Samples were washed 3 times with PBS after fixation.

Fluorescent labeling and immunocytochemistry

Actin was stained using Alexa-fluor-488 phalloidin (Invitrogen) in a 1:40 PBS/1%BSA/0.1%Triton dilution for 30 minutes according manufacturer's instructions. Afterwards samples were washed 3 times with PBS.

For the immunocytochemistry of cortactin, cells were incubated with the primary antibody in PBS/1%BSA/0.1%Triton (1:200) for 30 min at room temperature and afterwards washed 3 times with PBS. The cells were then incubated with the secondary antibody (1:200) diluted in PBS/1%BSA/0,1%Triton for 20 minutes at room temperature and then washed again 3 times with PBS. The primary antibody used was Anti-Cortactin (p80/85) (mouse), clone 4F11 (Millipore) and the secondary antibody was Alexa fluor 488 goat anti-mouse IgG (H+L) (Invitrogen). To remove any remaining salt residue, the cells were washed 3 times with dH_2O and left in dH_2O at 4˚C overnight.

Kinase activity analysis using Pepchip peptide arrays.

Kinome profiling using peptide arrays exploits the substrate specificity of individual kinases to infer their activity from changes in the phosphorylation of an array of immobilized peptide kinase substrates. In our approach, we employed peptide arrays containing 960 peptides each representing a fully annotated, disease-related kinase phosphorylation site, which were spotted on hydrogel-coated glass in triplicate (spotting procedure is described in [4]). The sequence of the peptides involved as well as the source proteins from which these peptides were derived can be found in more detail on: http://www.pepscan.com/presto/files/pepchip/PepChip-Kinomics-Map.zip). Procedures for performing kinome arrays were described in detail elsewhere [5, 6]. Briefly, cells were lysed in 50µl lysis buffer

(20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM $MgCl_2$, 1mM glycerophosphate, 1mM Na_3VO_4, 1mM NaF, 1µg/ml Leupeptin, 1µg/ml Aprotinin, 1mM PMSF) and 10 ml of the peptide array incubation mix (50% glycerol, 50µM ATP, 0.05% v/v Brij-35, 0.25mg/ml BSA, ^{33}P-γ-ATP (1MBq)) was added to the filtered lysates and the mixture loaded onto the microarray chip and allowed to phosphorylate the peptide substrates for 90 min at 37°C. The chips were washed twice with TBS 0.1% Triton X-100, twice in 2M NaCl, and twice in de-mineralized water and air-dried. The slides were then exposed to a phosphor-imager plate for 72h and acquisition of the peptide array performed using a phosphor-imager (Storm™, Amersham Biosciences, Sweden). Levels of incorporated radioactivity, which correspond to the phosphorylation status were quantified by array software Scanalyze (Eisen Software) and exported to Microsoft Excel. As a control, further slides were incubated with ^{33}P-γ-ATP (1MBq)) without cell lysate to demonstrate that the signal observed represented kinase-dependent covalent transfer of the terminal phosphor atom.

For data analysis every peptide was first given an "on" call or "off" call (Markov state analysis). To do this an average signal for each peptide was first calculated using the three replicates to yield an aggregate dataset for each of the experimental conditions. Subsequently, for each of the aggregate datasets, an "on" call or an "off" call was given to each peptide substrate (Markov state analysis). In order to do this, we assumed that the subset of signals representing the $1\text{-}e^{-1}$ fraction of peptides having the lowest phosphorylation of all peptides contained pure noise and did not represent meaningful phosphorylation. The distribution of this noise was fitted as a single exponent, using the amplitude-sorted row number of these substrates as the X domain of the distribution and this single exponent was assumed to describe noise for the entire dataset for this experimental condition. Now for all data points within the subset, when the actual amplitude observed minus 1,96 the standard deviation was in excess of the value expected from the distribution describing the noise, a substrate was given an "on" call. For further analysis, peptides were grouped in signal transduction categories and the number of "on" calls in each category was compared for the different experimental conditions and subjected to Galton statistics. Attribution of peptides to signal transduction categories as well as the number of "on" calls in each category can be found in ***Figure S3.***

Results were then grouped into 8 broad biological processes for first analysis by contrasting activities in these broad groups then mined for detailed elucidation of the signaling evoked by BMPR2 in SMAD4 negative cancer cells.

Kinase inhibitor screen

Cells were grown in 6 well plates. Cells were transfected with either pcDNA or BMPR2 when 60% confluent after which the cells were treated with kinase inhibitors (see scheme below for concentrations). After 24 hours of treatment the cells were lysed using Trizol and RNA was isolated. A RT-PCR was performed for the expression of Vimentin (see "Real Time PCR" paragraph for protocol).

Kinase inhibitors

A list of kinase inhibitors can be found in the supplementary section of this chapter. For the treatment of cancer cell line cells, the IC50 for each specific kinase was used.

Real Time PCR

Total RNA was isolated using Trizol (Invitrogen) according to manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using Random primers (Promega) and MMLV-reverse transcriptase (Invitrogen). PCR was performed using the iCycler Thermal Cycler and iQ5 Multicolor Real Time PCR Detection System (Biorad, Santa Cruz, California). GAPDH expression was used to normalize for variance. A list of the primers can be found in the supplementary section of this chapter.

Immunoblotting

Cells were grown in 6-well plates. Cells were washed with PBS and cell lysis buffer (Cell Signaling Technology, Beverly, USA) plus protease inhibitor (Pierce Biotechnology, Rockford, IL) was added. The cells were lysed on ice for 15 minutes, scraped into 1.5 mL tubes (Eppendorf, Hamburg, Germany), sonicated and centrifuged for 10 min at 14000 rpm, 4°C. Protein levels were measured with a BCA-kit (Pierce Biotechnology, Rockford, IL) using clear lysates. The protein levels in each sample were equalized, the lysates were mixed with 3x SDS sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 3% β-mercaptoethanol; 20% glycerol,

bromphenol blue) and were boiled at 97°C for 5 minutes. The proteins were separated by SDS-PAGE gel electrophoresis and blotted onto PVDF membrane (Millipore, Billerica, US). The blots were blocked in blocking buffer containing 5% non-fat dry milk (Biorad) dissolved in Tris Buffered Saline supplemented with 0.05% Tween-20 (TBST) and washed three times in TBST. Both primary and secondary antibodies were dissolved in 0.5% non-fat dry milk. Primary antibody was incubated overnight at 4°C (see table below for antibodies used and dilution) in 0.1% non-fat dry milk-TBST. Secondary HRP-linked antibody was incubated for 1 hour at room temperature 0,1% non-fat dry milk-TBST. Blots were then exposed to Lumilight+ (Roche, Basel, Switzerland) chemiluminescent substrate and visualized using a chemiluminescence imager (Biorad). A list of primary antibodies can be found in the supplementary section of this chapter.

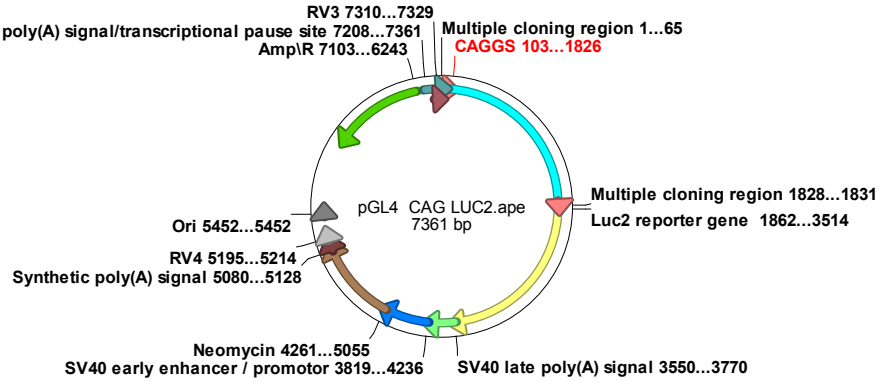
Immunoprecipitation

HA-tag antibodies (Cell signaling) were added to the cell lysate (1:150) and incubate with gentle rocking overnight at 4°C. Afterwards protein A agarose beads (Cell signaling) (20 µl) were added and lysate was incubated with gentle rocking for 3 hours at 4°C. Lysate was spun down and the pellet was washed five times with 500 µl of cell lysis buffer. The pellet was resuspended in 20 µl 3X SDS sample buffer and heated to 97°C 5 minutes. Subsequently the samples were loading on a SDS-PAGE gel.

Orthotopic metastasis mouse model

Development of luciferase expressing cell line

HT29-cells were stably transfected with a pGL4 vector expressing firefly luciferase controlled by a CAGGS-promoter. The transfected cells were selected using G418 (500 µg/ml).



Intrasplenic cell injection

Liver metastasis were generated by injecting 0.5 *10^6 HT29 cells expressing luciferase dissolved in 50 µl of medium:matrigel (1:1) into the spleens of 6 week old immunocompromised CD1 nude mice (males). Mice were purchased from Charles River Laboratories International, Inc (Wilmington, MA, USA).

The mice were anesthetized with isoflurane. A dorsal subcostal incision was made to expose the spleen. The spleen was exteriorized and placed on gauze soaked in PBS. 50µL volume of tumor cell suspension was injected slowly into the spleen using a 25-gauge needle.

Treatment and analysis

The mice were treated by intraperitoneal injection (i.p.) with 2.5mg/kg Y-27632 (R&D systems) dissolved in PBS (n=9) or with PBS (n=9) every other day. Weekly scans were performed by i.p. injection of 100µl luciferin (25mg/ml) and tumor formation was visualized using the IVIS Lumina Imaging system (Xenogen, Hopkinton, MA, USA). Living Image (software made by Caliper, Hopkinton, MA, USA) was used to analyze and quantify the scans. After seven weeks the mice were euthanized. The weight and volume of the livers was determined for each mouse.

Tissue Microarray (TMA)

Immunohistochemistry for SMAD4, BMPR1A, BMPR1B, BMPR2, TGFβR1 and TGFβR2 was performed in colorectal cancer tissue of 145 patients included on a

tissue microarray. Each individual tumor was represented by 3 separate 0.6mm cores on the TMA as well as one core of normal colon mucosa.

	Total n=145 (%)
Mean age±SD (y)	67,2±12,1
Gender	
Female	63 (43,4)
Male	82 (56,6)
Dukes	
I	50 (34,5)
II	55 (37,9)
III	32 (22,1)
IV	8 (5,5)
Grade†	
Poorly differentiated	15/102 (14,7)
Moderately differentiated	66/102 (64,7)
Well differentiated	21/102 (20,6)
Location‡	
Left sided*	80/97 (82,5)
Right sided**	17/97 (17,5)

† known for 102 of the 145 patients

‡ known for 97 of the 145 patients

*Left sided is defined as rectosigmoid, sigmoid and decending colon

** Right sided is defined as splenic flexure, transverse colon, hepatic flexure, ascending colon and caecum

Immunohistochemistry

SMAD4, BMPR2 and TGFβR2

Sections were deparaffinized, rehydrated in graded ethanol and immersed in 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity and antigen retrieval was performed in 1xTris/EDTA, pH 9.0, for 30 min at 97˚C. Nonspecific binding sides were blocked with 10% normal goat serum for 20 min. Primary antibody incubation was performed for 1 h at room temperature (diluted in PBS/0.1%triton/1%BSA; see table below for concentration). Subsequently the Dako detection system LSAB+ System-HRP (Dako) was used. Peroxidase activity was detected with fastDAB (Sigma-Aldrich).

BMPR1A, BMPR1B and TGFβR1

Sections were deparaffinized, rehydrated in graded ethanol and immersed in 0.3%

hydrogen peroxide in methanol for 30 min. Antigen retrieval was performed by heating for 10 min to 97˚C in sodium citrate, pH 6.0, and nonspecific binding sides were blocked by incubation with TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% Tween 20, pH 8.0) for 10 min. Antibodies were diluted in PBS/0.1%triton/1%BSA; see table below for concentration. Incubation took place overnight at 4˚C and subsequently the Dako detection system LSAB+ System-HRP (Dako) was used. Peroxidase activity was detected with fastDAB (Sigma-Aldrich). List of primary antibodies can be found in the supplementary section of this chapter.

TMA analysis

Analysis was performed in a blinded fashion by two investigators independently. SMAD4 expression was scored according to previously described methods [7]. SMAD4 staining was scored as follows: 0, no staining, 1, weak nuclear staining or negative nuclear and weak intracytoplasmic staining in less than 10% of the cells; 2, moderate to strong nuclear staining in more than 10% of the cells. The scoring for of TGFβ and BMP receptor staining (BMPR1A, BMPR1B, BMPR2, TGFβR1 and TGFβR2) was based on both staining intensity and the percentage of tumor cells stained as outlined below:

Scoring system for BMP/ TGFβ receptor immunohistochemistry

	Percentage of cells stained		
Intensity of staining	<10%	10-30%	>30%
No	0	0	0
Weak	0	0	1
Moderate	0	1	2
Strong	0	2	4

A BMP/TGFβ receptor expression score of 0 or 1 is considered negative. A score of 2 or 3 is considered positive.

Statistical analysis

Statistical analysis was performed using 2-tailed Student's t test, and $p < 0.05$

was considered statistically significant. Significant differences are presented as following: *p < 0.05; **p < 0.01 *** p < 0.001. All experiments were done with a minimum of 3 independent experiments. Data are shown as mean ± SEM. Statistical analysis was performed with Prism 5 for Windows (GraphPad Software, Inc.) using the student's t-test and chi-square test and with PASW Statistics 18 for Windows (SPSS, Inc.) for the Kaplan-Meier analysis and Cox regression. Student's t-tests were 2-tailed. A p-value of <0.05 is considered significant.

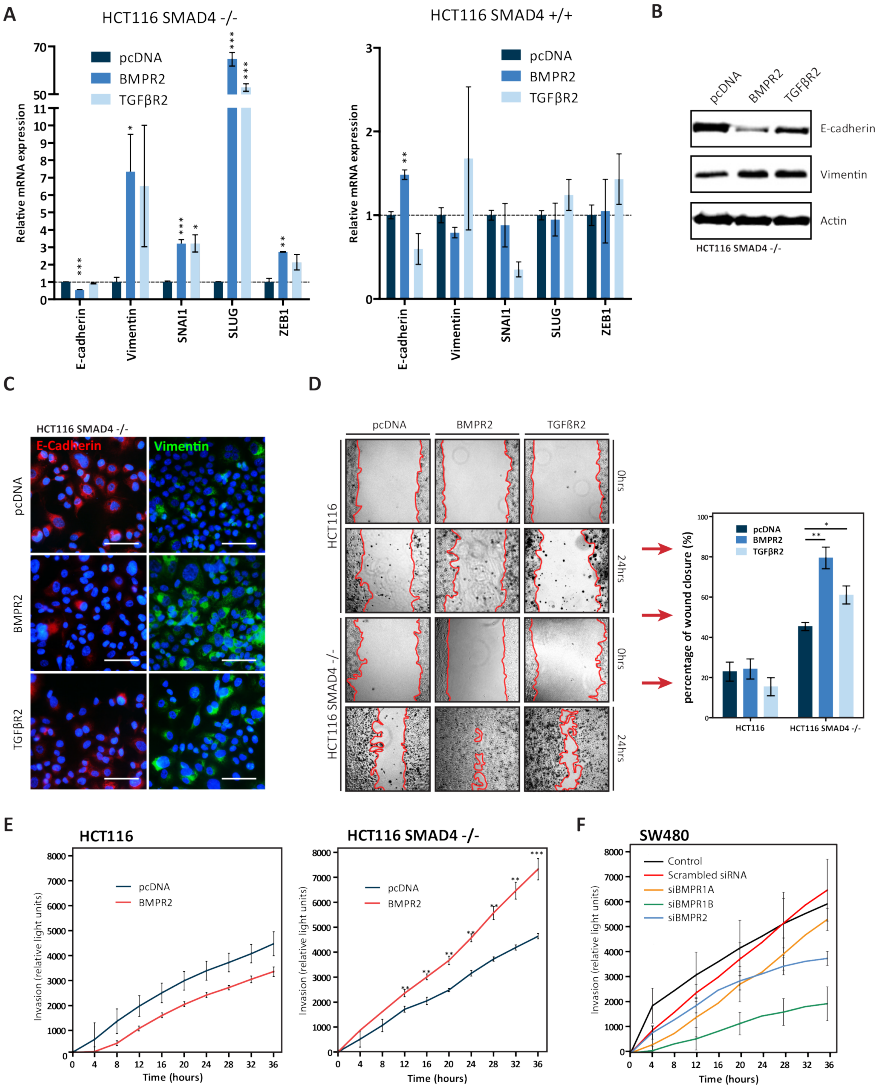
Results

SMAD4 loss switches BMP signaling from tumor suppressive to pro-metastatic

In order to investigate the effects of BMP signaling in the context of SMAD4 loss we used the isogenic CRC cell lines HCT116 and HCT116 SMAD4-/-. HCT116 cells are known to express low levels of BMPR2 and transfection of BMPR2 results in a major increase in SMAD4-dependent (canonical) BMP signaling activity measured using a BRE-luc reporter construct as we have shown previously[1]. Transfection of BMPR2 in HCT116 SMAD4 -/- cells does not lead to an increase in BRE-luciferase activity (***Figure S1A&B***). Activation of BMP signaling in SMAD4 -/- cells using BMPR2 transfection results in a decrease in E-cadherin mRNA expression and an increase in Vimentin and the transcription factors SNAI1, SLUG1 and ZEB1 suggesting induction of Epithelial-to-Mesenchymal Transition (EMT) (***Figure 1A***). In contrast, activation of BMP signaling in the SMAD4 expressing HCT116 cells does not result in EMT, but increases E-cadherin expression suggesting a change towards a more epithelial like state. TGFβR2 transfection is used as a control as it is known to induce EMT. To confirm the changes seen at mRNA level we checked for protein expression changes in E-cadherin and Vimentin using immunoblot analysis and fluorescent immunocytochemistry and saw similar

Figure 1. SMAD4-independent BMP signaling induces EMT, migration and invasion

(A) Activation of BMP and TGFβ signaling in HCT116 SMAD4-/- and HCT116 cells using BMPR2 and TGFβR2 transient transfection. q-PCR results, corrected for GAPDH expression, are presented and pcDNA control values are set to one. (B) Immunoblot analysis of E-cadherin and Vimentin protein expression. Actin is used as a loading control. (C) Immunofluorescence staining of E-cadherin and Vimentin protein expression. DAPI is used to visualize cell nuclei. Scale bars are 50μm. (D) Wound healing assay showing the wound healing after 24 hrs. HCT116 and HCT116 SMAD4-/- cells were transiently transfected with pcDNA, BMPR2 or TGFβR2 and a scratch was made in confluent



cells. (E) Invasion of HCT116 and HCT116 SMAD4-/- cells measured over a 36 hour time period in a matrigel-transwell system. Cells were transiently transfected with pcDNA or BMPR2, labeled with the fluorescence label Cell tracker, and seeded in the upper chamber of a transwell system. (F) Invasion of SW480 cells transfected with either siRNA against BMPR1A, BMPR1B, BMPR2 or scrambled siRNA. Scrambled siRNA was used as a control. Non-transfected SW480 cells were used as an additional control. *Student t-tests were performed using the corrected means of the individual experiments (*<0.05, **<0.001, ***< 0.001). Data is presented as mean ±SEM.*

effects at the protein level as seen at mRNA level (***Figure 1B&C***). Inhibition of BMP signaling using the small molecule BMPR1A inhibitor LDN-193189 in SMAD4 -/- cells results in a change to more epithelial-like cells (***Figure S1C***). We also reconstituted SMAD4 expression in SMAD4 negative CRC cells and observed a change into more epithelial-like cells based on E-cadherin and Vimentin RNA expression (***Figure S1D***).

To assess changes in the functional aspects of the cancer cells we used scratch wound healing and matrigel-transwell-invasion assays. After BMPR2 transfection an increase in migration and invasion can be seen in the HCT116 SMAD4-/- cells, but not in the SMAD4 positive cells (***Figure 1D&E***). We also reduced BMP signaling by silencing individual BMP receptors in the SMAD4 negative cell line SW480 and measured invasion over 36 hours. SW480 cells, in contrast to HCT116 cells, express high levels of BMP receptors, making them more suitable for BMP receptor silencing experiments[1]. Knockdown of BMP receptor expression results in a decrease in invasion (Invasion assay shown in ***Figure 1F***, ***Figure S1*** shows western blots of BMP receptor knockdown). Knockdown of BMPR1B leads to the most pronounced decrease in invasion, followed by knockdown of BMPR2.

Critical experiments were repeated using different BMP activation methods and different SMAD4 negative cell lines to avoid cell line specific bias (***Figure S1E-H***). Overall, these data suggest that BMP signaling switches to a pro-metastatic pathway upon SMAD4 loss.

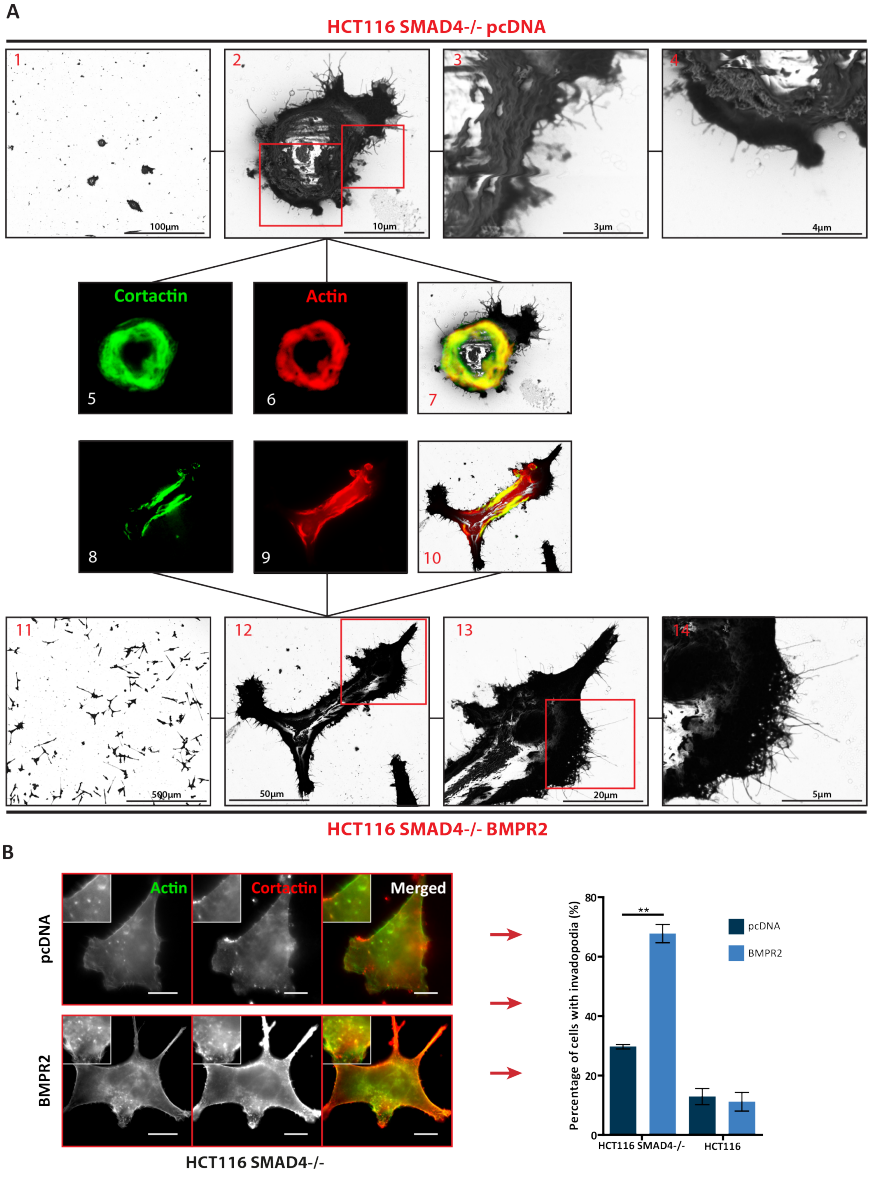
SMAD4-independent BMP signaling changes cell shape and increases invadopodia

Phase-contrast images of SMAD4-independent BMP activation showed changes in cell-shape with an increase in small cell-protrusions (data not shown). To analyze these protrusions we used a new technique where scanning-electron-microscopy

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Figure 2. SMAD4-independent BMP signaling increases invadopodia formation.

(A) HCT116 SMAD4-/- cells were grown on ITO slides coated with poly-L-lysin and gelatin. Cells were transiently transfected with either pcDNA or BMPR2. After 24 hrs the cells were fixed and stained for cortactin (mouse-anti-cortactin and anti-mouse-AlexaFluor 488nm) and actin (phalloidin-594nm). The ITO slides were placed in a Simultaneous Correlative Scanning Electron and High numerical aperture (High-NA) Fluorescence microscope. Figures 1-7 are pcDNA samples. Figures 8-14 are BMPR2 samples. Figures 1-4 and 11-14 show that BMPR2 transfected cells exhibit more cell protrusions. Figures 5-10 show the change in cell shape and actin/cortactin distribution after BMPR2 transfection. (B) Phalloidin-488 was used to stain for actin and mouse-anti-cortactin plus Anti-mouse-AlexaFluor 594 for visualization for cortactin. Prior to staining HCT116 and HCT116



SMAD4-/-cells were transiently transfected with either pcDNA or BMPR2. Scale bar is 5µM. This figure only shows the HCT116 SMAD4-/- cells. (C) Quantification of the experiment presented in figure 2B. Cells with co-localisation of cortactin and actin were counted as having invadopodia. A cell showing one or more invadopodium was counted as positive.100 cells per condition were analyzed. *Student t-test was performed using the means of the individual experiments (*<0.05, **<0.01, ***< 0.001). Data is presented as mean ±SEM.*

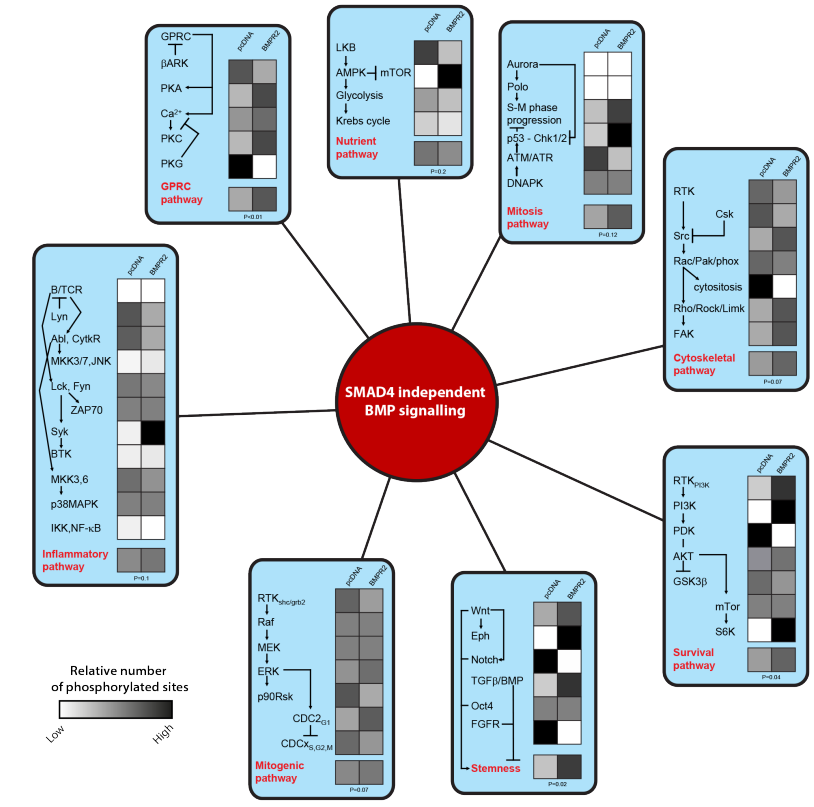
(SEM) and wide-field immunofluorescence can be combined simultaneously.[18, 19] SMAD4-independent BMP signaling was activated by transfection of BMPR2 in HCT116 SMAD4-/- cells which resulted in more spindle like cells (***Figure 2A***) with polarized distribution of actin and cortactin. There is also an increase in the number and size of protrusions. To confirm this observation we used an established and recognized technique in which co-localization of actin and cortactin molecularly identifies the presence of invadopodia[20]. We calculated the amount of cells with invadopodia in the BMPR2 transfected cells and compared this to the control. A higher percentage of cells with invadopodia are seen in the condition where SMAD4-independent BMP signaling is activated (***Figure 2B&C***). A separate analysis of cell shape changes shows that BMP activation in the SMAD4-/- cells results in more spindle-shaped cell morphology typical of mesenchymal cells (***Figure S2***).

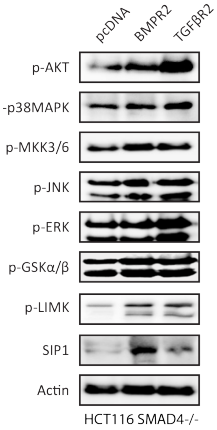
Comprehensive analysis of the SMAD4-independent BMP signaling cascade using kinome profiling

In order to assess the kinase activity changes associated with SMAD4-independent BMP signaling, HCT116 SMAD4-/- cells were transfected with BMPR2 or the empty pcDNA vector and subsequently a 'PepChip' array was performed [21, 22] (***Figure S3***). The most prominent effects seen in the peptide arrays were activation of signaling pathways responsible for cell migration, cell survival and proliferation (***Figure 3A***). To establish the validity of the kinome profiling data the most consistent kinase activity changes were confirmed by immunoblotting with

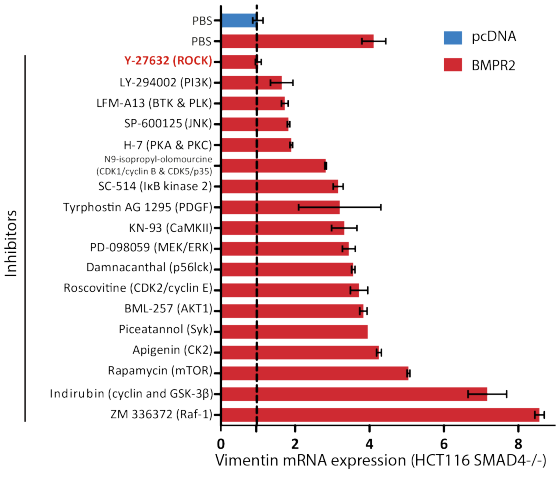
Figure 3. Peptide array analysis of SMAD4-independent BMP signaling

(A) HCT116 SMAD4-/- cells were transiently transfected with either pcDNA or BMPR2. A PepChip peptide array was performed as described in the supplementary methods. In the peptide array phosphorylation changes in the substrates of 960 kinases were measured. The results were categorized according to well-known signaling pathways. A selection of the individual changes of specific targets within the pathway, as well as the changes in the whole pathway, is presented. For a full list of the kinome profiling changes see Figure S4. (B) Validation of the results of Kinome profiling using immunoblotting. (C) HCT116 SMAD4-/- cells were transiently transfected with either pcDNA or BMPR2. Subsequently cells were treated with different kinase inhibitors from a commercial inhibitor screening kit. The IC50 (can be found in supplementary methods) was used for each inhibitor. RNA was isolated and q-PCR was performed for Vimentin expression. GAPDH was used as a control. The Vimentin expression is presented as relative to the Vimentin expression in the control condition. *Student t-tests were performed using the corrected means of the individual experiments (*<0.05, **<0.001, ***< 0.001). Data is presented as mean ±SEM.*


SMAD4 independent BMP signalling
GPRC
βARK
PKA
Ca2+
PKC
PKG
GPRC pathway
LKB
AMPK
mTOR
Glycolysis
Krebs cycle
Nutrient pathway
Aurora
Polo
S-M phase progression
p53 - Chk1/2
ATM/ATR
DNAPK
Mitosis pathway
RTK
Csk
Src
Rac/Pak/phox
cytositosis
Rho/Rock/Limk
FAK
Cytoskeletal pathway
B/TCR
Lyn
Abl, CytkR
MKK3/7,JNK
Lck, Fyn
ZAP70
Syk
BTK
MKK3,6
p38MAPK
IKK,NF-κB
Inflammatory pathway
RTKPDK
PI3K
PDK
AKT
GSK3β
mTor
S6K
Survival pathway
RTKShc/grb2
Raf
MEK
ERK
p90Rsk
CDC2G1
CDCXG,G2,M
Mitogenic pathway
Wnt
Eph
Notch
TGFβ/BMP
Oct4
FGFR
Stemness
pcDNA
BMPR2
Relative number of phosphorylated sites
Low
High


pcDNA
BMPR2
TGFβR2
p-AKT
-p38MAPK
p-MKK3/6
p-JNK
p-ERK
p-GSKα/β
p-LIMK
SIP1
Actin
HCT116 SMAD4-/-

C


pcDNA
BMPR2
Inhibitors
PBS
PBS
Y-27632 (ROCK)
LY-294002 (PI3K)
LFM-A13 (BTK & PLK)
SP-600125 (JNK)
H-7 (PKA & PKC)
N9-isopropyl-olomoucine (CDK1/cyclin B & CDK5/p35)
SC-514 (IκB kinase 2)
Tyrphostin AG 1295 (PDGF)
KN-93 (CaMKII)
PD-098059 (MEK/ERK)
Damnacanthal (p56lck)
Roscovitine (CDK2/cyclin E)
BML-257 (AKT1)
Piceatannol (Syk)
Apigenin (CK2)
Rapamycin (mTOR)
Indirubin (cyclin and GSK-3β)
ZM 336372 (Raf-1)
0
2
4
6
8
Vimentin mRNA expression (HCT116 SMAD4-/-)

activation-specific antibodies against pAKT, pGSKα/β, pp38MAPK, pMKK3/6, pJNK, pERK, p-LIMK and SIP1 (***Figure 3B***).

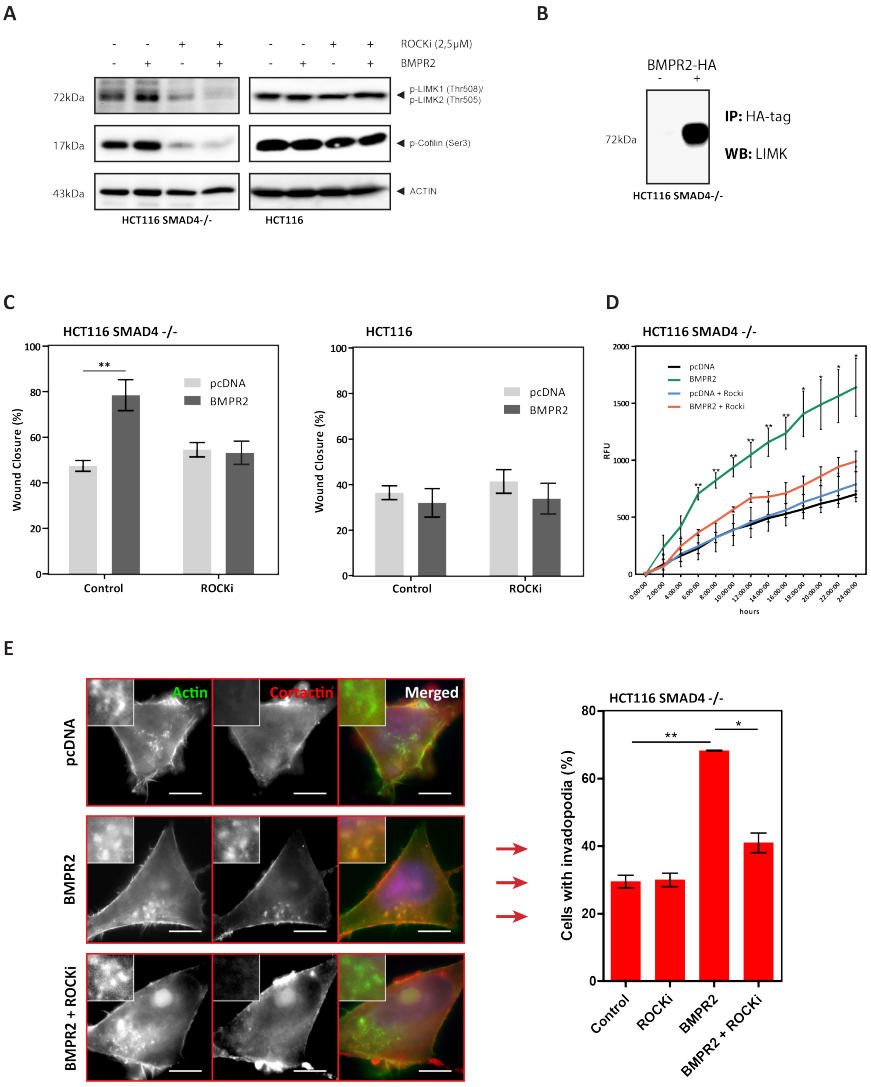
We also activated non-canonical SMAD4-independent TGFβ signaling by transfecting TGFβR2 into HCT116 SMAD4-/- cells. Our result show that SMAD4-independent BMP signaling is similar to SMAD4-independent TGFβ signaling although the activation and phosphorylation of AKT is more prominent in cells transfected with TGFβR2 than with BMPR2. Both signaling pathways are comparable in activating the WNT pathway, but SMAD4-independent BMP signaling has more effect on the expression of SIP1/ZEB2, a member of the δEF-1 family of two-handed zinc finger nuclear factors associated with EMT.

SMAD4-independent BMP signaling induces EMT and invasion via the ROCK pathway

Having established that SMAD4-independent BMP signaling induces EMT, migration and invasion, and identified the downstream signaling pathways involved, we set out to screen for which of these pathways is primarily responsible for the induction of EMT. We induced SMAD4-independent BMP signaling using BMPR2 transfection in SMAD4 negative cells, treated the cells with a series of kinase inhibitors and assessed Vimentin expression by q-PCR. The use of pharmacological inhibitors has the advantage that inhibitors can be relatively easily further tested as potential new CRC treatment options. Based on the reduction of Vimentin expression (***Figure 3C***) the ROCK inhibitor Y-27632 is the most potent inhibitor of BMP induced EMT.

Figure 4. SMAD4-independent BMP signaling activates the Rho/ROCK/LIMK pathway

(A) Immunoblot analysis of HCT116 and HCT116 SMAD4-/- cells. Cells were transiently transfected with either BMPR2 or pcDNA vector. Subsequently cells were treated with 2.5μM of ROCK inhibitor or PBS. Blots were incubated with antibodies against pLIMK1/2 and p-Cofilin. Actin was used as a loading control. (B) HCT116 SMAD4-/- cells were transfected with BMPR2-HA (plasmid encoding both BMPR2 and HA-tag). Cells were lysed and immunoprecipitation was performed using HA-tag antibodies and protein A agarose beads. Subsequently western blot analysis was performed and blots were exposed to LIMK antibodies. (C) Wound healing assay of HCT116 and HCT116 SMAD4-/- cells transiently transfected with either pcDNA or BMPR2 and treated with either 2.5μM ROCK inhibitor or PBS. After 24hrs wound healing was quantified. (D) Invasion of HCT116 SMAD4-/- transfected with either pcDNA or BMPR2. Cells were labeled with Celltracker, seeded in the upper chamber of a transwell system and invasion was measured



every two hours for 24 hrs. (E) Immunofluorescence pictures visualizing invadopodia by showing co-localization of actin and cortactin. Actin was visualized using phalloidin-488. Mouse-anti-cortactin plus Anti-mouse-AlexaFluor 594 was used to visualize cortactin. HCT116 SMAD4-/- cells were transiently transfected with either pcDNA or BMPR2. Subsequently cells were treated with either 2.5µM of Rock inhibitor or PBS for 24 hrs. For quantification, 100 cells per condition were counted. If a cell contained co-localization of cortactin and actin it was counted as invadopodia positive. *Student t-tests were performed using the corrected means of the individual experiments (*<0.05, **<0.01, ***< 0.001). Data is presented as mean ±SEM.*

SMAD4-independent BMP signaling activates the Rho/ROCK/LIMK pathway

Our next step was to investigate the connection between the BMP pathway and the ROCK pathway and to further evaluate the effects of ROCK inhibition.

First we further assessed the role of SMAD4 in BMP-induced ROCK pathway activation. Activation of the BMP pathway in HCT116 SMAD4 -/- cells leads to an increase the phosphorylation of the Rho/ROCK downstream targets LIMK1/2 and Cofilin (***Figure 4A***), but not in SMAD4 positive cells. Treatment with the ROCK inhibitor Y-27632 (2.5μM) leads to a decrease of phosphorylated ROCK downstream targets and an abolition of the effect of BMP pathway activation on the ROCK pathway in SMAD4 -/- cells. Previous in vitro studies using Y-27632 have used various concentrations depending on the cell type (1-20 μM)[23]. In our experimental set-up 2.5 μM was the lowest concentration in which a major p-Cofilin downregulation could be seen (***Figure S4A***). A direct connection between the BMP and ROCK pathway was investigated by transfecting cells with an HA-tagged BMPR2 construct, immunoprecipitating BMPR2 using anti-HA antibodies, and immunoblotting for LIMK (***Figure 4B***). This suggests a direct association between LIMK and BMPR2 in these cells.

Y-27632 abolishes the increase in wound healing, invasion and invadopodia seen after the activation of SMAD4-independent BMP signaling, showing that the effects of the BMP pathway on cell shape and migration/invasion are ROCK dependent (***Figure 4C-E***). We see the same effects using different cell lines and both ligand and receptor-based BMP activation (***Figure S4B-E***).

The ROCK inhibitor Y-27632 reduces metastases in an orthotopic liver metastasis model of SMAD4 negative colorectal cancer

To test the anti-metastatic effects of ROCK inhibition we used a mouse model of liver metastasis in which firefly-luciferase expressing human CRC cells are injected into the spleens of immunocompromised CD-1 Nu mice. The development of liver metastases was evaluated weekly using bioluminescence scans and after seven weeks the mice were sacrificed. We used the SMAD4 negative CRC cell line HT-29 that results more consistently in liver metastases in our hands than HCT116 and SW480. Activation of BMP signaling in HT-29 also leads to the same increase in invasion without changes in cell viability in vitro comparable to other SMAD4 negative cell lines (***Figure S5A-C***). Mice received either 2.5mg/kg

of Y-27632 dissolved in PBS or PBS i.p. every other day. 8/9 of the control mice compared to 3/9 of the Y-27632 treated mice developed liver metastases (***Figure 5A***). There is a significant difference in total luminescence at week 7 between the control mice and the Y-27632 treated mice (***Figure 5B***). Quantification of the total whole mouse scans is closely correlated with the quantification of ex-vivo liver scans (R^2=0.95) showing that liver metastasis formation can be followed using in vivo whole mouse scans in this model (***Figure S5D***). Multiple random sections per liver were analyzed microscopically using haematoxylin-eosin. Metastases could be found in 8/9 of the control mice compared to 3/9 of the Y-27632 treated mice (***Figure S5G***). Mice treated with Y-27632 had a significantly lower liver weight and volume (***Figure S5E&F***).

SMAD4-independent BMP signaling leads to a poorer prognosis

Several studies have consistently shown that loss of SMAD4 expression in CRC is associated with a worse prognosis in patients[24, 25]. Our in vitro data suggest that this could be the result of SMAD4-independent BMP signaling. In a previous analysis of the expression of elements of the BMP pathway in CRC we observed

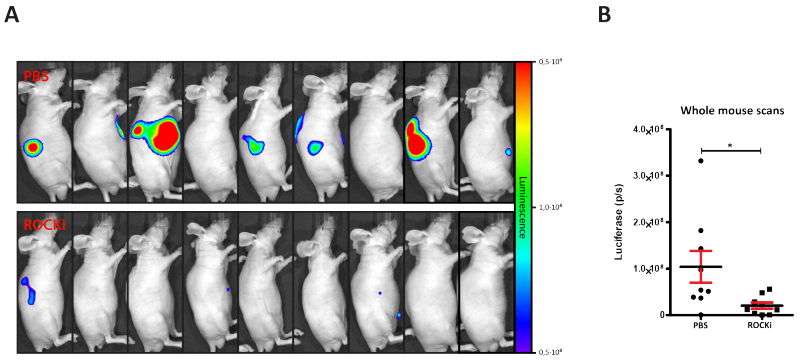


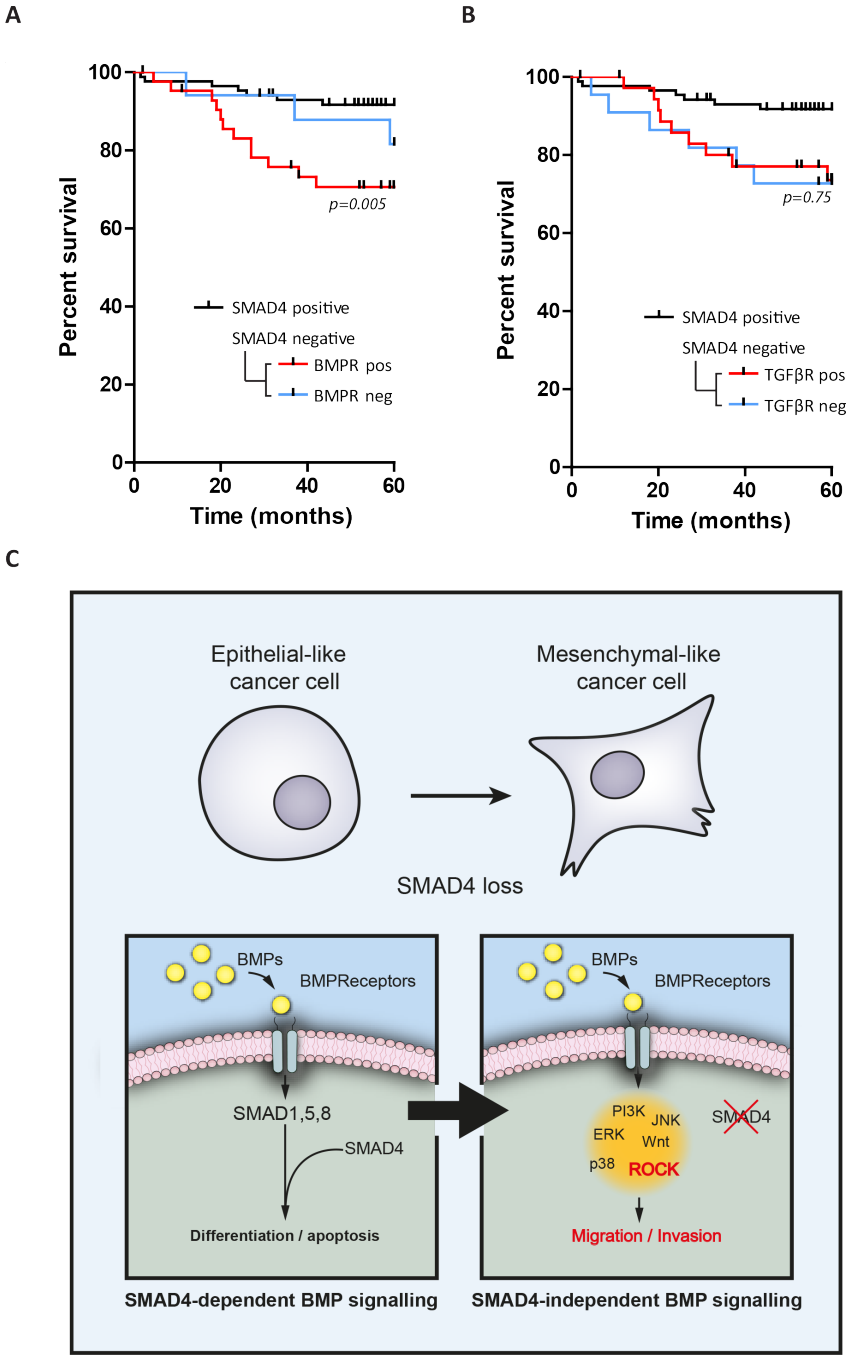
Figure 5. The ROCK inhibitor Y-27632 reduces colorectal cancer metastases in an orthtopic mouse model of liver metastasis. 10^5 HT-29 cells, which naturally express no SMAD4, stably transfected with an optimized firefly luciferase construct were injected into spleens of CD-1 Nu mice suspended in 50µl of medium:matrigel (50:50). Mice were treated with i.p. injection of either PBS (n=9) or Rock inhibitor (2.5mg/kg) (n=9) every other day. Weekly bioluminescence scans were used to visualize liver metastasis formation. 7 weeks after the intrasplenic injection the mice were sacrificed. (A) Bioluminescence whole mouse scans at week 7. (B) Quantification of the whole mouse scans at week 7. Comparison is made using a Student t-test.

frequent aberrant expression of the BMP receptors[1]. We would therefore expect that concurrent loss of BMP receptor expression would lead to a better prognosis in patients with SMAD4 negative cancers by reducing this SMAD4-independent BMP signalling. In other words, the poor prognosis associated with loss of SMAD4 would be expected to be dependent on the presence of normal BMP receptor expression in the tumor.

We mapped the expression patterns of SMAD4 and BMP receptors in patients using immunohistochemistry for SMAD4, BMPR1A, BMPR1B and BMPR2 in a tissue microarray in 145 CRCs. Immunostaining was scored according to the system described in the supplementary methods and cancers were subsequently ascribed to one of four categories based on the expression of SMAD4 and BMP receptors. Cancers were divided into SMAD4 positive and SMAD4 negative and these two groups were then subdivided into BMP receptor positive and BMP receptor negative. A patient was considered BMP receptor negative if any one of the three BMP receptors had aberrant expression.

40.7% of the tumors had negative SMAD4 immunostaining, which is consistent with other studies (***Figure S6A&B***). Subdivision of the SMAD4 negative cancers into BMP receptor positive and BMP receptor deficient revealed that the presence of normal BMP receptor expression in the SMAD4 negative tumors is associated with a poor prognosis (p=0.005) (***Figure 6A***), suggesting that SMAD4-independent BMP signaling leads to a worse prognosis in patients. Conversely patients with SMAD4 negative tumors with impaired BMP receptor expression have a survival similar to those with normal SMAD4 expression. The poor prognosis found in cancers with SMAD4-independent BMP signaling is not independent of tumor stage. This is probably because BMP receptor expression in SMAD4 negative cancers is weakly associated with stage (***Figure S6C***). Interestingly, there is no correlation between the poor prognosis seen in SMAD4 negative cancers and TGFβ

Figure 6. The influence of BMP receptor expression in SMAD4 negative cancers. (A) CRC tissue from 145 patients was stained for SMAD4, BMPR1A, BMPR1B and BMPR2 in a tissue microarray. Three cores per cancer were evaluated. The graph shows a Kaplan-Meier survival curve based on SMAD4 and BMP receptor expression. SMAD4 loss leads to a poorer survival. Closer investigation of the SMAD4 negative group shows that a subgroup of patients with cancers that express BMP receptors have a poorer prognosis than patients that have reduced BMP receptor expression. (C) Similar analysis as in (B), but based on the expression levels of TGFβ receptors (1&2) instead of BMP receptors.


A
Percent survival
100
80
60
40
20
0
0
20
40
60
Time (months)
p=0.005
SMAD4 positive
SMAD4 negative
BMPR pos
BMPR neg
B
Percent survival
100
80
60
40
20
0
0
20
40
60
Time (months)
p=0.75
SMAD4 positive
SMAD4 negative
TGFβR pos
TGFβR neg
C
Epithelial-like cancer cell
Mesenchymal-like cancer cell
SMAD4 loss
BMPs
BMPReceptors
SMAD1,5,8
SMAD4
Differentiation / apoptosis
SMAD4-dependent BMP signalling
BMPs
BMPReceptors
PI3K
JNK
ERK
Wnt
p38
ROCK
SMAD4
Migration / Invasion
SMAD4-independent BMP signalling

receptor expression (p=0.75) (***Figure 6C***). ***Figure S6C&E*** show TGFβ receptor expression frequencies and stage distribution). These findings are consistent with the conclusion that the poor prognosis seen in patients with SMAD4 negative cancers is due to active SMAD4-independent BMP signaling rather than TGFβ signaling.

Discussion

The data we present show that the BMP signaling pathway, which is a well-known tumor suppressive pathway, changes into a pro-metastatic pathway upon loss of SMAD4. The current paradigm is that the poor survival associated with loss of SMAD4 is the result of effects on TGFβ signaling. We show that BMP signaling in the absence of SMAD4 leads to the activation of a broad range of signaling pathways and enhances invasion and metastasis.

BMP signaling has been previously associated with invasiveness. For example BMP2 increases the motility and invasiveness of gastric cancer cell lines[26] and BMP4 induces EMT in ovarian cancer cell lines[27], but the accepted view of BMP signaling is that of a tumor suppressor[28] and a barrier to tumor progression and metastasis (Reviewed by[29]). Here we show that BMP signaling can be tumor promoting by increasing EMT and invasiveness in CRC and we show that this depends on the expression of SMAD4. A similar dual role has been seen with TGFβ which is initially tumor suppressive but switches to tumor promoting in the later stages of cancer progression[30].

The activation of SMAD4-independent BMP signaling also results in an increase in invadopodia. Invadopodia are transformed podosomes with the ability to degrade the extracellular matrix[31] allowing a transition to a more mesenchymal-like state[32]. Identification of invadopodia can be performed by co-localization of cortactin and actin[20] which we used in our assay. We performed imaging in the same cells using a newly developed combined simultaneous correlative scanning electron microscope and high numerical aperture (high-NA) fluorescence microscope that allowed us to visualize the distribution of actin and cortactin using fluorescent microscopy and the cell shape protrusions using scanning

electron microscopy simultaneously[19]. The fluorescent image does not show the typical spots of co-localized actin and cortactin seen at the tip of invadopodia with confocal microscopy because of the use of a wide-field fluorescent microscope that doesn't eliminate the light outside the focal plane. The combined fluorescent and electron microscope images do show an increase in cell protrusions and a change in the actin/cortactin distribution, implying a change in cell polarity associated with EMT[33] after activation of SMAD4-independent BMP signaling.

Kinome profiling analysis revealed that SMAD4-independent BMP signaling activates a complex network of kinases. Among others, p38MAPK, ERK, JNK, PI3K, AKT, GSK and LIMK are activated, targets of pathways involved in inflammation, mitosis, cell survival, proliferation, stemness and cytoskeleton rearrangement.

The activation of p38MAPK, ERK and JNK is consistent with previous reports of SMAD4-independent BMP7 signaling[34]. Other changes have not been described before and include inhibition of Glycogen synthase kinase (GSK) α/β which is part of the AXIN/APC/GSK complex responsible for degrading β-catenin. Inhibiting GSK leads to an increase in β-catenin and thereby an increase in WNT signaling activity. BMP signaling is known to oppose WNT signaling[15], but not to enhance it as we observe here in SMAD4 negative cells. While we show activation of AKT due to activation of BMP signaling in SMAD4 negative cells, BMP signaling has been shown to inhibit proliferation via dephosphorylation of AKT in adrenocortical carcinomas[35].

The Rho/ROCK/LIMK pathway is involved in the reorganization of the cytoskeleton and in cell migration. BMP7 is known to activate the Rho/ROCK pathway in fibroblasts[36]. It is also known to act downstream of BMP in neural development[37] and osteogenesis[38], but is not known to be a part of the BMP signaling cascade in epithelial cells. BMPR2 is known to directly interact with LIMK[39], which we confirmed in our experimental set-up. This may explain the connection between SMAD4-independent BMP signaling and the Rho/ROCK/LIMK pathway in CRC. We show that the Rho/ROCK/LIMK pathway is a critical component of the SMAD4-independent BMP signaling driving invasion and metastasis and represents a sensitive pharmacological target by which to inhibit this. Clinically ROCK is an interesting target for treatment as ROCK inhibitors, known by the

name Fasudil, have already been used in patients. The use of Fasudil in patients with pulmonary hypertension and cerebral vasospasm did not lead to severe side effects[40, 41]. However, further safety assessment is imperative considering the fact that ROCK inhibition in mouse embryos resulted in developmental defects.[42]
Loss of SMAD4 protein expression is one of the best molecular prognostic markers in CRC[24]. This is thought to be due to loss of antimetastatic SMAD4-dependant TGFβ signaling[9]. However, when we subdivide patients with SMAD4 negative CRC into two groups according to the expression of BMP receptors, only the SMAD4 negative tumors that express high levels of BMP receptors have a poorer prognosis. Those with low BMP receptor expression have a prognosis similar to patients with SMAD4 positive CRC. In a similar analysis TGFβ receptor expression shows no correlation with prognosis. BMPR and TGFβ receptor expression in SMAD4 negative CRC are both associated with more advanced stage, so the association with prognosis is not independent of stage. Nevertheless, we feel this provides preliminary evidence in humans that the BMP and not the TGFβ pathway is responsible for the poor prognosis associated with loss of SMAD4. It also suggests that SMAD4 is a good prognostic marker due to its central role in the BMP pathway and not simply because it is a surrogate marker for aneuploidy as has been suggested[43]. Furthermore SMAD4 can potentially be refined as a prognostic marker by analysis of BMP receptor expression.

To conclude we have shown that upon SMAD4 loss BMP signaling becomes pro-metastatic through activation of a broad program of SMAD4-independent BMP signaling. This consists of the activation of various downstream signaling pathways including the Rho/ROCK/LIMK pathway. Our patient data suggest that BMP signaling and not TGFβ signaling is responsible for the poor prognosis associated with SMAD4 loss and that concurrent analysis of BMPR expression can potentially improve SMAD4 as a prognostic marker. These results would suggest that ROCK inhibitors might be a novel therapeutic approach in the subgroup of colorectal cancers that have lost SMAD4, a subgroup with a poor prognosis that respond poorly to current therapy.

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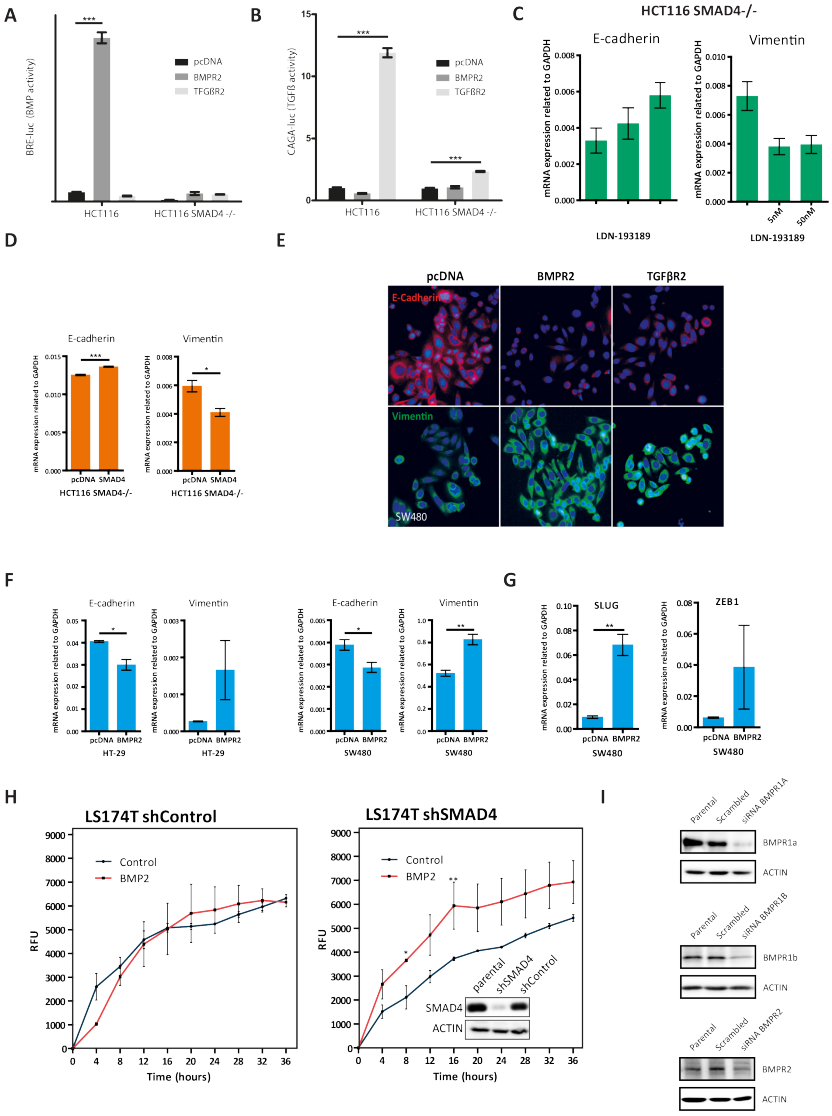


Figure S1: (A+B) HCT116 and HCT116 SMAD4-/- cells were transiently transfected with either BMPR2, TGF-βR2 or the empty control plasmid pcDNA. After 24 hours BMP (BRE-luc) and TGF-β (CAGA-luc) activity was measured using luciferase reporter constructs.
(C) HCT116 SMAD4-/- cells were treated with 5nM and 50nM of LDN-193189 for 24hrs and compared with a control. Subsequently mRNA expression levels of E-cadherin and Vimentin were measured using qRT-PCR. GAPDH was used a control. (D) HCT116 SMAD4-/- cells were transfected with either pcDNA or a plasmid expressing WT SMAD4.
(Figure S1 continued) mRNA levels of E-cadherin and Vimentin were measured using qRT-

PCR. (E) Fluorescent immunocytochemistry for E-cadherin and Vimentin in SW480 cells. Cells were transfected with either pcDNA or BMPR2. (F) mRNA expression levels of E-cadherin and Vimentin measured using qRT-PCR in SW480 and HT-29 (SMAD4 negative) cells transfected with either pcDNA or BMPR2. GAPDH was used as a control. (G) SW480 (SMAD4 negative) cells were transfected with either pcDNA or BMPR2. mRNA expression levels of the EMT associated transcription factors SLUG and ZEB1 were measured using qRT-PCR. GAPDH was used as a control. (H) Invasion of LS174T and LS174t shSMAD4 cells measured over a 36 hour time period in a matrigel-transwell system. Cells were labeled with a fluorescence label Cell tracker, and seeded in the upper chamber of a transwell system and subsequently treated with 100ng/ml BMP2 or PBS (control). (I) Western blot analysis of the siRNA knockdown of BMPR1A, BMPR1B and BMPR2 in SW480 cells. Actin is used as a loading control. Cells transfected with non-targeting scrambled siRNA were used as a control.
Student t-tests were performed as appropriate (<0.05, **<0.01, ***< 0.001). Data is presented as mean ±SEM.*

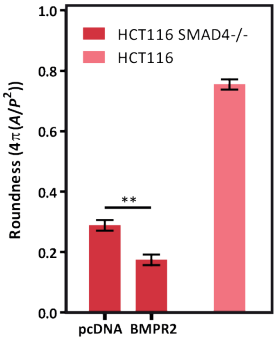


Figure S2: HCT116 SMAD4-/- cells were transfected transiently with either BMPR2 or control pcDNA. The roundness was calculated as described in the supplementary methods. HCT116 cells were used as a SMAD4 positive control.
Student t-tests were performed as appropriate (<0.05, **<0.01, ***< 0.001). Data is presented as mean ±SEM*

Figure S4 ***(next page)*****:** (A) SW480 cells were treated with different concentrations of the ROCK inhibitor Y-27632 to evaluate the optimal treatment concentration. Cells were lysed and samples were analyzed by immunoblotting using antibodies against p-Cofilin. Actin was used as a loading control. At 2.5μM the ROCK inhibitor leads to near maximal inhibition of p-Cofilin. (B) SW480 cells were transfected with either BMPR2 or pcDNA. Subsequently the cells were treated with 2,5μM of the ROCK inhibitor Y-27632 in PBS or PBS alone. Cells were lysed and samples were analyzed by immunoblotting. Blots were exposed to antibodies against p-LIMK1/2 and p-Cofilin. Actin was used as a loading control. (C) SW480 cells were treated with Noggin (500ng/ml), LDN-193189 (5nM) or BMP-2 ligands (100ng/ml) for 24hrs. Afterwards cells were lysed and immunoblotting for protein expression of p-Cofilin was performed. Actin was used as a loading control. (D) SW480 cells were transfected with either pcDNA or BMPR2. Afterwards a wound-healing assay was performed to measure cell migration. The image shows a quantification of the wound closure after 24 hours. (E) Invasion assay of SW480 cells treated with either PBS (control), BMP-2 (100ng/ml), ROCK inhibitor (2.5μM) or a combination of BMP-2 (100ng/ml) plus a ROCK inhibitor (2.5μM).

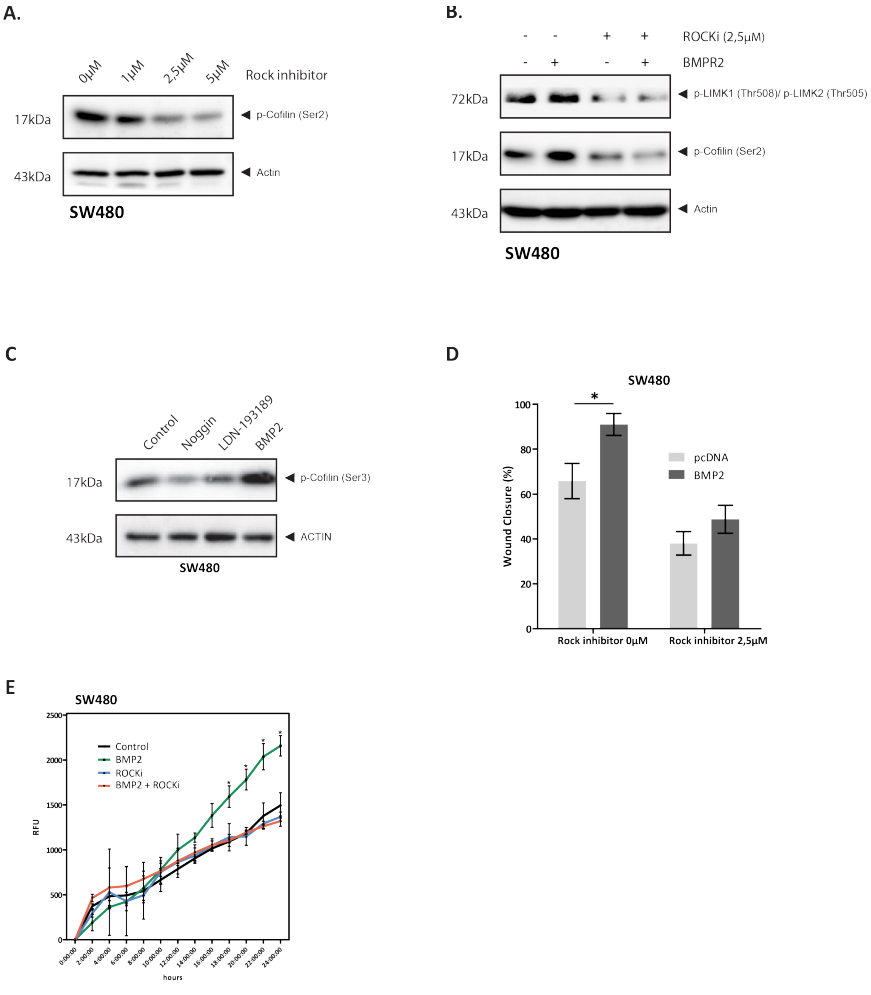
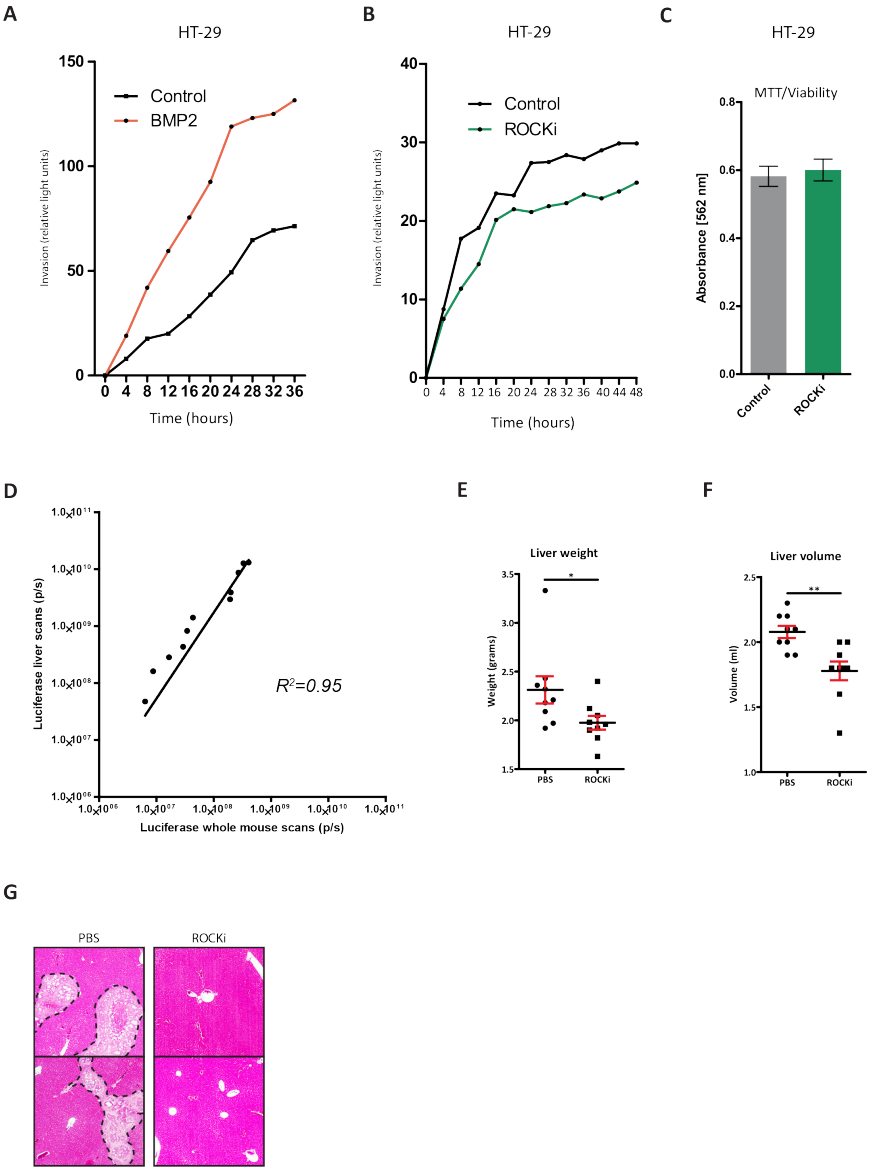


Figure S5 ***(right page)*** **:** (A) HT-29 cells were labeled with Celltracker and seeded in the upper chamber of a transwell system. Cells that had migrated through matrigel into the lower chamber were measured every two hours for 48 hrs in total. During the invasion assay cells were treated with PBS or 100ng/ml BMP-2. (B) HT-29 cells were labeled with Celltracker and seeded in the upper chamber of a transwell system. Cells that had migrated through matrigel into the lower chamber were measured every two hours for 48 hrs in total. During the invasion assay cells were treated with PBS or 2,5µM of the ROCK inhibitor Y-27632 in PBS. (C) HT-29 cells were treated with 2,5µM of the ROCK inhibitor Y-27632 in PBS or PBS alone for 48hrs. Cell viability/



proliferation was measured using a MTT assay. (D) Correlation between the whole mouse scans and the ex-vivo liver scans. The association between the quantification of the two scans is a log regression which follows: *y = 10^(1.51*log(x) -2.86)* (E) Measurement of the weight of the livers. (F) Measurement of the liver volume. (G) Haematoxylin-eosin histology liver sections. Two examples without metastases from the PBS treated group and two examples with metastases from the ROCK inhibitor group are shown.

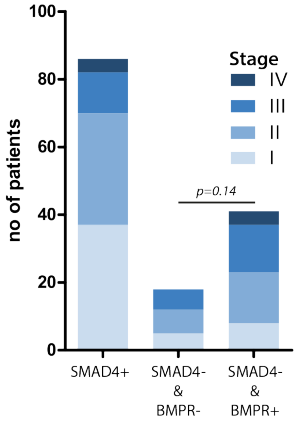
A

		Distribution BMP components		
		BMPReceptor positive	BMPReceptor negative	Total
SMAD4	Negative	36 (24.8%)	23 (15.9%)	59 (40.7%)
	Positive	46 (31.7%)	40 (27.6%)	86 (59.3%)
	Total	82 (56.6%)	63 (43.5%)	145 (100%)

B

		Distribution TGFβ components		
		TGFβReceptor positive	TGFβReceptor negative	Total
SMAD4	Negative	37 (25.5%)	22 (15.2%)	59 (40.7%)
	Positive	56 (38.6%)	30 (20.7%)	86 (59.3%)
	Total	94 (64.1%)	52 (35.9%)	72 (100%)

C



D

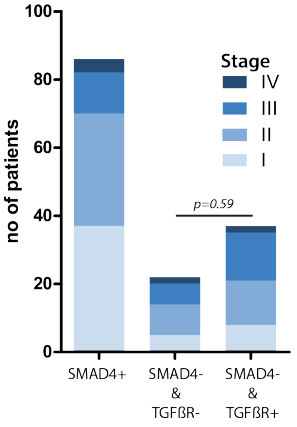


Figure S6: (A) Colorectal cancer tissue of 145 patients was stained for SMAD4, BMPR1A, BMPR1B and BMPR2 using immunohistochemistry. The table shows the distribution of the BMP signaling groups. The cancers were first divided in SMAD4 negative versus SMAD4 negative. Then the groups were subdivided into "BMPR positive" or "BMPR negative" subgroups. (B) Analysis similar to (A) only using TGFβ receptor expression instead of BMP receptor expression. (C) Distribution of the tumor stages within the groups based on SMAD4 and BMP receptor expression. (D) Distribution of the tumor stages within the groups based on SMAD4 and TGFβ receptor expression.

Supplementary tables Kinase inhibitors

Substance	Function/inhibits	Concentration	IC50	Company
hBMP-2	BMP ligand	100ng/ml	-	R&D systems
hNoggin	BMP	500ng/ml	-	R&D systems
LDN-193189	BMPR1A	5nM	5nM	AxonMedchem
Y-27632	ROCK	2.5μM	2-10μM	Cayman/Millipore
PD-098059	MEK/ERK	10μM	10μM	Cayman/Sigma
SP-600125	JNK	20μM	5-20 μM	Cayman/Sigma
LY-294002	PI3K	10μM	10μM	Cayman/Sigma
H-7	PKA and PKC	10μM	10μM	Cayman
SC-514	IκB kinase 2 (IKK2)	12μM	3-12μM	Cayman
Tyrphostin AG	PDGF	0.5μM	0.5μM	Cayman
Damnacanthal	p56lck	620nM	620nM	Cayman
Piceatannol	Syk	10μM	10μM	Cayman
AG-490	JAK-2	10μM	10μM	Cayman
KN-93	CaMKII	370nM	370nM	Cayman
ML-7	myosin light chain kinase	0.5μM	0.5μM	Cayman
N9-isopropyl-olomourcine	CDK1/cyclin B en CDK5/p35	3μM	2μM and 3μM	Cayman
Roscovitine	CDK2/cyclin E	0.1μM	0.1μM	Cayman
ZM 336372	Raf-1	70nM	70nM	Cayman
LFM-A13	BTK and Polo-like kinase	10μM	2.5μM and 10μM	Cayman
Indirubin	cyclin-dependent kinases and GSK-3β	75nM	75nM and 0.19μM	Cayman
Apigenin	CK2 activity	30μM	30 μM	Cayman
Rapamycin	mTOR	0.1nM	0.1nM	Cayman
BML-257	AKT1	100nM	64nM and 98nM	Cayman

Primers

Gene	Forward (‘5-‘3)	Reverse (‘5-‘3)
hCDH-1	*Purchased from Qiagen cat No: PPH00135F*	
hVimentin	CTGGATTTCCTCTTCGTGGA	CGAAAACACCCTGCAATCTT
hSNAI1	CCTTCTCTAGGCCCTGGCT	AGGTTGGAGCGGTCAGC
hSLUG	TGGTTGCTTCAAGGACACAT	GTGCAGTGAGGGCAAGAA
hZEB1	AAGAATTCACAGTGGAGAGAAGCCA	CGTTTCTTGCAGTTTGGGCATT
hGAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT

List of primary antibodies for immunoblotting:

Protein	Phospho site	Clone	Company	#	Source	Size (kD)	Dilution
E-cadherin	-	G-10	Santa Cruz	sc-8226	mouse monoclonal	120	1:200
Vimentin	-	R28	Cell Signaling	3932	rabbit polyclonal	57.5	1:500
ACTIN	-	I-19	Santa Cruz	sc-1616	rabbit polyclonal	43	1:10000
p-AKT	Thr308	-	Cell Signaling	9275	rabbit polyclonal	60	1:500
p-p38 MAPK	Thr180/Tyr182	3D7	Cell Signaling	9215	rabbit polyclonal	43	1:500
p-MKK3/6	Ser189/Ser207	-	Cell Signaling	9231	rabbit polyclonal	40/41	1:500
p-JNK	Thr183/Tyr185	-	Cell Signaling	9251	rabbit polyclonal	46/54	1:500
p-ERK1/2	Thr202/Tyr204	20G11	Cell Signaling	4376	rabbit monoclonal	42/44	1:500
p-GSKα/β	Ser21/9	-	Cell Signaling	9331	rabbit polyclonal	46/51	1:500
SIP1	-	-	Sigma-Aldrich	SAB1406675	mouse polyclonal	31.6	1:500
p-Cofilin	Ser3	77G2	Cell Signaling	3313	rabbit monoclonal	19	1:500
p-LIMK1/2	Thr508/Thr505	-	Cell Signaling	3841	rabbit polyclonal	72	1:500
BMPR1A	-	H-60	Santa Cruz	sc-20736	rabbit polyclonal	66	1:200
BMPR1B	-	H-44	Santa Cruz	sc-25455	rabbit polyclonal	50	1:200
BMPR2	-	G-25	Santa Cruz	sc-130704	rabbit polyclonal	115	1:200
SMAD4	-	B-8	Santa Cruz	sc-7966	mouse monoclonal	61	1:500

List of primary antibodies for immunohistochemistry:

Protein	Company	#	Source	Antigen retrieval	Blocking	Incubation	Dilution
BMPR1A	R & D systems	MAB24061	mouse monoclonal	Tris/EDTA	goat serum	1 hour at RT	1:100
BMPR1B	R & D systems	MAB5051	mouse monoclonal	Tris/EDTA	goat serum	1 hour at RT	1:100
BMPR2	R & D systems	AF811	goat polyclonal	Citrate	TENG-T	Overnight at 4˚C	1:100
TGFβR1	Santa Cruz	H-100	rabbit polyclonal	Tris/EDTA	goat serum	1 hour at RT	1:100
TGFβR2	Santa Cruz	L-21	rabbit polyclonal	Citrate	TENG-T	Overnight at 4˚C	1:100
SMAD4	Santa Cruz	B-8	mouse monoclonal	Citrate	TENG-T	Overnight at 4˚C	1:100

Chapter 4

The BMP pathway either enhances or inhibits the Wnt pathway depending on the SMAD4 and p53 status in CRC.

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A***im***

Constitutive Wnt activation is essential for colorectal cancer (CRC) initiation but also underlies the cancer stem cell phenotype, metastasis and chemosensitivity. Importantly Wnt activity is still modulated as evidenced by higher Wnt activity at the invasive front of clonal tumours termed the β-catenin paradox. SMAD4 and p53 mutation status and the Bone Morphogenetic Protein (BMP) pathway are known to affect Wnt activity. The combination of SMAD4 loss, p53 mutations and BMP signalling may integrate to influence Wnt signalling and explain the β-catenin paradox.

Methods

We analysed the expression patterns of SMAD4, p53 and β-catenin at the invasive front of CRCs using immunohistochemistry. We activated BMP signalling in CRC cells in vitro and measured BMP/Wnt activity using luciferase reporters. MTT assays were performed to study the effect of BMP signalling on CRC chemosensitivity.

Results

84% of CRCs with high nuclear β-catenin staining are SMAD4 negative and/or p53 aberrant. BMP signalling inhibits Wnt signalling in CRC only when p53 and SMAD4 are unaffected. In the absence of SMAD4, BMP signalling activates Wnt signalling. When p53 is lost or mutated BMP signalling no longer influences Wnt signalling. The cytotoxic effects of 5-FU are influenced in a similar manner.

Conclusion

The BMP signalling pathway differentially modulates Wnt signalling dependent on the SMAD4 and p53 status. The use of BMPs in cancer therapy, as has been proposed by previous studies, should be targeted to individual cancers based on the mutational status of p53 and SMAD4.

Introduction

Mutations in APC (Adenomatous Polyposis Coli) or *CTNNB1 (*β-catenin) result in constitutive activation of the Wnt pathway, an essential first step in the molecular sequence of events underlying the adenoma-carcinoma sequence. However, while *APC/CTNNB1* mutations are identical throughout a clonal tumour, immunohistochemical analysis reveals heterogeneous expression of nuclear β-catenin. Nuclear β-catenin accumulation, indicative of high levels of Wnt pathway activity, is found in tumour cells at the invasive front with lower nuclear β-catenin in the center of the tumour. 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). This results in high levels of Wnt signalling specifically in cells at the invasive front, which underlies the cancer stem cell properties and metastatic potential of these cells (Vermeulen *et al.*, 2010). What these Wnt modulating factors are remains unknown but these nuclear β-catenin expressing cells at the invasive front only arise after an adenoma becomes a carcinoma and in the proximity of the stroma and are thought to be the result of interaction between tumour cell intrinsic and extrinsic factors. Likely tumour cell intrinsic factors are therefore mutations occurring at this point in the adenoma to carcinoma sequence, the commonest of which are SMAD4 and p53 mutations (Cho and Vogelstein, 1992;Sjoblom *et al.*, 2006). SMAD4 and p53 have both already been shown to influence Wnt signalling *in vitro* (Freeman *et al.*, 2012;Kim *et al.*, 2011). How these two molecules influence the Wnt pathway is unknown but is likely to involve modulation of a Wnt interacting pathway, as neither is directly involved in Wnt signalling. The main Wnt-antagonizing pathway in the intestine is the BMP pathway (Wakefield and Hill, 2013). Tumour cell extrinsic, stromal factors that have been proposed from indirect evidence to influence tumour cell Wnt activity include the Forkhead transcription factors acting via, among others, the BMP pathway, HGF, PDGF and COX2 (Fodde and Brabletz, 2007). However, recent research in several tumour types has identified BMP pathway components as stromally produced factors influencing tumour progression (Karagiannis *et al.*, 2013;McLean *et al.*, 2011;Sneddon *et al.*, 2006). While the BMP pathway is classically thought to be tumour suppressive in nature[1] ;Beck *et al.*, 2006;Hardwick *et al.*, 2004),

our previous work in pancreatic cancer would suggest that BMPs can promote invasion in the context of SMAD4 loss (Voorneveld *et al.*, 2013) and others have shown that related molecules (TGFβ) can do the same in the context of aberrant p53.[2] Since high levels of Wnt signalling promote invasion we hypothesized that this may be due to these mutations altering the influence of stromally produced BMPs on Wnt signalling.

The critical interaction between BMP and Wnt signalling in the intestine is exemplified by their roles in crypt-villus homeostasis. In normal intestine the BMP and Wnt pathways interact to control cell fate (Radtke and Clevers, 2005). BMPs that induce differentiation are produced at the top of the villus and antagonize Wnts, which are responsible for a progenitor phenotype, and are produced in the crypt. BMP antagonists from stromal myofibroblasts near the crypt base further ensure that stem cells are not exposed to BMPs, thus both epithelial and stromal cells contribute to BMP signalling (Kosinski *et al.*, 2007). Experiments in transgenic mice show the importance of the BMP pathway in intestinal neoplasia and suggest that this occurs through the influence of the BMP pathway on the Wnt pathway (He *et al.*, 2004).

In summary, we hypothesized that a combination of SMAD4 loss, p53 mutations and BMP signalling may explain the high levels of Wnt signalling seen in CRC cells at the invasive front that is thought to be responsible for the stem-like phenotype, metastatic potential and chemoresistance of these cells. We therefore set out to investigate the influence of BMP signalling on Wnt signalling in CRC, how two of the most common mutations occurring late in the adenoma-carcinoma sequence influence this and how this influences CRC chemosensitivity.

Methods

Patient information (stage I/II CRC)

Formalin-fixed, paraffin-embedded tissues from 94 patients with stage I/II CRC who received surgery between 1980 and 2001 were used for the study. We studied stage I/II cancers because these cancers are at a stage prior to metastasizing. In these cancers one might expect to find both tumours with a low metastatic potential

as tumours with a high metastatic potential. Using cancers with more advanced stage is likely to select for the more aggressive invasive/metastatic tumours where there will be less difference in metastatic potential. Blocks were selected from the archives of the Pathology Department at the Leiden University Medical Center, Leiden, the Netherlands. All samples were handled in a coded fashion, according to National ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

Immunohistochemistry

SMAD4 & β-catenin

Sections were deparaffinized, immersed in 0.3% hydrogen peroxide in methanol for 30 min to block for endogenous peroxidase activity and antigen retrieval was performed in 1xTris/EDTA, pH 9.0, for 30 min at 97˚C. Nonspecific binding sites were blocked with 10% normal goat serum for 10 min. SMAD4 primary antibodies (Santa Cruz, sc-7966, B-8, mouse monoclonal) were diluted in PBS/1%BSA/0.1%Triton (1:400) and incubated for 1h at room temperature. β-catenin primary antibodies (Biosciences, 610154, mouse monoclonal) were diluted in PBS/1%BSA/0.1%Triton (1:400) and incubated overnight at 4˚C. Poly-HRP-Goat-α-Mouse (Immunologic) antibodies were used as secondary antibodies. Peroxidase activity was detected with fastDAB (Sigma-Aldrich). Every staining included a negative control where we used the same protocol but without the primary antibody.

P53, BMP2, BMP4, BMP6 & BMP9

Sections were deparaffinized and immersed in 0.3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was achieved by heating for 10 min to 97˚C in sodium citrate, pH 6.0, and nonspecific binding sides were blocked by incubation with TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% Tween 20, pH 8.0) for 10 min. P53 (sc-126, DO-1, mouse monoclonal), BMP2 (sc-6895, N-4, goat polyclonal), BMP4 (sc-393329, D-6, mouse monoclonal) and BMP6 (sc-27409, D-19, goat polyclonal) primary antibodies were obtained from Santa Cruz. BMP9 antibodies (ab35088, rabbit polyclonal) were obtained from Abcam. All primary antibodies were diluted in PBS/1%BSA/0.1%Triton (1:100). Incubation took place overnight at 4˚C and

the Dako detection system LSAB+ System-HRP was used. Peroxidase activity was detected with fastDAB (Sigma-Aldrich). Every staining included a negative control where we used the same protocol but without the primary antibody. Antibody specificity was tested using western blot analysis on colorectal cancer cell lines and colorectal cancer samples (***Supplementary figure 1***).

Tissue analysis

Analysis was performed in a blinded fashion by two investigators independently. Scoring was done according to the scoring systems supplied in the supplementary methods.

Cell lines

HCT116, DLD-1, SW480, RKO, LS174T, HT-29, and HEK-293 cells were obtained from the ATCC. HCT116 SMAD4-/-, HCT116 p53 R248, HCT116 p53-/- and DLD-1 SIL/+ cells were the kind gift of dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA). A description of the generation of the cell lines is previously published (Sur *et al.*, 2009;Zhou *et al.*, 1998). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland) with 4.5g/l glucose and with 580mg/l L-glutamine. This was supplemented with penicillin (50U/ml) and streptomycin (50µg/ml) and with 10% fetal calf serum (FCS) (Gibco) unless stated otherwise. All in vitro experiments were performed on cells growing exponentially.

Reagents

Stock solutions of recombinant human BMP-2 ligands (R&D systems, Minneapolis, MN, USA) were prepared in Phosphate-buffered saline (PBS) and subsequently dissolved in culture medium (100ng/ml) containing 0.5% FCS. Stock solutions of LDN-193189 (AxonMedchem BV, Groningen, the Netherlands) were prepared in dimethyl sulfoxide (DMSO) and subsequently dissolved in culture medium containing 10% FCS (5nM).

BMPR2 transfection

Cells were transiently transfected with either a pcDNA4/TO*BMPR2 plasmid or pcDNA4/TO control vector (Invitrogen, Breda, the Netherlands). pcDNA4/

TO*BMPR2 plasmid was constructed by digesting a previously developed pcDNA3.1*BMPR2 construct (Rosenzweig *et al.*, 1995) using HindIII and BamHI restriction enzymes and subsequently placing the BMPR2 sequence into a pcDNA4/TO plasmid. The efficiency of transfection was evaluated by co-transfection with pmaxGFP control vector (from Amaxa GmbH, Cologne, Germany)). Efficiency of transfection was determined by the measurement of GFP-positive cells and was at least 70%. All experiment where BMPR2 transfection was used, were done in normal culture conditions, which includes 10%FCS.

Luciferase reporter assays

Transcriptional activity of canonical BMP, TGF-β and Wnt signalling was measured by transfection of BRE-Luc, CAGA-Luc or WRE-luc/MRE-luc respectively (Korchynskyi and Ten, 2002;Dennler *et al.*, 1998;van *et al.*, 2011). Transfection efficiency was corrected by co-transfection of a CMV promoter-driven Renilla luciferase vector (Promega, Leiden, The Netherlands). Transfections were performed using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega) on a Luminometer (Berthold Technologies, Bad Wildbad, Germany).

Stable knock-down of SMAD4

Lentiviral constructs expressing shRNAs targeting SMAD4 (TRCN0000040028) and a non-targeting control construct (SHC002) were obtained from the Sigma MISSION shRNA library (Sigma-Aldrich, St. Louis, MO). Production of lentiviruses by transfection into 293T cells has been described earlier (Carlotti *et al.*, 2004). Cells were selected using puromycin. The shSMAD4 transduced CRC cell lines were constructed and used previously.(Voorneveld *et al.*, 2014)

Real Time PCR

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using Random primers (Promega) and MMLV-reverse transcriptase (Invitrogen). PCR was performed using the iCycler Thermal Cycler and iQ5 Multicolour Real Time PCR Detection System (Bio-Rad). GAPDH expression was used to normalize for variance. Primer sequences and protocols can be provided upon request.

Wnt specific RT-PCR array

HCT116 and HCT116 SMAD4-/- cells were transfected with a pcDNA4/TO*BMPR2 plasmid or pcDNA4/TO control vector and after 24 hrs the cells were lysed and RNA was isolated using RNeasy (Qiagen). The Human WNT Signalling Targets RT² Profiler PCR Array was purchased from SABIOsciences and used according to the manufacturers' instructions.

Immunofluorescence

Cells were allowed to adhere to poly-l-lysine (Sigma-Aldrich) coated coverslips, fixed in 4% paraformaldehyde and stained in permeabilisation buffer (PBS containing 0.05% Triton X-100) using mouse monoclonal β-catenin (1:100) and goat-anti-mouse 594nm (1:200) antibodies. Slides were embedded in SlowFade Gold (Invitrogen). Images were obtained using a Leica TCS SP2 confocal system (Leica, Mannheim, Germany) and processed using ImageJ software.

Chemosensitivity

Cells were transfected with either a pcDNA4/TO*BMPR2 plasmid or the pcDNA4/TO control vector 48hrs prior to the treatment with different concentrations of 5-fluorouracil (5-FU). After 24 hours of 5-FU treatment cell viability was measured by adding 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for two hours at 37˚C [0,5 mg/ml] after which the absorbance of the samples was measured at 562nm. In another experimental set-up cells were treated with 5nM LDN-198189 or control (DMSO) for 4 days prior to the treatment with different concentrations of 5-FU.

Statistical analysis

In vitro experiments were analysed using a 2-tailed Student's t test. Significant differences are presented as following: *$p < 0.05$; **$p < 0.01$ *** $p < 0.001$. All experiments were done with a minimum of 3 independent experiments. For the tissue analysis the Fisher's Exact Test was used as appropriate and performed using SPSS Statistics version 20 (IBM, New York, USA). A p-value of <0.05 was considered significant. The hierarchical cluster analysis was performed using JMP 10 (Cary, North Carolina, USA).

Results

BMPs are expressed abundantly in both the tumour and stroma at the invasive front.
To explore to what extent the BMP signalling pathway can potentially be activated by BMP ligands we assessed the levels of BMP ligand expression at the invasive front. We stained and scored BMP2, BMP4, BMP6 and BMP9 in the invasive front of 94 colorectal cancers using immunohistochemistry. BMP2, BMP4, BMP 6 and BMP9 ligands were expressed in the tumour in 45.2%, 10.7%, 50.0% and 90.5% respectively (***Supplementary figure 2***). In the surrounding stroma we detected one or more BMP ligands in 56.0% of the cases. Overall, in all of the cancer specimens there were one or more BMPs expressed in the tumour, stroma or both. From this we concluded that there are sufficient BMP ligands present at the invasive front to potentially activate BMP signalling.

Association between expression of β-catenin, SMAD4 and p53 at the invasive front in human colorectal cancer tissue.
We analysed the expression patterns of SMAD4, p53 and β-catenin at the invasive front of 94 CRCs using immunohistochemistry. We have made use of immunohistochemical analysis methodology that others have shown to correlate well with the mutation status p53 (see supplementary methods) (Curtin *et al.*, 2004;Yemelyanova *et al.*, 2011). Allelic loss of 18q and SMAD4 mutation also correlates with SMAD4 expression, but SMAD4 expression can also be reduced without the presence of mutations or allelic imbalance.[3] 59.6% of the cancers have a high expression of nuclear β-catenin, 55.3% have aberrant p53 expression and 40.4% have loss of expression of SMAD4 (***Table 1***). There is no association between age, gender, stage and immunohistochemical scoring for p53, nuclear SMAD4 and nuclear β-catenin, except that patients with SMAD4 positive cancers are younger (p=0.04). Left-sided tumours have significantly higher levels of nuclear β-catenin (p=0.001) and SMAD4 negative cancers are associated with Microsatellite stability (p=0.008) and left sided tumours (p=0.04) (***Supplementary table 1***). There is a strong association between loss of SMAD4 expression and high expression of nuclear β-catenin (p=0.001) and there is an association between aberrant p53 expression and high expression of nuclear β-catenin (p=0.04) (***Table 1***). To graphically visualize the relationship between the expression profiles of these three

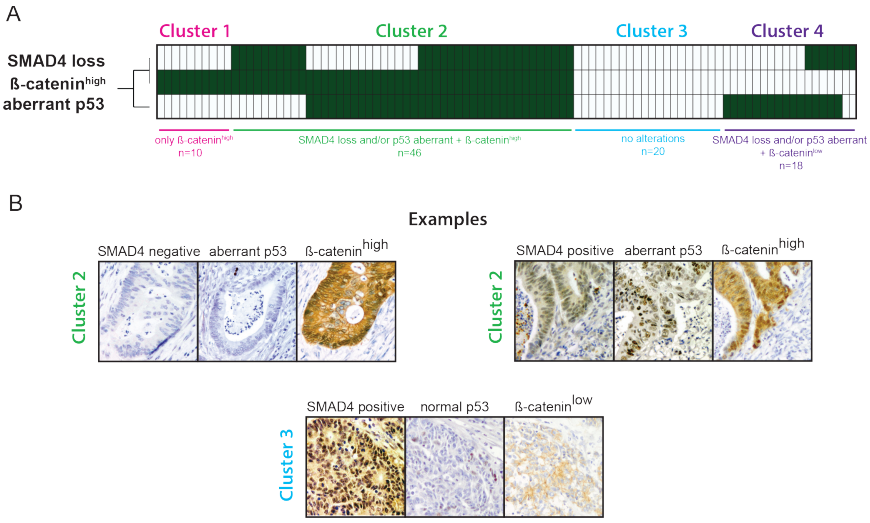


Figure 1 (above). The majority (82%, 46/56) of colorectal cancers exhibiting high levels of nuclear β-catenin at the invasive front have abnormal expression of SMAD4 or p53 or both. (A) Hierarchical cluster analysis of the expression patterns of β-catenin combined with SMAD4 and p53. The invasive front of 94 stage I/II cancers was stained and scored for nuclear SMAD4, p53 and β-catenin. (B) Examples of cluster 2 and 3 expression patterns. *(400X)*

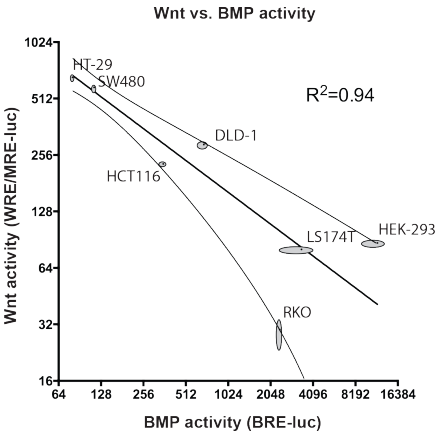


Figure 2 (left). Graph to show the relationship between basal levels of BMP and Wnt signalling in CRC cell lines HCT116, RKO, HT-29, SW480, DLD-1 and LS174T and the embryonic kidney cell line HEK-293 (used as non-cancer cell line) were transfected with either BRE-luc (BMP activity) or WRE-luc (Wnt activity). MRE-luc was used to control for transcriptional changes not associated with Wnt signalling. A cmv-Renilla plasmid was co-transfected to control for the transfection efficiency. The graph shows the BMP activity plotted against the Wnt activity in each cell line. Regression analysis reveals a Log regression with a correlation of R^2 = 0.94. (The experiment was performed three times. Grey areas depict the SD of each cell line. Dotted lines show the 95% confidence interval of the nonlinear curve fit)

molecules we used a hierarchical cluster analysis (***Figure 1A***). This shows that the majority of cancers with high nuclear β-catenin staining (n=56) are either SMAD4 negative and/or p53 aberrant (n=46/56, 82%). ***Figure 1B and supplementary figure 3*** show examples of the four clusters based on these expression patterns. When comparing patient characteristics cluster 1 and 2 cancers are more often left-sided compared to cluster 3 and 4 cancers (***Supplementary table 2***).

BMP/Wnt activity in colorectal cancer cell lines

We investigated the effects of BMP signalling on Wnt signalling activity in CRC cell lines. We measured the BMP and Wnt signalling activity in a panel of CRC cell lines and a control cell line HEK-293 (embryonic kidney cells) using luciferase reporter assays and found an inverse correlation between the activity of the two pathways (***Figure 2***): Low BMP signalling activity is associated with a high Wnt signalling activity and vice versa. Mathematically BMP and Wnt signalling show a log correlation following the equation WRE-luc = 10^(-0.564*log(BRE-luc) + 3.907) with a correlation coefficient of R^2=0.94

BMP activation results in paradoxical Wnt activation in a subset of CRC cell lines.

It is known that BMP signalling can inhibit Wnt signalling in normal intestinal epithelium, but it is not known whether the major mutations found in CRC influence this pathway interaction. We therefore activated BMP signalling in a panel of CRC cell lines and HEK-293 using a pcDNA4/TO plasmid expressing WT-BMPR2 and measured Wnt signalling activity using WRE/MRE-luciferase.

Table 1

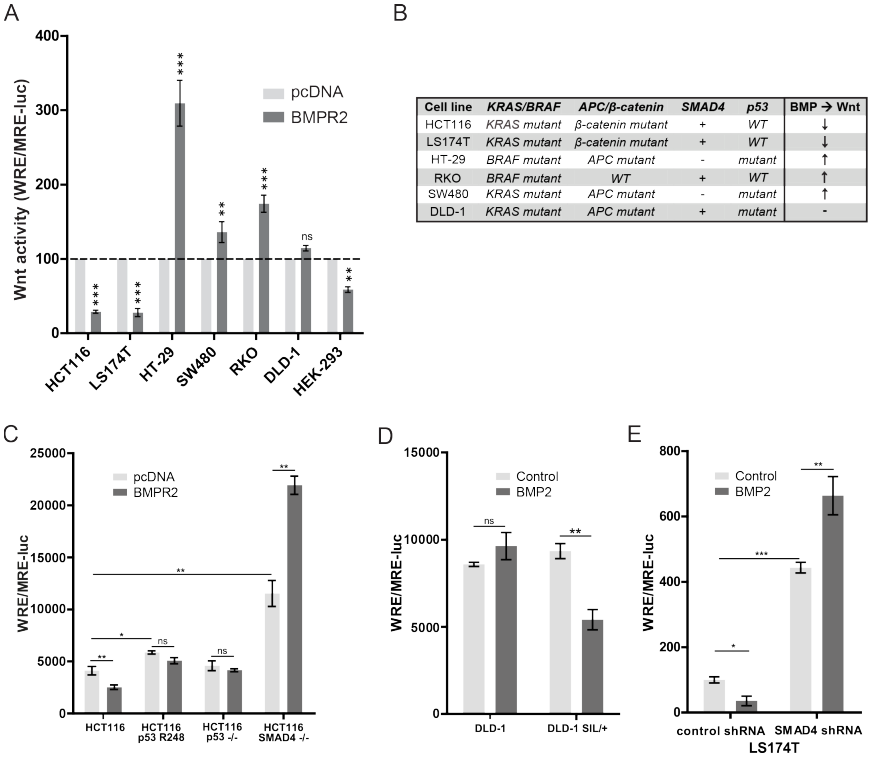
			β-catenin		
		Total	high	low	
		***n*=94 (%)**	**n=56 (59.6%)**	**n=38 (40.4%)**	***p***
SMAD4					
	positive	56 (59.6)	25 (44.6)	31 (81.6)	0.001
	negative	38 (40.4)	31 (55.4)	7 (18.4)	
p53					
	normal	42 (44.7)	20 (35.7)	22 (57.9)	0.04
	aberrant	52 (55.3)	36 (64.3)	16 (42.1)	

We have previously shown that transfection of BMPR2 results in a reliable and robust increase in BMP signalling activity (BRE-luc) (Kodach *et al.*, 2008) and it does not lead to activation of TGF-β signalling (CAGA-luc) (***Supplementary figure 4***). Activation of BMP signalling results in a reduction of Wnt signalling only in HCT116 and LS174T cells and the control cell line HEK-293. In HT-29, RKO and SW480 cells activation of BMP signalling results in an increase in Wnt signalling (***Figure 3A***). To elucidate whether mutations might affect the BMP-Wnt interaction we looked at the known mutation profile of these cell lines to see if this suggested a pattern (***Figure 3B***). Interestingly, the two cell lines in which BMP signalling has a negative effect on Wnt signalling (HCT116 and LS174T) are both SMAD4 positive and p53 WT. This would suggest that if either SMAD4 is lost and/or p53 is mutant, the BMP-Wnt interaction is either reversed or abolished.

The effect of BMP signalling on Wnt signalling is dependent on the SMAD4 and p53 status.

To investigate the influence of SMAD4 and p53 status on the BMP-Wnt signalling interaction we first compared several cell lines in which the p53 and SMAD4 status was manipulated. We activated BMP signalling in colorectal cancer cells that have intact p53 expression (HCT116) and compared this to p53 null cells (HCT116 p53-/-) or p53 mutant cells (HCT116 p53 R248). In p53 expressing cells BMP signalling reduces Wnt signalling activity, while a mutation in p53 or the absence of p53 abolishes the inhibiting effect of BMP signalling (***Figure 3C***). To further confirm the role of p53 in influencing the BMP-Wnt interaction we treated DLD-1 cells, that naturally contain one mutated and one wild type p53 allele, with 100ng/ml BMP-2 and compared these with DLD-1 p53 SIL/+ cells, where the mutant p53 allele has been silenced (***Figure 3D***). Activating BMP signalling does not lead to changes in Wnt signalling in the parental DLD-1 cells, but in the DLD-1 p53 SIL/+ cells Wnt signalling activity is decreased.

Next we investigated the role of SMAD4 in the BMP-Wnt interaction. First we compared the effect of BMP signalling on Wnt signalling activity in HCT116 (SMAD4 positive) versus HCT116 SMAD4-/- cells. Loss of SMAD4 leads to a major increase in Wnt signalling and reversal of the BMP/Wnt interaction with BMP activation now leading to an activation of Wnt signalling (***Figure 3C***). This was measured both by WRE-luc and nuclear localization of β-catenin. The reversal



Cell line	*KRAS/BRAF*	*APC/β-catenin*	*SMAD4*	*p53*	BMP → Wnt
HCT116	*KRAS mutant*	*β-catenin mutant*	+	*WT*	↓
LS174T	*KRAS mutant*	*β-catenin mutant*	+	*WT*	↓
HT-29	*BRAF mutant*	*APC mutant*	-	*mutant*	↑
RKO	*BRAF mutant*	*WT*	+	*WT*	↑
SW480	*KRAS mutant*	*APC mutant*	-	*mutant*	↑
DLD-1	*KRAS mutant*	*APC mutant*	+	*mutant*	-

Figure 3 Activation of BMP signalling differentially modulates Wnt signalling dependent on the SMAD4 and p53 status. (A) CRC cell lines HCT116, LS174T, HT-29, SW480, RKO and DLD-1 and the embryonic kidney cell line HEK-293 (used as non-cancer cell line) were transfected with WRE-luc and MRE-luc. The next day the cells were transfected with either BMPR2 (to activate BMP signalling) or the empty control vector pcDNA4/TO. After 24 hrs the cells were lysed and luciferase activity was measured. The control (pcDNA) condition was set to 1. (B) An overview of APC/β-catenin, KRAS/BRAF, p53 and SMAD4 mutation status in the cell lines. (C) HCT116, HCT116 SMAD4-/-, HCT116 R248 (p53 mutated) and HCT116 p53-/- cells were transfected with WRE-luc and MRE-luc. The next day the cells were transfected with either BMPR2 (to activate BMP signalling) or the empty control vector pcDNA4/TO. After 24 hrs the cells were lysed and luciferase activity was measured. (D) DLD-1 (harbouring a p53 mutation) and DLD-1 SIL/+ (one WT copy p53) were transfected with WRE-luc/MRE-luc. The next day the cells were treated with either 100ng/ml BMP2 or the control vehicle (both diluted in medium with 0,5% FCS). After 24 hrs the cells were lysed and luciferase activity was measured. (E) SMAD4 was stably knocked down in LS174T cell using lentiviral shRNA transduction. The empty shRNA SHC002 was used to create a control cell line. The cells were transfected with WRE-luc/MRE-luc. The next day the cells were treated with either 100ng/ml BMP2 or the control vehicle (both diluted in medium with 1% FCS). After 24 hrs the cells were lysed and luciferase activity was measured. (All experiments were performed three times. MEAN±SEM is shown, *p<0.05 ** p<0.01 ***p<0.001)

of the BMP-Wnt interaction due to loss of SMAD4 is further confirmed by the treatment of HCT116 SMAD4-/- cells with the BMP inhibitor noggin, which results in reduced nuclear and increased membranous β-catenin (***Supplementary figure 5A***).

The role of SMAD4 was further investigated by stable lentiviral shRNA-mediated knockdown of SMAD4 in LS174T cells and treatment with 100ng/ml BMP2. BMP inhibits Wnt signalling in the control shRNA LS174T cell line, which expresses SMAD4 (***Figure 3E***). Knocking down SMAD4 switches BMP signalling from inhibiting to enhancing Wnt signalling. The effect of BMP signalling on Wnt signalling in SMAD4 negative cells can also be seen by the increase in mRNA expression of the Wnt signalling components AXIN2 and c-MYC (***Supplementary figure 5B***). We conclude that the BMP-Wnt interaction is dependent on the SMAD4 and p53 status.

BMP activation influences Wnt target gene expression to a greater extent in SMAD4 deficient cells.

To obtain insight into which Wnt pathway-associated genes are affected by the reversal of the BMP-Wnt interaction due to SMAD4 loss, we performed a Wnt signalling RT-PCR array while activating the BMP pathway in 2 isogenic cell lines with or without SMAD4. ***Figure 4*** demonstrates that activation of BMP signalling in SMAD4 positive versus SMAD4 negative cells leads to a completely different expression pattern of Wnt signalling associated genes in the two cell types. It is also notable that BMP pathway activation leads to larger changes in Wnt gene expression in SMAD4 negative cells than in SMAD4 positive ones. One of the genes that is upregulated when activating BMP signalling in SMAD4 positive cells is CTNNBIP1. The CTNNBIP1 gene encodes for the β-catenin-interacting protein 1, which binds β-catenin to prevent interaction with TCF, thereby inhibiting Wnt signalling (Tago *et al.*, 2000; Yemelyanova *et al.*, 2011). Activation of BMP signalling in SMAD4 negative HCT116 cells leads to the upregulation of DVL1. *DVL1* encodes for the protein Segment polarity protein dishevelled homolog (DVL-1), which prevents the GSK3β/APC/Axin complex from degrading β-catenin.

BMP signalling influences 5FU chemosensitivity dependent on the SMAD4 and p53 status.

It has been shown, that patients with CRC with low levels of SMAD4 protein expression respond poorly to 5-fluorouracil (5-FU).[4] Also, inactivation of SMAD4 leads to an increase in 5-FU resistance (Papageorgis *et al.*, 2011). Inactivation of p53 has also been shown to result in a poorer response to chemotherapy in vivo (Lowe *et al.*, 1994) and in vitro (Bunz *et al.*, 1999), although some reports show otherwise(Hawkins *et al.*, 1996). The use of BMPs has been proposed as a means of combatting chemoresistance in several cancer types including CRC (Lombardo *et al.*, 2011;Piccirillo *et al.*, 2006). We tested the influence of BMP activation on 5-FU chemosensitivity in SMAD4 and p53 inactivated cell lines. In SMAD4 and WT p53 expressing parental HCT116 cell line an increase in chemosensitivity can be seen when BMP signalling is activated (***Figure 5A***). In the p53 mutated HCT116 R248 cell line a slight reduction in chemosensitivity can be seen when BMP is activated, but not in the HCT116 p53-/- cells (***Figure 5B&C***). In the HCT116 SMAD4-/- cells, BMP signalling activation results in less chemosensitivity, especially at high concentrations of 5-FU (***Figure 5D***). These results suggest that activation of BMP signalling can increase the effects of chemotherapy as others have suggested but only in cancers that express SMAD4 and WT p53.

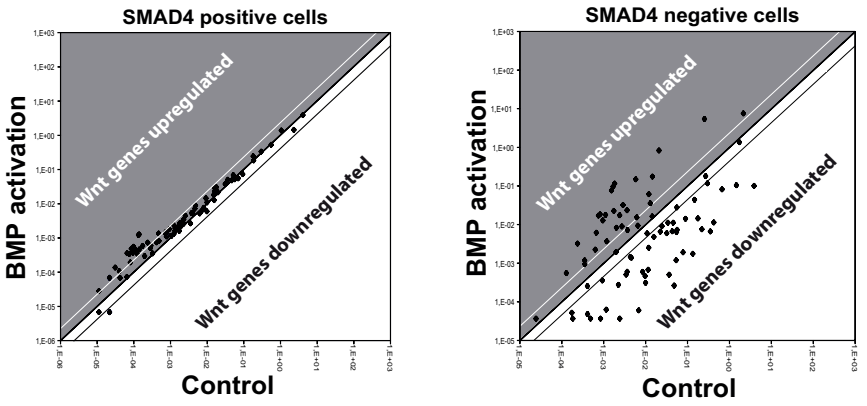


Figure 4. BMP activation influences Wnt target gene expression to a greater extent in SMAD4 deficient cells. HCT116 and HCT116 SMAD4-/- cells were transfected with either BMPR2 (to activate BMP signalling) or the empty control vector pcDNA4/TO. After 24 hrs RNA was isolated and a Human WNT Signalling Targets RT² Profiler PCR Array from SABIOsciences was used. The graphs show the expression patterns of the control samples (x-axis) versus the BMPR2 transfected samples (y-axis). The lines indicate a 2-fold change compared.

We subsequently investigated the effect of BMP pathway inhibition by treating cell lines with the BMPR1A (ALK3) inhibitor LDN-193189. After 4 days of treatment with 5nM LDN-193189 a significant decrease in viability can be seen in SMAD4-/- cells (***Figure 5E***). At a dose of 5nM the kinase inhibitor LDN-193189 is a highly specific inhibitor of BMPR1A and results in specific downregulation of BMP signalling compared to TGFβ signalling as measured by reporter assays. Higher concentrations only result in less specificity (***Supplementary figure 6A-C***). Pre-treating HCT116 SMAD4-/- cells for 4 days with 5nM of LDN-193189 followed by subsequent 5-FU treatment resulted in a stronger decrease in viability (***Figure 5F***).

In summary, in HCT116 cells in which both SMAD4 and p53 are wild type, activation of BMP signalling results in an increase in chemosensitivity, while BMP inhibition has no effect. In HCT116 SMAD4 wild type, p53 mutant cells, activating or inhibiting BMP signalling has no effect on chemosensitivity, while in HCT116 SMAD4 cells BMP activation increases chemoresistance and BMP inhibition increases chemosensitivity (***Figure 5G***).

Discussion

The β-catenin paradox cannot be explained by mutations within the Wnt signalling pathway, as *APC/CTNNB1* mutations are clonal within a tumour. Based on several observations made in previously published studies we hypothesized that the β-catenin paradox may at least in part be explained by the effects of SMAD4 loss or p53 mutations on the ability of BMP signalling to suppress Wnt signalling in colorectal cancer cells at the tumour invasive front.

Further evidence to support the likely involvement of the BMP pathway in determining the levels of Wnt signalling in tumour cells at the invasive front is the finding that the combination of extensive stroma at the invasive front and loss of SMAD4 in the tumour leads to a poorer prognosis (Mesker *et al.*, 2009). One potential explanation for this is that SMAD4-independent tumour-stroma signalling drives invasion and metastasis, both of which are driven by enhanced Wnt signalling.

We hypothesized that candidate mutations underlying the β-catenin paradox are likely to be those that occur at the same stage in tumour progression as the appearance of this phenomenon itself. High levels of Wnt signalling are seen in cells that have acquired the ability to invade and metastasize. This occurs at the transition from advanced adenoma to invasive carcinoma and the two most

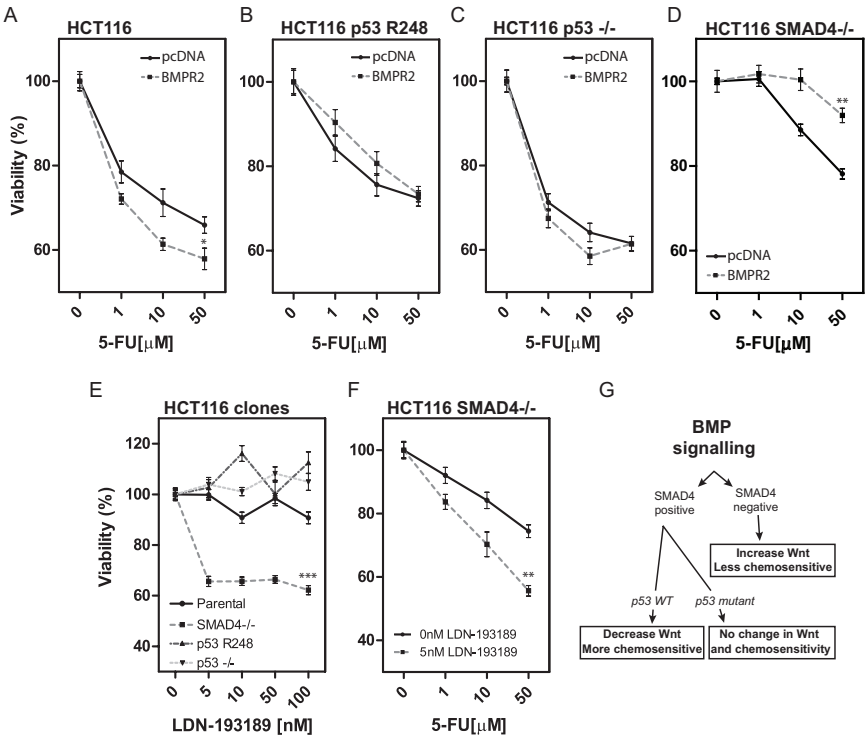


Figure 5. BMP pathway activation or inhibition differentially affects chemosensitivity dependent on the mutation status of p53 and SMAD4. (A-D) HCT116, HCT116 SMAD4-/-, HCT116 R248 (p53 mutated) and HCT116 p53-/- cells were transfected with BMPR2 (to activate BMP signalling) or the empty control vector pcDNA4/TO. The next day cells were treated with different concentrations of 5-FU. After 24 hrs of 5-FU treatment the cell viability was measured. (E) HCT116 SMAD4-/- cells were treated with different concentrations of LDN-193189 (BMP pathway inhibitor) for 4 days and afterwards cell viability was measured. (F) HCT116 SMAD4-/- cells were treated with 5nm LDN-193189 (BMP pathway inhibitor) for 4 days. Afterwards the cells were washed and serum starved for 8 hrs. Subsequently the cells were treated with different concentrations of 5-FU. After 24 hrs cell viability was measured. (G) A scheme showing how BMP signalling influences the chemosensitivity dependent on the p53 and SMAD4 status. (All experiments were performed in triplicate. MEAN±SEM is shown, *p<0.05 ** p<0.01 ***p<0.001)

frequently occurring mutations at this stage are SMAD4 and p53 mutations. This choice of candidate mutations is further supported by previous studies showing that SMAD4 loss is associated with elevated levels of Wnt activity in CRC cell lines (Freeman *et al.*, 2012) and that p53 mutations increase Wnt signalling activity *in vitro* (Kim *et al.*, 2011).

The BMP pathway counteracts Wnt signalling in the normal colonic epithelium as has been shown by BMP pathway manipulation in transgenic mouse models (Haramis *et al.*, 2004;He *et al.*, 2004). We hypothesized that SMAD4 or p53 mutations may influence the way BMP signalling activity modulates Wnt signalling activity despite APC/β-catenin mutations and provide a molecular explanation for the β-catenin paradox.

We performed our initial analysis in archival human colorectal cancer specimens in tissue sections at the invasive front. We assessed nuclear β-catenin, SMAD4 and p53 expression using immunohistochemistry and found an association between SMAD4 loss and/or aberrant p53 expression and a high level of nuclear β-catenin (representing high Wnt activity). This provides evidence *in vivo* to support previous evidence *in vitro* that SMAD4 and p53 can alter Wnt signalling activity, revealing for the first time a connection between SMAD4, p53 and Wnt signalling in the invasive front of CRC tissue.

To investigate this further we activated BMP signalling using transient transfection of BMPR2 in a set of isogenic cell lines in which p53 and SMAD4 have been genetically manipulated. Although transient transfection of BMPR2 is a rather artificial method to activate the BMP pathway, we have previously shown that it is a reliable way of achieving robust activation of BMP signalling in all CRC cell lines by circumventing the loss of BMPR2 seen in a proportion of CRCs and variations in expression levels of BMP ligands and inhibitors (Kodach *et al.*, 2008). To avoid bias based on the use of a single method of BMP activation we also performed similar experiments using BMP2 ligands in cells we have previously shown to have normal BMP receptor levels.

We found an interesting correlation between the p53 and SMAD4 status and the ability of the BMP signalling pathway to modulate Wnt signalling activity. Wild-type p53 is necessary for BMP to inhibit Wnt signalling and loss of SMAD4 completely reverses the BMP-Wnt interaction switching inhibition into activation. It has been previously described that the effect of cancer signalling pathways can

change due to the mutation or loss of oncogenes outside the main signalling pathway. This is illustrated by studies showing that the Transforming Growth Factor-β (TGF-β) signalling pathway can either inhibit or promote migration/invasion dependent on the presence of wild-type or mutant p53.[2]

We also found that the BMP signalling pathway can modulate the chemosensitivity of 5-FU based on the SMAD4 or p53 status. The similarity between the effects of BMP signalling on both Wnt signalling activity and chemosensitivity can be explained by the previously observed correlation between β-catenin levels and chemoresistance (Sinnberg *et al.*, 2011).

Our study has several limitations. The study in patient tissue is limited by the fact that it is difficult to assess BMP signalling activity in CRC tissue especially when this is SMAD-independent. We observed an abundance of BMP ligand expression at the invasive front both in the stroma and the tumour cells, which would suggest that active BMP signalling is more or less ubiquitous at the invasive front of CRC. However, we have not analysed the expression of a large number of other BMP ligands and inhibitors as it is currently impossible to deduce the integrated effect on pathway activity even with a much more comprehensive analysis. Many of the effects of BMP signalling on Wnt signalling seem to be SMAD4-independent, as we describe in our study. While canonical SMAD-dependent BMP activity can be assessed by nuclear pSMAD1,5,8 localization using immunohistochemistry as we have performed previously, there is no equivalent for the assessment of SMAD-independent BMP activity.

Our study is necessarily highly reductionist in nature. As we have outlined, there are many other molecular pathways and many other mutations that could be explored. In fact, one of the cell lines we used (RKO) is SMAD4 positive and p53 WT, but showed an increase in Wnt signaling upon BMP activation suggesting the involvement of other pathways. However, we feel that the study of the two most important signalling pathways in conjunction with two of the commonest mutations is a good starting point.

Cancer therapy is increasingly focused on the targeted pharmacological modulation of the specific molecular pathways underlying carcinogenesis. Understanding how the major pathways interact and how mutations influence this is thus of critical importance. The clinical implications of this study are that the use of BMPs in cancer therapy, as has been proposed by previous studies, could

have deleterious effects and should be targeted to individual cancers. However, interventions to modulate BMP signalling have the potential to enhance the effect of conventional chemotherapy dependent on the mutational status of p53 and SMAD4. This may be especially important in SMAD4 negative CRCs, which are aggressively invasive and metastatic cancers with a poor prognosis that respond poorly to current therapy.

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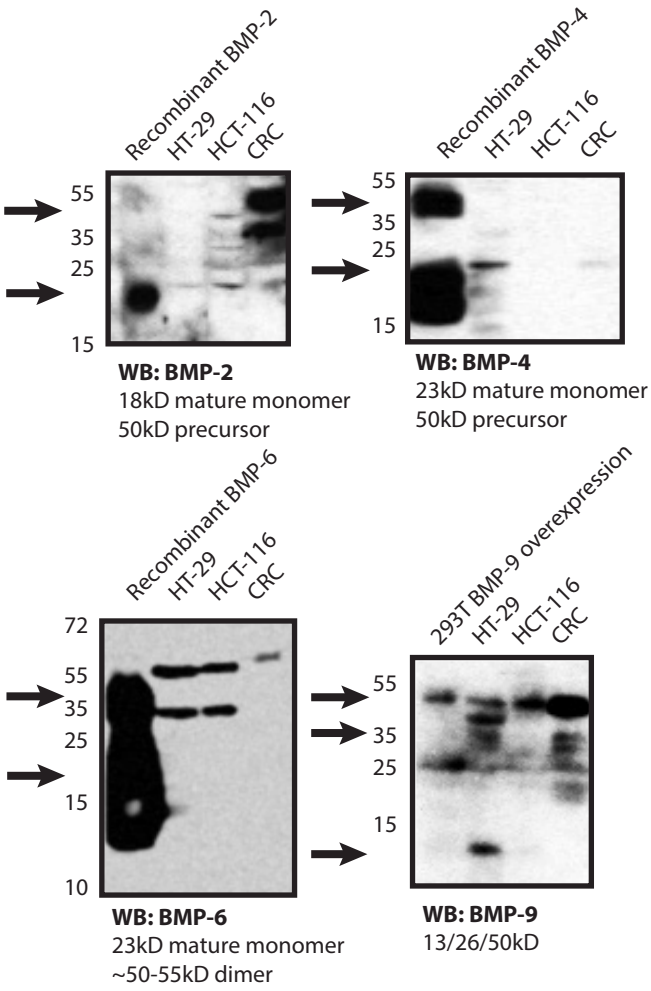


Figure S1 (left, upper) Western blot analysis using antibodies against BMP-2. A band is expected at 18kD; this is the mature monomer form of BMP-2. Another band is expected at 50kD; this is the precursor of BMP-2. The figure shows a clear band at 18kD in the recombinant BMP-2 and HCT116 sample, and at 50kD in the CRC sample. (right, upper) Western blot analysis using antibodies against BMP-4. A band is expected at 23kD; this is the mature monomer form of BMP-4. Another band is expected at 50kD; this is the precursor of BMP-4. A clear band is seen at 23kD in the recombinant BMP-4, HT29 and CRC samples, recombinant BMP-4 also shows a band at 50kD. (left, lower) Western blot analysis using antibodies against BMP-6. A band is expected at 23kD; this is the mature monomer form of BMP-6. Also, a BMP-6 dimer would be seen at around 50-55kD. A clear band is seen at 23kD in the recombinant BMP-6 sample. The HT29 sample shows a slight band. Clear bands can be seen at 50-55kD in all the samples. (right, lower) Western blot analysis using antibodies against BMP-9. Bands are expected at 13kD, 26kD and 50kD. HEK-293T that overexpress BMP-9, HCT116 and CRC samples show a clear band at 26kD and 50kD, HT29 also shows a band at 13kD. In several samples multiple bands can be observed both lower and higher than expected. BMP ligands can form dimers; this could be an explanation for the bands at higher molecular weights. Ligands can also degrade; this could explain the bands found at lower molecular weights.

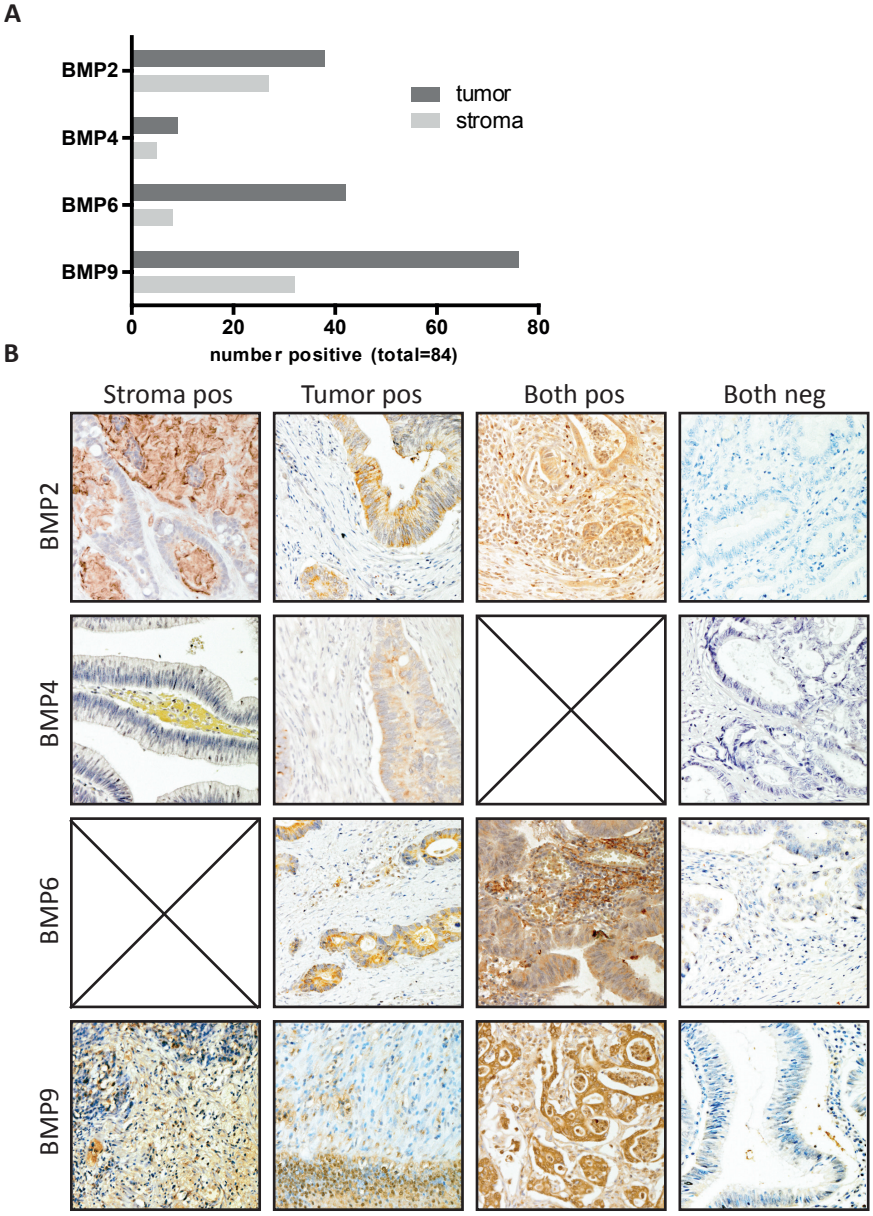


Figure S2. (A) BMP ligand expression was measured in colorectal cancer tissue using immunohistochemistry. The graph shows the percentage of tumours or the stroma surrounding the tumours that express BMP2, BMP4, BMP6 and/or BMP9. (B) Examples of tissue scored positive or negative for BMP2, BMP4, BMP6 and BMP9.

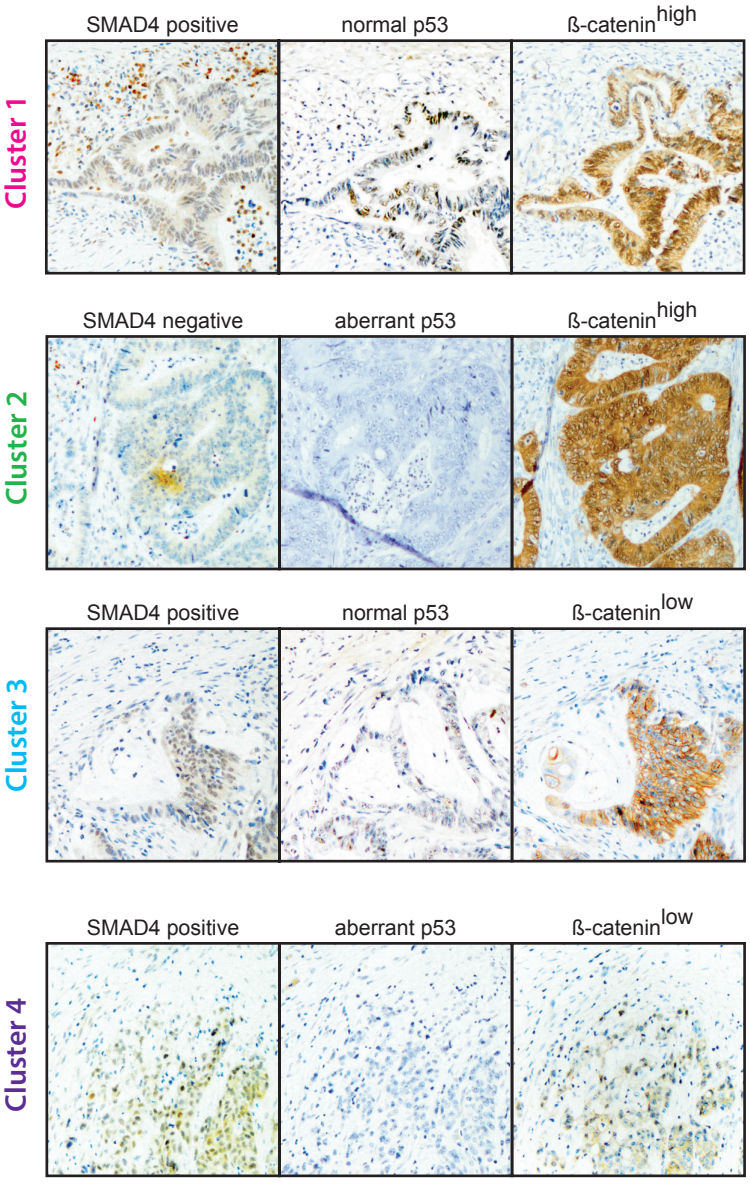


Figure S3. Examples of cluster expression patterns. (100X)

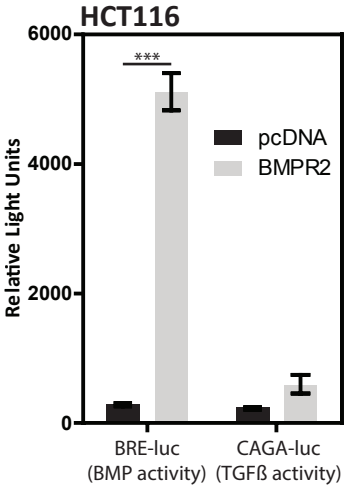


Figure S4 (left).
Transient transfection with BMPR2 leads to robust activation of the BMP pathway but not the TGF-β pathway. HCT116 cells were transfected with either BRE-luc (BMP signalling) or CAGA-luc (TGFbeta signalling). The next day the cells were transfected with either BMPRII (to activate BMP signalling) or the empty control vector pcDNA4/TO. After 24 hrs the cells were lysed and luciferase activity was measured. A cmv-Renilla plasmid was co-transfected to control for the transfection efficiency. (All experiments were performed three times. MEAN±SEM is shown, *p<0.05 ** p<0.01 ***p<0.001)

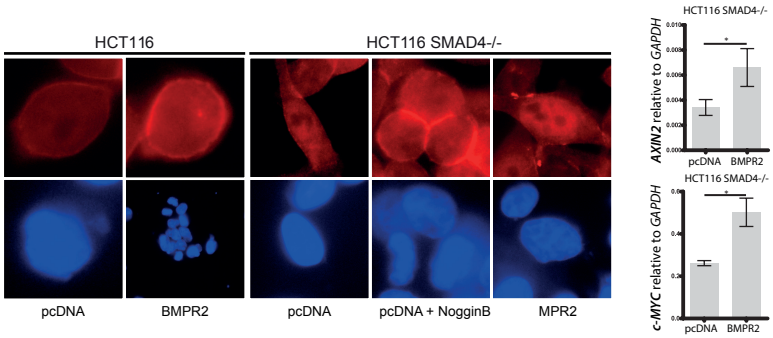
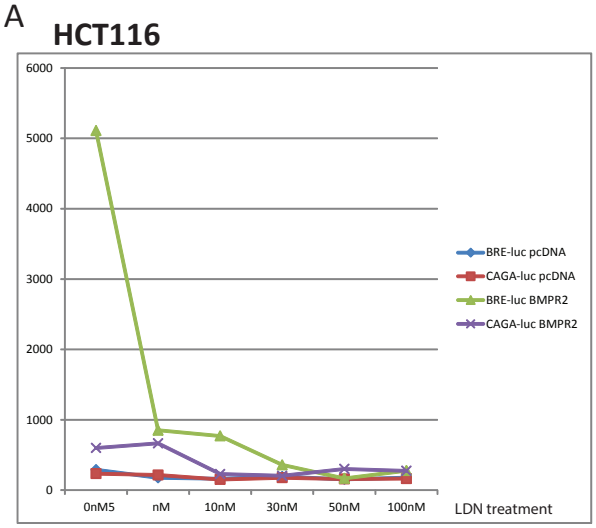
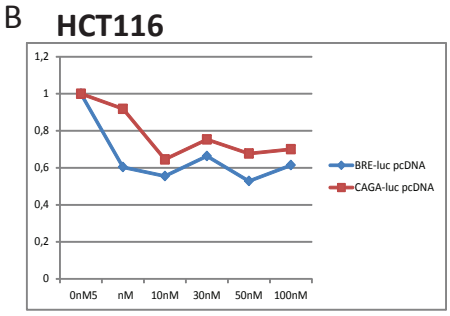
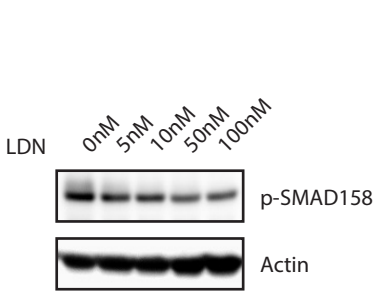


Figure S5. Activation of the BMP pathway leads to increased nuclear accumulation of β-catenin only in SMAD4 deficient CRC cells. (left) HCT116 and HCT116 SMAD4-/- cells were transfected with either BMPR2 (to activate BMP signalling) or the empty control vector pcDNA4/TO. HCT116 SMAD4-/- cells were also treated with 500ng/nl Noggin (BMP inhibitor). After 24 hrs the cells were fixed and stained for β-catenin using mouse monoclonal β-catenin (1:100) and goat-anti-mouse 594nm (1:200) antibodies. DAPI was used to visualize the nuclei. BMP pathway activation has no effect on nuclear β-catenin expression in SMAD4-expressing cells. In SMAD4 deficient cells, BMP pathway inhibition with Noggin leads to a slight increase in β-catenin at cell-cell junctions, while BMP pathway activation leads to increased nuclear β-catenin compatible with increased Wnt pathway activity (right) HCT116 SMAD4-/- cells were transfected with either BMPR2 (to activate BMP signalling) or the empty control vector pcDNA4/TO. After 24 hrs RNA was isolated and a RT-PCR for the Wnt targets AXIN2 and c-MYC was performed. GAPDH was used as a control. BMP pathway activation in SMAD4 deficient cells leads to increased expression of the Wnt targets AXIN2 and c-MYC. (All experiments were performed three times. MEAN±SEM is shown, *p<0.05 ** p<0.01 ***p<0.001)







Reduction Ratio (*reduction BRE-luc/reduction CAGA-luc)*

0nM5	nM	10nM	30nM	50nM1	00nM
1	,849532	1,255514	1,364287	1,460491	,287971

Optimal concentration in favor of BMP-signalling reduction

Figure S6. The small molecule BMPRIA inhibitor LDN-193189 inhibits BMP signalling specifically at 5nM (compared to TGF-β signalling). (A&B) HCT116 cells were transfected with either BRE-luc (BMP signalling) or CAGA-luc (TGFbeta signalling). The next day the cells were treated with different dosages of LDN-193189. After 24 hrs the cells were lysed and luciferase activity was measured. (C) HCT116 cells were treated with different dosages of LDN-193189. After 24hrs the cells were lysed and a western blot analysis visualizing pSMAD1,5,8 protein expression was performed. Actin was used as a loading control. (All experiments were performed three times.)

	Total	SMAD4			p53			β-catenin		
		negative	positive		aberrant	normal		high	low	
	***n*=94 (%)**	***n*=38 (40.4%)**	***n*=56 (59.6%)**	***p***	***n=52* (55.3%)**	***n=42* (44.7%)**	***p***	**n=56 (59.6%)**	**n=38 (40.4%)**	***p***
Mean age±SD (y)	67.6±11.47	70.6±8.7	65.5±13.0	**0.04**	66.2±12.0	69.3±11.1	0.20	66.1±12.2	69.7±10.6	0.14
Gender										
Female	46 (48.9)	17 (44.7)	29 (51.8)	0.53	25 (48.1)	21 (50.0)	1.0	27 (48.2)	19 (50.0)	1.0
Male	48 (51.1)	21 (55.3)	27 (48.2)		27 (51.9)	21 (50.0)		29 (51.8)	19 (50.0)	
Stage										
I	16 (17.0)	9 (23.7)	7 (12.5)	0.15	7 (13.5)	9 (21.4)	0.41	11 (19.6)	5 (13.2)	0.49
II	78 (83.0)	29 (76.3)	49 (87.5)		45 (86.5)	33 (78.6)		45 (80.4)	33 (86.8)	
Location										
Left sided*	44 (46.8)	23 (60.5)	21 (37.5)	**0.04**	26 (50.0)	18 (42.9)	0.53	34 (60.7)	10 (26.3)	**0.001**
Right sided**	50 (53.2)	15 (39.5)	35 (62.5)		26 (50.0)	24 (57.1)		22 (39.3)	28 (73.7)	
MSI status										
MSS	71 (79.8)	35 (94.6)	36 (69.2)	**0.008**	42 (87.5)	29 (70.7)	0.08	46 (86.8)	25 (69.4)	0.14
MSI	18 (20.2)	2 (5.4)	16 (30.8)		6 (12.5)	12 (29.3)		7 (13.2)	11 (30.6)	
Unknown	5	1	4		4	1		3	2	

p-values are based on Fisher's Exact tests and are two-sided

*Left sided is defined as rectosigmoid, sigmoid and decending colon

** Right sided is defined as splenic flexure, transverse colon, hepatic flexure, ascending colon and caecum

Table S1

	Total	cluster 1	cluster 2	cluster 3	cluster 4	
	n=94 (%)	*n*=10 (10.6%)	*n*=46 (49%)	*n*=20 (21.3%)	*n*=18 (19.1%)	*p*
Mean age±SD (y)	67.6±11.47	61.3±15.6	67.2±11.3	70.6±8.4	68.7±12.8	0.21
Gender						
Female	46 (48.9)	6 (60.0)	21 (45.7)	9 (45.0)	10 (55.6)	0.77
Male	48 (51.1)	4 (40.0)	25 (54.3)	11 (55.0)	8 (44.4)	
Stage						
I	16 (17.0)	3 (30.0)	8 (17.4)	3 (15.0)	2 (11.1)	0.49
II	78 (83.0)	7 (70.0)	38 (82.6)	17 (85.0)	16 (88.9)	
Location						
Left sided*	44 (46.8)	6 (60.0)	28 (60.9)	5 (25.0)	5 (27.8)	**0.01**
Right sided**	50 (53.2)	4 (40.0)	18 (39.1)	15 (75.0)	13 (72.2)	
MSI status						
MSS	79 (79.8)	7 (70.0)	39 (90.7)	12 (63.2)	13 (76.5)	0.07
MSI	18 (20.2)	3 (30.0)	4 (9.3)	7 (36.8)	4 (23.5)	
Unknown	5	0	3	1	1	

p-values are based on Chi-square tests and are two-sided

*Left sided is defined as rectosigmoid, sigmoid and decending colon

** Right sided is defined as splenic flexure, transverse colon, hepatic flexure, ascending colon and caecum

Table S2

Supplementary methods

SMAD4 scoring system

(according to previously described methods(1))

Table 1. Scoring system for pSMAD1,5,8				
Intensity of staining	Percentage of cells stained			
	<10%	10-30%	30-50%	>50%
No staining	0	0	0	0
Weak staining	0	0	1	1
Moderate staining	0	1	2	3
Strong staining	1	2	3	3

Only nuclear staining was considered positive.

Dichitomised:

0 & 1 → negative

2 & 3 → positive

P53 scoring system

Mutations in *p53* can result in the absence of p53 or in very high expression of p53(2). Therefore we scored the percentage of tumour cells stained positive for p53. The complete absence (0%) and a very high expression (61-100%) of p53 was considered aberrant. Is has been used previously in a similar manner as a surrogate marker for the *p53* mutational status in ovarian cancer (3). The table below also describes the number of tumors within each group.

p53 scoring

pos. nuclei (%)	n (%)	Status
0	33 (35,1)	Aberrant
1-20	12 (12,8)	Normal
21-40	13 (13,8)	Normal
41-60	17 (18,1)	Normal
61-80	8 (8,5)	Aberrant
81-100	11 (11,7)	Aberrant

β-catenin scoring system

Tissue was scored and divided in beta-catenin low and beta-catenin high according to previously described methods (4).

BMP scoring system

BMP expression was scored as either positive or negative. ≥30% expression is positive and <30% is negative.

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Chapter 5

Fibroblasts promote invasion in SMAD4 negative colorectal cancers by producing BMP-2

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Aim

Colorectal cancers (CRC) surrounded by large quantities of tumour stroma have a worse prognosis, especially the molecular subgroup of cancers that have lost SMAD4. SMAD4 is the central critical component of the Bone Morphogenetic Protein (BMP) and Transforming Growth Factor (TGF)-β signalling pathways, which play an important role in CRC. We set out to investigate the role of BMP/TGFβ signalling in the tumour-stroma interaction, especially in SMAD4 negative cancers.

Methods

We performed matrigel invasion assays to monitor CRC cell migration towards 18-CO (normal colonic) fibroblasts and compared SMAD4 negative and positive cancer cell line cells. An orthotopic mouse metastasis model was used to measure effects of fibroblasts on metastasis formation. In reverse experiments we treated fibroblasts with SMAD4 positive and SMAD4 negative CRC Conditioned Medium (CM) and performed a BMP/TGFβ specific PCR array. Finally, we stained and scored the invasive front of 146 stage III CRCs for SMAD4 and BMP2 expression and correlated this with patient survival.

Results

SMAD4 negative CRC cell line cells migrate more towards fibroblasts than SMAD4 positive CRC cells. Co-injection of SMAD4 negative CRC cells and fibroblasts in an orthotopic mouse model results in an 88-fold increase in liver metastases compared to injection of SMAD4 negative CRC cells alone while co-injection of SMAD4 positive cells with fibroblasts leads to a 4-fold increase. A BMP/TGFβ pathway qPCR screen revealed that BMP2 expression is upregulated in fibroblasts when treated with CM from SMAD4 negative CRC cells, and reduced when treated with CM from SMAD4 positive cells. SMAD4 negative CRC cells treated with BMP2 showed an increase in invasion, which can be reduced by using BMP inhibitors. We observed high stromal BMP2 expression in 40% of human CRC specimens. High stromal BMP2 expression is associated with poor patient survival only in SMAD4 negative cancers (p=0.03). In SMAD4 positive cancers stromal BMP2 expression is not associated with a poor prognosis (p=0.72).

Conclusion

SMAD4 negative CRC cells produce unknown soluble factors that stimulate BMP2 expression in fibroblasts. BMP2 has prometastastic effects specifically in SMAD4 negative CRC cells. This is supported by the fact that stromal BMP2 expression at the invasive front of CRC tissue is associated with a poorer patient survival only in SMAD4 negative cancers. This would suggest that patients with SMAD4 negative CRC and high stromal BMP2 expression might benefit from inhibition of BMP signalling.

Introduction

For many years the focus of cancer research has been on the epithelial tumour cells themselves but recently the influence of the tumour microenvironment, also known as tumour stroma, has received increasing attention. Colorectal cancers surrounded by large quantities of tumour stroma (also known as Stroma^{High} cancers) have a worse prognosis compared to Stroma^{Low} cancers.[1] The exact mechanism of this specific CRC tumour/stroma interaction remains to be elucidated. What is known about the tumour/stroma interaction in general is that tumour epithelial cells produce growth factors which can activate the surrounding fibroblasts, inflammatory cells and endothelial cells in a paracrine way. In response, the fibroblasts, inflammatory cells and endothelial cells produce proteases, growth factors and extracellular matrix components that can promote angiogenesis and malignant tumour growth.[2,3] Interestingly, Stroma^{High} CRC are associated with a poor prognosis specifically in a subset of colon cancers with SMAD4 loss.[4] This suggests that the tumour/stroma interaction may not only depend on the organ of origin but also on the specific genomic subtype of the tumour cells.

SMAD4 is the central critical component of the Transforming Growth Factor (TGF)- β/Bone Morphogenetic Protein (BMP) signalling pathways. BMP signalling is important in differentiation and apoptosis in normal colonic tissue. The clinical relevance of the BMP pathway can be observed in hereditary colorectal

cancer syndromes. For example, BMPRIA and SMAD4 mutations are found in Juvenile Polyposis[5] and Gremlin-1 mutations are found in Hereditary Mixed Polyposis.[6] Recently we have shown that activation of the Bone Morphogenetic Protein (BMP) signalling pathway increases migration, invasion and metastasis formation in SMAD4 negative CRC cells through activation of non-canonical, SMAD4 independent BMP targets; a reverse effect was observed in SMAD4 positive CRC cells.[7] Other groups have also shown a central role for SMAD4 in metastasis formation. [8,9] There is also extensive evidence linking stromal fibroblasts with metastasis formation.[3,10,11] This led to our hypothesis that stromal cells might act on SMAD4 negative cancer cells through the activation of non-canonical, SMAD4-independent BMP signalling.

Our aim was to investigate the effects of fibroblasts, a major component of the tumour stroma, on the invasiveness of SMAD4 negative colorectal cancer cells and the role of BMP signalling in the tumour-stroma interaction in CRC.

Methods

Cell culture

Studies were performed using two pairs of isogenic cell lines differing only in their expression of SMAD4. HT29 (naturally SMAD4 mutant) and HCT116 (naturally SMAD4 wt) colon cancer cell lines, and 18CO colonic fibroblasts, were obtained from ATCC. HCT116 SMAD4-/- cells, with a targeted homozygous deletion of SMAD4, were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA). We used lentiviral constructs expressing pcDNA3.1_SMAD4 to stably express SMAD4 in HT29 CRC cells. Production of lentiviruses was performed by transfection into 293T cells. HT29 cells were transduced in the presence of 8 mg/mL polybrene (Sigma-Aldrich) and cells were selected using puromycin. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland) with 4.5g/l glucose and with 580mg/l L-glutamine. This was supplemented with penicillin (50U/ml) and streptomycin (50µg/ml) and with 10% fetal calf serum (FCS) (Gibco) unless stated otherwise. All *in vitro* experiments were performed on cells growing exponentially and all *in vitro* experiments were performed thrice.

Invasion assay

Cells were labelled with 5 μM CellTracker Green CMFDA (Invitrogen) after which they were detached using trypsin with 2 mM ethylenediaminetetraacetic acid (EDTA). 10^5 cells/insert were transferred to 8 μM pore size HTS FluoroBlok Cell Culture Inserts (BD Falcon, Breda, the
Netherlands) in medium containing 0.5% FCS. The inserts were coated with 100 μl of 1:1 mix of serum free-medium and Matrigel (BD Biosciences, Breda, the Netherlands). Inserts were placed in 24-well plates (Corning Incorporated) in which 600μl of 10% FCS-containing media without phenol red were present. Fluorescence of the invaded cells at the bottom well of the transwell was measured every 4 hours using the BioTek Flx800 (BioTek, Winooski, VT, USA). Data was corrected for background fluorescence and migration start points were set to zero. Variations on this model were used throughout the study.

Orthotopic metastasis mouse model

HT29-cells and $HT29^{SMAD4}$ cells were stably transfected with a pGL4 vector expressing firefly luciferase controlled by an optimised CAGGS-promoter (see supplementary figure 2 for vector map). Transfected cells were selected using G418 (500 μg/ml). Liver metastasis were generated by injecting 5 *10^5 HT29 or $HT29^{SMAD4}$ cells expressing luciferase with or without 10^5 18CO fibroblasts dissolved in 50 μl of medium into the spleens of 6 week old immunocompromised CD1 nude mice (males). Mice were purchased from Charles River Laboratories International, Inc (Wilmington, MA, USA). The mice were anesthetized with isoflurane. A dorsal subcostal incision was made to expose the spleen. The spleen was exteriorized and placed on gauze soaked in PBS. 50μL volume of tumour cell/fibroblasts suspension was injected slowly into the spleen using a 25-gauge needle. *In vivo* whole body bioluminescence scans were performed by intraperitoneal injection of 100 mL luciferin (25 mg/mL) and tumour formation was visualized using the IVIS Lumina Imaging system (Xenogen, Hopkinton, MA). Living Image software (Caliper, Hopkinton, MA) was used to analyse and quantify the scans. After 7 weeks the mice were euthanized. The weight and volume of the livers was determined for each mouse.

Conditioned medium from CRC cell lines

HT-29 or HT29^{SMAD4} cells were incubated with DMEM supplemented with 1% FCS for 24 hours. After incubation medium was centrifuged for 5 minutes at 1200 rpm.

TGF-β / BMP specific qPCR array

Fibroblasts were treated with 2 ml conditioned medium of cancer cells and incubated overnight. The next day the cells were harvested and RNA was isolated using Trizol (Invitrogen) according manufacturer's instructions. Then a 96 well TGF-β / BMP Signalling Pathway PCR array for 84 genes (SABiosciences) was performed according manufacturer's instructions.

Fold-Change was calculated by dividing the normalized gene expression of the treated samples (2^(- Delta Ct)) by the normalized gene expression of the control samples. p values were calculated based on a Student's t-test of the replicate 2^(- Delta Ct) values for each gene in the control group and treatment groups.

CRC tissue

Formalin-fixed paraffin-embedded primary tumour tissues from 146 stage III colorectal cancer patients between the years 1995 and 2011 were collected. Blocks were obtained from the archives of the pathology department at Leiden University Medical Center, Leiden. Patient characteristics and survival data were available from the LUMC dept. of Surgery colorectal cancer database. Embedded tumours were sectioned and stained for BMP2 and SMAD4. Analysis was performed in a blinded fashion by two investigators independently. SMAD4 expression was scored according to previously described methods.[12] BMP expression was scored as either positive or negative. ≥30% expression is positive and <30% is negative.[13]

SMAD 4 immunohistochemistry

Slides were deparaffinised in xylene, rehydrated in graded alcohol and blocked for endogenous peroxidase activity in methanol containing 0,3% hydrogen peroxide for 30 minutes. Antigen retrieval was performed using Tris EDTA (pH =9.0) at 98^{0}C for 45 minutes. Slides were blocked 10 minutes using 10% goat serum, afterwards the slides were incubated with mouse-anti-SMAD4 antibodies (Santa Cruz Biotechnology) dissolved in SkyTek diluent (SkyTek Laboratories,

Logan, Utah) (1:400) for 60 minutes. Secondary antibody goat-anti-mouse poly HRP (Immunologic, Duiven, The Netherlands) was incubated for 60 minutes. Peroxidase activity was detected by FastDAB (Sigma-Aldrich, Zwijndrecht, The Netherlands) and slides were counterstained using Haematoxylin (Sigma-Aldrich).

BMP 2 immunohistochemistry

Slides were deparaffinised in xylene, rehydrated in graded alcohol and blocked for endogenous peroxidase activity in methanol containing 0,3% hydrogen peroxide for 30 minutes. Antigen retrieval was performed using Sodium Citrate (pH =6.0) at 98^{0}C for 20 minutes. The slides were blocked for 30 minutes using TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 0,15 mol/L NaCl, 0,25% gelatin, 0,05% (vol/vol), afterwards the slides were incubated with goat-anti-BMP2 antibodies (Santa Cruz Biotechnology) dissolved in PBS, 1% FBS, 1% Triton (1:200) for 60 minutes. Next the slides were incubated with biotinylated secondary antibodies for 30 minutes and subsequently incubated with streptavidin-biotin-horseradish peroxidase (Dako, Glostrup, Denmark) for 30 minutes. Peroxidase activity was detected by FastDAB (Sigma-Aldrich, Zwijndrecht, The Netherlands) and slides were counterstained using Haematoxylin (Sigma-Aldrich).

Statistics

Statistical analyses were performed using SPSS (version 20.0 for Windows, IBM SPSS statistics). A *p*-value of <0.05 was considered statistically significant. Cox regression analyses were performed for Overall Survival (OS) and Disease Free Survival (DFS). Mean follow up was 6.0 years (range: 0.2-29.9). In the multivariate analysis adjustments for potential confounders were made for sex, age, adjuvant therapy (yes/no), microsatellite status, lymph node status (N1/N2) and tumour location (right/left). Chi-square tests were used to compare groups. All statistical tests were performed 2-sided.

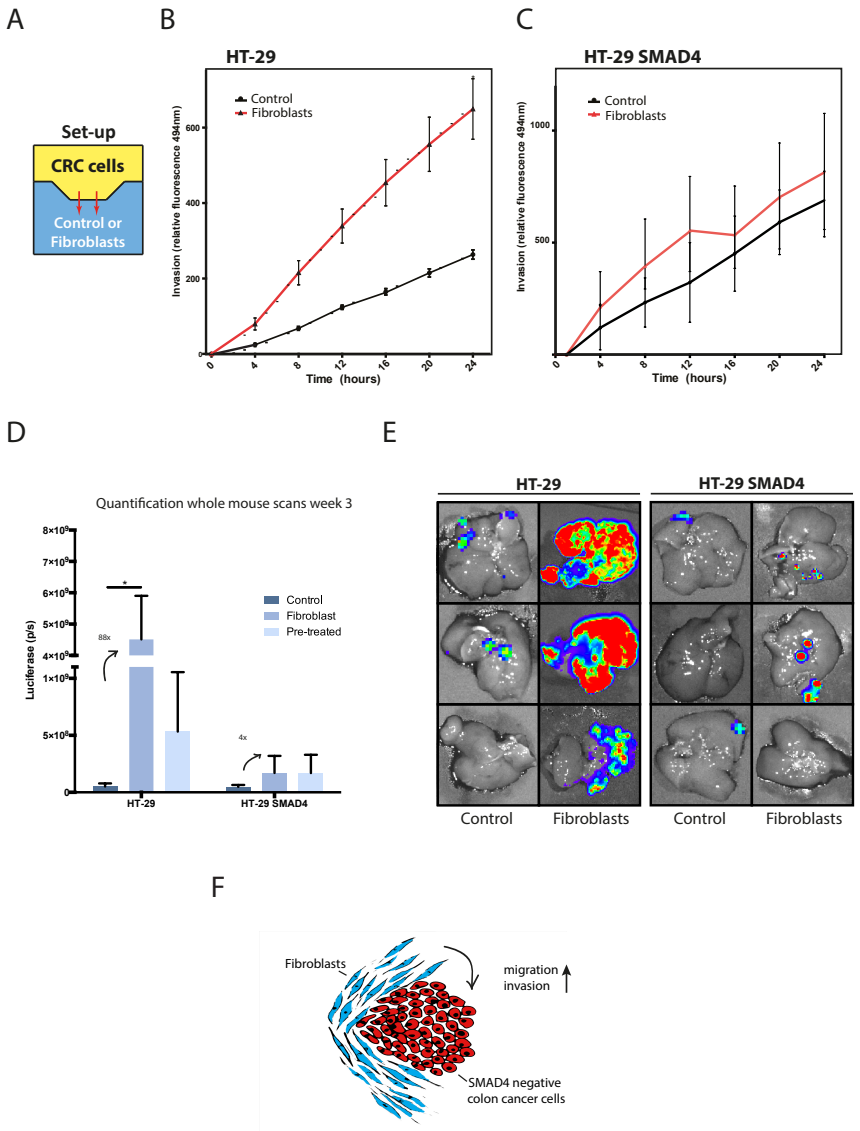
Results

Fibroblasts increase migration and metastasis formation of SMAD4 negative colon cancer cells

In order to investigate possible pro-migratory effects of fibroblasts on colon cancer cells we performed *in vitro* matrigel invasion assays and *in vivo* rodent metastasis models. In the introduction we describe that a combination of large quantities of stroma surrounding SMAD4 negative tumours is associated with a worse prognosis as was demonstrated by Mesker et al; we hypothesise that this is the result of increased invasion and metastatic capacity of the SMAD4 negative cells caused by the presence of fibroblasts. We further hypothesised that this will lead to SMAD4 negative colon cancer cells tending to migrate and invade faster towards fibroblasts than their SMAD4 positive counterparts. To test this, we used a pair of isogenic cell lines differing only in their expression of SMAD4 (HT29 and HT29^{SMAD4}) and placed each separately in the upper well of a transwell system (***Figure 1A)***; in the bottom well we used the normal colonic fibroblast cell line 18-CO or no cells. The CRC cell line cells were labelled with Celltracker in order to measure fluorescence in the bottom well in real time as they pass through the matrigel coated transwell filter. Using SMAD4 negative HT-29 cells we observed a significant increase in migration towards fibroblasts (***Figure 1B***). SMAD4 positive HT-29 cells did not migrate faster towards fibroblasts (***Figure 1C***). We confirmed these results by repeating the experiments with a second pair of isogenic cell lines (HCT116 and HCT116 SMAD4-/-) where we again saw that SMAD4 negative cells migrate faster towards fibroblasts.

In an orthotopic liver metastasis model we injected SMAD4 positive and negative

Figure 1. Fibroblasts increase migration and metastases formation in SMAD4 negative colon cancer cells (A) Schematic of experimental set-up of experiments shown in figure 1B and 1C. Fluorescent labelled cancer cells are seeded in the upper chamber of a transwell system in medium containing 0.5% FCS. Fibroblasts or no cells are seeded in the lower chamber in medium containing 10% FCS. Fluorescence was measured in the lower chamber. Invasion through the matrigel coated transwell inserts was measured every 4 hours for a period of 24 hours. Values were corrected for background fluorescence and starting point was set to zero. (B) Invasion of SMAD4 negative HT-29 cells towards fibroblasts or control (no cells). (C) Invasion of SMAD4 positive HT-29 cells towards)*(figure 1 continued)* fibroblasts or control (no cells). (D) Quantification of *in vivo* bioluminscence scans of whole mice three weeks after splenic injection of cancer cells. 5x10^5 SMAD4 positive and SMAD4 negative HT-29 cells were injected using 3 different treatment conditions. 1. after pre-treatment with fibroblast conditioned medium for 24 hours before injection 2. co-injected with 1x10^5 fibroblasts 3. injected alone without any pre-



treatment or fibroblasts (control). n=5 per group. The luciferase (p/s) from the HT-29 SMAD4 negative cells co-injected with 18C0 fibroblasts is 88 times stronger than that from the HT-29 SMAD4 negative cell alone (*p=0.02*), compared to a 4-time difference between HT29 SMAD4 positive cells co-injected with 18C0 fibroblasts and the mono-injection of HT29 SMAD4 positive cells (*p=0.5*). (E) Visualization of liver metastases using bioluminescence scans of the liver *ex vivo* just after sacrifice (three weeks after injection).(F) Proposed model *(*<0.05, **<0.001, ***< 0.001). Data is presented as mean ±SEM.*

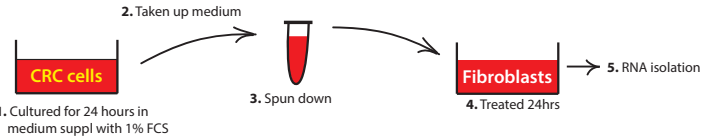
HT-29 CRC cells into the spleens of immunodeficient CD-1 nude mice (n=5) in combination with 18-CO fibroblasts or vehicle control. Both the SMAD4 positive and SMAD4 negative HT29 cells were stably transfected with a CAGGS-luc vector expressing luciferase to monitor metastases formation over time by *in vivo* bioluminescence imaging. Splenic injection of SMAD4 positive HT-29 cells together with fibroblasts led to the development of slightly more metastases as shown by higher levels of *in vivo* bioluminescence than when SMAD4 positive HT-29 cells were injected alone without fibroblasts. However, this difference was not statistically significant (p=0.5) (***Figure 1D***).***Figure 1E*** shows representative liver scans). In contrast co-injection of SMAD4 negative HT-29 cells together with fibroblasts led to a significant 88-fold increase in metastasis formation as shown by *in vivo* bioluminescence compared to injection of SMAD4 negative HT29 cells without fibroblasts (p=0.02). We also included a population of HT-29 cells that were pre-treated with fibroblast conditioned medium for 24 hours to observe whether cancer cells need direct contact of fibroblasts or just their secreted factors. ***Figure 1D*** shows that factors in fibroblasts conditioned medium increase SMAD4 negative cancer cell metastasis formation (not statistically different p=0.3), but co-injection with fibroblasts has a far greater effect on metastasis formation.
From the experiments presented in figure 1 we conclude that fibroblasts increase the migration and metastasis formation of colon cancer cells, primarily that of SMAD4 negative CRC cells (***Figure 1F***).

Fibroblasts show increased BMP-2 expression upon contact with SMAD4 negative colon cancer cell CM

In order to elucidate the mechanism by which fibroblasts increase SMAD4

Figure 2. BMP2 expression is up regulated in fibroblasts after treatment with conditioned medium from SMAD4 negative colon cancer cells. (A) Experimental set-up. SMAD4 positive and SMAD4 negative HT29 cells are cultured in 1%FCS medium for 24 hours. Subsequently, medium was taken up, spun down and added to 18CO fibroblasts that were in culture. Standard medium with 1% FCS was used as a control. After 24 hours, RNA was isolated from the fibroblasts and a TGFβ/BMP specific qPCR array was performed. (B) Top ten genes that were up regulated and down regulated in fibroblasts after treatment with conditioned medium from SMAD4 negative HT29 cells. (C) Top ten genes that were up regulated and down regulated in fibroblasts after treatment with conditioned medium from SMAD4 positive HT29 cells. (D) Proposed model. *Fold-Change was calculated by dividing the normalized gene expression of the treated samples (2^(- Delta Ct)) by the normalized gene expression of the control samples. p values were calculated based on a Student's t-test of the replicate 2^(- Delta Ct) values.*

A



B

HT-29 CM vs Control medium

Top 10 upregulated genes in fibroblasts

Gene	Fold change	p-value
BMP2	2,73	0,037
IGF1	2,42	0,048
B2M	2,02	0,000
IL6	1,76	0,125
IGFBP3	1,62	0,039
BMP6	1,40	0,325
JUNB	1,40	0,227
BMP4	1,40	0,234
TGFB1	1,39	0,346
FST	1,34	0,598

Top 10 downregulated genes in fibroblasts

Gene	Fold change	p-value
SMAD2	0,66	0,117
SMAD3	0,64	0,091
TGFB3	0,63	0,204
ACVR2A	0,59	0,122
GDF6	0,57	0,099
HIPK2	0,55	0,087
DLX2	0,52	0,329
LTBP4	0,49	0,046
CHRD	0,45	0,320
COL3A1	0,44	0,353

C

HT-29 SMAD4^{+} CM vs Conrol medium

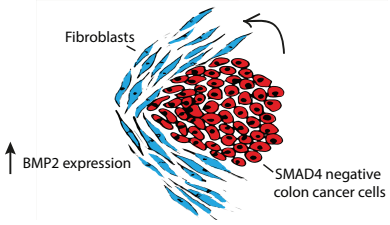
Top 10 upregulated genes in fobriblasts

Gene	Fold change	p-value
LTBP4	8,22	0,005
IL6	6,11	0,003
SMAD4	5,88	0,008
BAMBI	4,28	0,049
RPL13A	4,27	0,008
FST	4,08	0,008
ID1	3,69	0,016
FKBP1B	3,26	0,016
TGIF1	3,08	0,033
SMAD3	2,82	0,017

Top 10 downregulated genes in fibroblasts

Gene	Fold change	p-value
TGFB2	0,15	0,002
JUNB	0,11	0,004
BMP4	0,09	0,029
COL1A2	0,07	0,004
BMP2	0,07	0,000
SOX4	0,07	0,048
BMPER	0,06	0,030
COL1A1	0,05	0,004
COL3A1	0,05	0,019
IGFBP3	0,03	0,017

D



negative cancer cell migration, we first investigated the effects that SMAD4 negative cancer cells have on fibroblasts. As the SMAD4 status of the cancer cells is important in the interaction between fibroblasts and cancer cells, and SMAD4 is a key protein in the TGF-β/BMP signalling pathways, we hypothesized that fibroblasts excrete factors that may activate or inhibit TGF-β/BMP signalling pathways. Furthermore, results of our orthotopic mouse experiments suggest that direct presence of SMAD4 negative cancer cells is needed for excretion of TGF-β/BMP signalling associated factors.

We treated 18CO fibroblasts with SMAD4 positive and SMAD4 negative HT-29 conditioned medium for 24 hours, isolated RNA from the fibroblasts and performed a TGF-β/BMP specific qPCR array (***Figure 2A***). ***Figure 2B*** shows the top 10 up regulated and top 10 down regulated genes in fibroblasts after treatment with HT-29 CM. We compared this with the up and down regulated genes after treatment with HT-29_SMAD4 CM (***Figure 2C***). *BMP2* was the most highly upregulated gene when fibroblasts were treated with SMAD4 negative tumour cell CM. Interestingly, while *BMP2* expression is up regulated after treatment with the SMAD4 negative HT-29 CM, it is down regulated after treatment with SMAD4 positive HT-29 CM.

Migration of SMAD4 negative cancer cells towards fibroblasts is BMP pathway dependent

We have previously published that BMP2 treatment of SMAD4 negative colon cancer cells increased cell invasion.[7] This was not observed when SMAD4 positive cancer cells were treated with BMP2. When SMAD4 negative cells are serum starved for 24 hours, they start expressing high levels of BMP2 (***Supplementary Figure 1***); we hypothesized that cancer cells might also migrate towards BMP2 and that this might in part explain their migration towards fibroblasts. SMAD4 positive and SMAD4 negative HT-29 cells were seeded in the upper well of a matrigel transwell system. The lower well was filled with medium supplemented with 100 ng/ml BMP2 or control (***Figure 3A***). ***Figure 3B*** shows that SMAD4 negative HT-29 cells migrate faster towards BMP2 than towards the control condition. In contrast, SMAD4 positive HT-29 cells migrate equally fast towards BMP2 and the control condition (***Figure 3C***). We repeated these experiments using HCT116 and HCT116 SMAD4-/-.

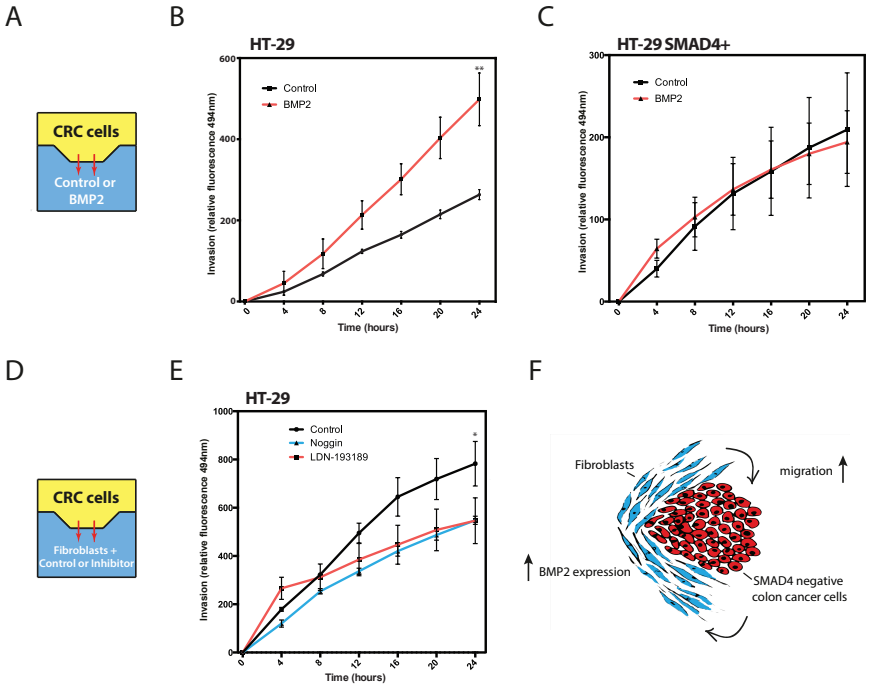


Figure 3. Fibroblasts increase migration in SMAD4 negative colon cancer cells through activation of BMP signalling. (A) Schematic diagram of the experimental set-up of experiments shown in figure 3B and 3C. Fluorescent labelled cancer cells are seeded in the upper chamber of a transwell system in medium containing 0.5% FCS. 100ng/ml BMP2 was added to medium with 0.5% FCS and placed in the lower chamber. Fluorescence was measured in the lower chamber. Invasion through the matrigel coated transwell inserts was measured every 4 hours for a period of 24 hours. Values were corrected for background fluorescence and the starting point was set to zero. (B) Invasion of SMAD4 negative HT-29 cells towards BMP2 or control. (C) Invasion of SMAD4 positive HT-29 cells towards BMP2 or control. (D) Schematic of experimental set-up of the experiment shown in figure 3E. Fluorescent labelled cancer cells are seeded in the upper chambers of a transwell system in medium containing 0.5% FCS. Fibroblasts were seeded in 0.5%FCS medium in the lower chambers in combination with 5nM LDN-193189 (small molecule BMPR1a inhibitor), 500ng/ml of Noggin or a control vehicle (PBS). Invasion through the matrigel coated transwell inserts was measured every 4 hours for a period of 24 hours. Values were corrected for background fluorescence and starting point was set to zero. (E) Invasion of SMAD4 negative HT-29 cells towards fibroblasts treated with LDN-193189, Noggin or control. (F) Proposed model *Student t-tests were performed using the corrected means of the individual experiments (*<0.05, **<0.001, ***< 0.001). Data is presented as mean ±SEM. All experiments were performed three times.*

If expression of BMP2 by fibroblasts is the reason SMAD4 negative cells migrate towards fibroblasts, then this should be impeded by inactivation of BMP2. To test this, we repeated the matrigel invasion assay depicted in ***figure 1A***, in which we seeded cancer cells in the upper wells and fibroblasts in the lower wells, only this time we also used conditions where we added 500ng/ml of Noggin or 5nM of the small molecule LDN-193189 to the fibroblasts (***Figure 3D***). Adding Noggin or LDN-193189 decreases the migration of SMAD4 negative HT-29 cells towards the fibroblasts (***Figure 3E***), implying that the tendency of SMAD4 negative cancer cells to migrate towards fibroblasts is partly BMP dependent.

Stromal BMP-2 expression is associated with poor patient survival in SMAD4 negative colon cancer

We stained and scored SMAD4 and BMP2 in the invasive front of 146 stage III colorectal cancers using immunohistochemistry. SMAD4 expression was scored in the tumour and BMP2 expression was scored in the non-epithelial stromal cells at the invasive front. SMAD4 expression is aberrant in 61% (89/146) of the cases as has been shown previously. There were no significant differences in patient characteristics between SMAD4 positive and SMAD4 negative cancers, except for mean age at operation (60.9y vs. 66.0y, *p=0.015*) (***Supplementary table 1***). SMAD4 loss is associated with a poorer Overall Survival (OS) (univariate: HR=1.7, *p=0.026* and multivariate: HR=1.41, *p=0.043*) and a poorer Disease Free Survival (DFS) (univariate: HR=1.75, *p=0.017* and multivariate: HR=1.62, *p=0.042*) (***Table 1***).
High stromal BMP2 expression at the invasive front was found in 39.7% (58/146) of the cancers. No significant differences in patient characteristics were observed, although there was a tendency for cancers with high stromal BMP2 expression to occur more in the right colon (60.3%, *p=0.065*) (***Supplementary table 1***). High

Table 1

	Overall survival				Disease Free Survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR	*P*-value	HR	*P*-value	HR	*P*-value	HR	*P*-value
Age (≥65y vs. <65y)	2.87	**<0.001**	2.02	**0.009**	2.33	**<0.001**	1.81	**0.015**
Sex (male vs. female)	1.37	0.16	-	-	1.35	0.17	-	-
Adjuvant therapy (no vs. yes)	2.86	**<0.001**	2.44	**0.005**	2.29	**0.002**	1.95	**0.016**
Location (right vs. left)	1.45	0.10	1.41	0.16	1.22	0.35	-	-
Microsat. status (MSI vs. MSS)	1.07	0.85	-	-	1.08	0.82	-	-
Lymphnodes (N2 vs. N1)	1.60	0.067	1.50	0.12	1.59	0.052	1.56	0.066
SMAD4 tumour (neg vs. pos)	1.70	**0.026**	1.41	**0.043**	1.75	**0.017**	1.62	**0.042**
BMP2 stroma (high vs. low)	1.42	0.12	-	-	1.15	0.52	-	-

Table 2

	Stromal BMP2 expression		
	Low	**High**	***P*-value**
All cancers			
Overall Survival			
Patients	88	58	
Deaths	41	38	
Hazard Ratio (95%)	1.0 (reference)	1.42 (0.91-2.20)	0.12
Adjusted Hazard Ratio (95%)*	1.0 (reference)	1.36 (0.66-2.79)	0.40
Disease Free Survival			
Patients	88	58	
Deaths	49	38	
Hazard Ratio (95%)	1.0 (reference)	1.15 (0.75-1.76)	0.52
Adjusted Hazard Ratio (95%)*	1.0 (reference)	1.27 (0.62-2.61)	0.51
SMAD4 negative cancers			
Overall Survival			
Patients	58	31	
Deaths	30	25	
Hazard Ratio (95%)	1.0 (reference)	1.81 (1.06-3.08)	**0.03**
Adjusted Hazard Ratio (95%)*	1.0 (reference)	2.70 (1.00-7.35)	**0.05**
Disease Free Survival			
Patients	58	31	
Deaths	35	25	
Hazard Ratio (95%)	1.0 (reference)	1.58 (0.94-2.64)	0.08
Adjusted Hazard Ratio (95%)*	1.0 (reference)	2.01 (0.73-5.49)	0.17
SMAD4 positive cancers			
Overall Survival			
Patients	30	27	
Deaths	11	13	
Hazard Ratio (95%)	1.0 (reference)	1.16 (0.52-2.63)	0.72
Adjusted Hazard Ratio (95%)*	1.0 (reference)	1.22 (0.25-5.92)	0.80
Disease Free Survival			
Patients	30	27	
Deaths	14	13	
Hazard Ratio (95%)	1.0 (reference)	0.84 (0.39-1.79)	0.65
Adjusted Hazard Ratio (95%)*	1.0 (reference)	0.96 (0.22-4.18)	0.95

*Adjusted for sex, age, adjuvant therapy, lymphnode status, left vs right sided and microstatellite status

stromal BMP2 expression in isolation is not associated with a poorer OS (HR=1.4, *p=0.10*) and DFS (HR=1.15, *p=0.52*) (***Table 1***). Contrary to what we might have expected from the *in vitro* studies, we found no association between the level of expression of SMAD4 in tumour cells and the level of expression of BMP2 in stromal cells (data not shown).

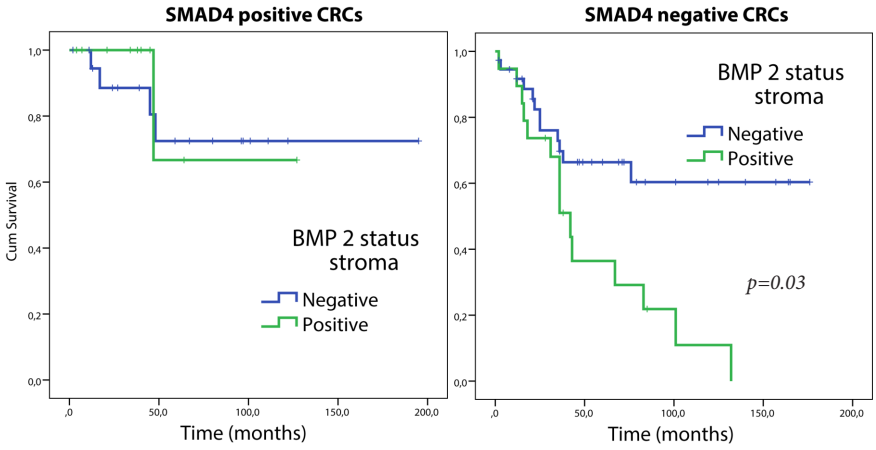
The *in vitro* and *in vivo* experiments show that BMP2 and fibroblasts increase

migration and metastasis formation specifically in SMAD4 negative cancer cells. We hypothesized that if the same were true in patients we would expect high stromal BMP2 expression in combination with tumour SMAD4 loss to negatively influence patient survival. Therefore, we analysed whether stromal BMP2 expression and tumour epithelial SMAD4 expression were associated with differences in patient survival. High stromal BMP2 expression was observed in 31 of the 89 SMAD4 negative cancers (34.8%). No significant differences in patient characteristics between BMP2 high and BMP2 low expression within SMAD4 negative cancers were found (***Supplementary table 2***). High stromal BMP2 expression in SMAD4 negative cancers is associated with a poor patient OS (HR 1.81; *p=0.03*, adjusted HR 2.70; *p=0.05*), and shows a poorer trend in DFS (HR 1.58; *p=0.08*, adjusted HR 2.01; *p=0.17)* (***Table 2***). In SMAD4 positive cancers high stromal BMP2 expression was not associated with differences in survival (OS: HR 1.16; *p=0.72*, adjusted HR 1.22; *p=0.80* and DFS: HR 0.84; *p=0.65*, adjusted HR 0.96; *p=0.95*).

Discussion

Previous studies have shown that stromal components contribute to tumour progression and that the presence of large quantities of stromal cells at the invasive front is associated with poorer patient survival in several solid tumour types. Interestingly, Mesker et al. published that a combination of SMAD4 loss in CRCs, observed through immunohistochemical staining and scoring of invasive fronts, and the abundant presence of stroma, is associated with a poor prognosis. We attempted to address why stromal cells contribute to a poorer prognosis in SMAD4 negative CRCs. Many cell types can be found in the stroma; we chose to focus on fibroblasts as a starting point, because stroma mainly consists of fibroblast-like cells.

We hypothesized that these associations observed in patients could be explained by a two-way interaction between tumour cells and fibroblasts with a central role for the TGF-β/BMP pathway. Firstly, we looked at how the fibroblasts influence the cancer cells. Our results show that SMAD4 negative colon cancer cells respond differently to the presence of fibroblasts than SMAD4 positive colon cancer cells; they show a more pronounced tendency to migrate towards fibroblasts and



show an increase in metastasis formation. SMAD4 loss in colon cancer cells has previously been shown to increase migration, invasion and metastasis formation independently *in vitro* and *in vivo* [9,14] and fibroblasts are also known to contribute to the migration of cancer cells.[10] Several mechanisms by which the tumour cell/fibroblast interaction may contribute to tumour progression have been proposed. These include structural support by secretion of extracellular matrix components, the production and secretion of matrix metalloproteases for tissue remodelling and matrix degradation through invadopodia.[10,15,16] It should be noted that these are just a few of the many mechanisms that have been explored to date, though none of them explain why fibroblasts would specifically enhance tumour cell migration in SMAD4 negative cancer cells. Furthermore, most of these studies have focused on the direct effect of fibroblasts when in the proximity of cancer cells, but they have not addressed why cancer cells would migrate towards fibroblasts as we have observed.

Next we looked at how the cancer cells might influence the fibroblasts. We investigated whether fibroblasts react differently to SMAD4 negative cancer cells than to SMAD4 positive cancer cells. SMAD4 is the central critical component of the TGFβ/BMP signalling pathways. Hawinkels et al have shown that TGFβ signalling activity is increased in Cancer Associated Fibroblasts after co-culture with colon cancer cell line cells or treatment with colon cancer cell line CM, leading

to the production of proteinases and TGFβ1, creating a positive cancer progressing feedback loop[17]. This suggests that the TGFβ superfamily plays an important role in tumour-stroma interaction, but no distinction was made here between SMAD4 positive and SMAD4 negative cancer cells. We treated fibroblasts with CM from SMAD4 positive and negative versions of a CRC cell line, performed a TGF-β/BMP specific qPCR array and found that *STAT1, BMP2*, *IGF1* and *IGFBP3* were significantly up regulated in fibroblasts after SMAD4 negative CM exposure and *LTBP4* was significantly down regulated. Insulin-like Growth Factor 1 (IGF1) enhances invasion in colon cancer cells through Akt [18] and Insulin-like Growth Factor Binding Protein-3 (IGFBP3) inhibits IGF1 by competing for the same receptors. Despite some promising results that serum levels of IGF1 could be used as a predictive marker for colon cancer, a large study conducted in 2009 including over 2000 cases and controls showed that IGF1 and IIGFBP3 serum levels are not associated with increased CRC risk.[19] Stat1 is part of the Signal Transducers and Activators of Transcription family, can be activated by a wide range of cytokines and growth factors, such as interferon (IFN), interleukin-6 (IL-6) and IL-10, and negatively influences metastasis of tumour cells[20]. Latent Transforming growth factor beta Binding Protein 4 (LTBP4) is responsible for correct folding and secretion of TGFβ-1[21]. LTBP4 knockout mice develop colorectal carcinomas[22] and LTBP4 expression is down regulated in various gastrointestinal cancers.[23]
In our view, the most interesting change is in BMP2 expression, which is up regulated when fibroblasts are treated with SMAD4 negative CM and down regulated when fibroblasts are treated with SMAD4 positive CM. LTBP4 expression is affected in a similar way, although reversed, but we have previously shown evidence for the importance of BMP2 in colorectal cancer cell invasion supporting the validity of this finding.[7]

Next we investigated to what degree BMP2 expression by fibroblasts could explain the migration of SMAD4 negative cancer cells towards fibroblasts. We show that adding BMP inhibitors reduces this effect. LDN-193189 is a small molecule BMPR1a specific inhibitor; the classic view is that BMP ligands bind to a complex of BMPR1 and BMPR2, but BMP2 could theoretically still signal through other receptors of the TGF-β/BMP family. Noggin inhibits BMP signalling by binding various BMP ligands so in this assay the reduction in migration seen in our

experimental set-up could be the consequence of Noggin binding BMP ligands other than BMP2. Our data confirms that the migration of SMAD4 negative cancer cells towards fibroblasts is at least partly BMP dependent.

Finally, we set out to investigate whether the mechanisms revealed in our *in vitro* and *in vivo* experiments might have any clinical significance in patients. Stromal BMP2 expression and SMAD4 expression in tumour cells were scored at the invasive front of stage III CRCs. High stromal expression of BMP2 is associated with a poor prognosis in SMAD4 negative CRC. Loss of SMAD4 has proven to be a reliable prognostic marker in CRC[24]. To confirm that our cohort is representative we included an analysis in which we show that SMAD4 loss is associated with a poorer OS and DFS as expected. Adding stromal BMP2 expression to SMAD4 loss is an even stronger prognostic marker in our cohort, although the group sizes are necessarily smaller reducing statistical power.

There are several limitations to the study. Because of the previously observed synergistic effect of SMAD4 status and a large amount of tumour stroma on patient survival, we focussed purely on SMAD4 loss and TGFβ/BMP signalling. SMAD4 positive cancer cells also benefited, although less so, from co-injection with fibroblasts in the metastasis model. Other signalling pathways are probably also involved in the interaction between SMAD4 positive cancer cells and fibroblasts. From previous research we know that p53 mutations combined with SMAD4 loss play a role in BMP-Wnt interaction and others have shown that TGF-β signalling pathway can either inhibit or promote migration/invasion dependent on the presence of wild-type or mutant p53. [13,25] Wnt signalling is mostly active at the invasive front despite the clonal occurrence of the same Wnt-activating APC/beta-catenin mutation throughout the tumour, indicating modulations of Wnt signalling by extrinsic factors in the microenviroment.[26] Future studies should focus on the involvement of other mutations in combination with SMAD4 loss and the possible involvement of Wnt signalling.
Based on the results of the BMP/ TGF-β specific qPCR array we focussed on BMP2, but multiple other proteins of the TGF-β /BMP superfamily could be involved. As mentioned previously, it has already been shown that TGF-β1 is involved in cancer cell-fibroblast interaction. LTBP4 is responsible for correct folding and secretion

of TGF-β1 and was down regulated in fibroblasts after treatment with SMAD4 negative CM (and up regulated after treatment with SMAD4 positive CM. LTBP4 is therefore an interesting target for future studies.
Lastly, we used 'normal' colonic fibroblasts in our models. It has been shown extensively that 'normal' fibroblasts can become Cancer Associated Fibroblasts (CAFs) after contact with cancer cells. In the exploratory phase of the study we did include CAFs extracted from human cancers and observed that SMAD4 negative cancer cells migrate towards CAFs with the same velocity as towards 'normal' fibroblasts (data not shown). All other experiments were performed only using 'normal' fibroblasts as has been performed by other leading groups .[27] 18CO fibroblasts have the advantage of being standardised and well characterised so that our work can easily be corroborated.

To conclude, this study is the first to show that the tumour/stroma interaction is not only bidirectional but highly dependent on the molecular subtype of the tumour with an important role specifically for the BMP arm of the TGF-β superfamily. We show that cancer cells react differently to fibroblasts dependent on the SMAD4 status of the cancer cells and that fibroblasts react differently to cancer cells also dependant on the SMAD4 status of the cancer cells. Fibroblasts produce more BMP2 when in proximity to SMAD4 negative cancer cells, which leads to an increase in migration and metastasis formation specifically in SMAD4 negative cancer cells. High levels of BMP2 expression in the stroma at the invasive front of human colon cancer specimens correlates with a poorer prognosis, specifically in SMAD4 negative cancers, providing clinical evidence to support the relevance of our preclinical studies. These results suggest that the targeted use of BMP specific inhibitors could potentially impede the proinvasive fibroblast/ cancer cell interaction.

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Supplementary table 1

	Total	SMAD4 pos	SMAD4 neg		Stroma BMP2 high	Stroma BMP2 low	
	n=146 (%)	*n*=57 (39.0%)	*n*=89 (61.0%)	*p-value*	*n*=58 (39.7%)	*n*=88 (60.3%)	*p-value*
Mean age±SD (y)	64.0±12.5	60.9±13.1	66.0±11.7	**0.015**	64.8 (11.7)	63.4 (13.0)	0.51
Gender							
Female	65 (44.5)	23 (40.4)	42 (47.2)	0.50	28 (48.3)	37 (42.0)	0.50
Male	81 (55.5)	34 (59.6)	47 (52.8)		30 (51.7)	51 (58.0)	
Lymphnode							
N1	105 (72.4)	43 (75.4)	62 (70.5)	0.57	39 (68.4)	66 (75.0)	0.45
N2	40 (27.6)	14 (24.6)	26 (29.5)		18 (31.6)	22 (25.0)	
Unknown	1	0	1		1	0	
Location							
Left sided*	72 (49.3)	25 (43.9)	47 (52.8)	0.31	23 (39.7)	49 (55.7)	0.065
Right sided**	74 (50.7)	32 (56.1)	42 (47.2)		35 (60.3)	39 (44.3)	
MSI status							
MSS	51 (79.7)	18 (72)	33 (91.7)	0.34	18 (69.2)	33 (86.8)	0.12
MSI	23 (20.3)	7 (28)	6 (8.3)		8 (30.8)	5 (13.2)	
Unknown	82	32	50		32	50	
Adjuvant therapy							
Yes	56 (58.2)	23 (40.4)	33 (37.1)	0.73	17 (29.3)	39 (44.3)	0.83
No	90 (61.6)	34 (59.6)	56 (62.9)		41 (70.7)	49 (55.7)	

p-values are based on Chi-square tests and are two-sided

*Left sided is defined as rectosigmoid, sigmoid, decending colon and splenic flexure

** Right sided is defined as transverse colon, hepatic flexure, ascending colon and caecum

Supplementary table 2

	SMAD4 negative tumour		
	Stroma BMP2 pos	Stroma BMP2 neg	
	n=31 (34.8%)	*n*=58 (65.2%)	*p*
Mean age±SD (y)	68±10.2	64.9±12.4	0.2
Gender			
Female	18 (58.0)	24 (41.4)	0.2
Male	13 (42.0)	34 (58.6)	
Lymphnode			
N1	19 (63.3)	43 (74.1)	0.3
N2	11 (16.7)	15 (25.9)	
Unknown	1	0	
Location			
Left sided*	14 (45.2)	33 (56.9)	0.4
Right sided**	17 (54.8)	25 (43.1)	
MSI status			
MSS	12 (85.7)	21 (84.0)	1.0
MSI	2 (16.3)	4 (16.0)	
Unknown	17	33	
Adjuvant therapy			
Yes	9 (29.0)	24 (41.4)	0.4
No	22 (71.0)	34 (58.6)	

p-values are based on Chi-square tests and are two-sided

*Left sided is defined as rectosigmoid, sigmoid, decending colon and splenic flexure

** Right sided is defined as transverse colon, hepatic flexure, ascending colon and caecum

Chapter 6

A meta-analysis of SMAD4 immunohistochemistry as a prognostic marker in colorectal cancer

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Aim

SMAD4 immunohistochemistry is considered a valuable prognostic marker in colorectal cancer, but individual studies have often been small and the results variable. A meta-analysis could potentially clarify these findings.

Methods

In September 2014 a Pubmed and Google Scholar search was conducted to find publications that reported the prognostic value of SMAD4 expression. A meta-analysis was performed to clarify the association between SMAD4 expression and survival outcomes.

Results

137 studies were found of which 13 were considered eligible. The studies consisted of a total of 3800 patients. Three different endpoints were taken into account, namely overall survival (OS), disease free survival (DFS) and cancer specific survival (CSS). Also, the studies were divided into univariate and multivariate analyses. The pooled hazard ratios were as followed; univariate CSS: 1,75 (95%CI: 0,93–3,32; z=1,69; p=0,09); multivariate CSS: 2,17 (95%CI: 1,56–3,01; z=4,65; p=0,000); univariate DFS: 2,11 (95%CI: 1,36–3,28; z=3,32; p=0,001); multivariate DFS: 2,15 (95%CI: 1,56–3,01; z=4,65; p=0,000) univariate OS DFS 2,30 (95%CI: 1,41–3,73; z=3,36; p=0,001); univariate OS 2,28 (95%CI: 1,30–4,00; z=2,89; p=0,004).

Conclusion

The results of the presented meta-analyses indicate that SMAD4 expression status using immunohistochemistry is a prognostic marker for patient survival.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer death in men and women combined in the US[1]. Colorectal cancer is thought to result from the accumulation of genetic alterations, which give cells a survival advantage over surrounding cells. An important genetic change in CRC is mutation of *SMAD4* leading to loss of SMAD4 protein expression. SMAD4 protein expression is lost in approximately 30-40% of the CRCs[2-4] and is associated with metastasis formation and poor response to chemotherapy[5-8]. *SMAD4* is the common mediator of the Transforming Growth Factor-β (TGF- β) and Bone Morphogenetic Protein (BMP) pathways and is located on chromosome 18q21**.** SMAD4 immunohistochemistry correlates very well with the genetic status as is shown in Juvenile Polyposis[9]. SMAD4 immunohistochemistry is therefore frequently used to ascertain the SMAD4 status of a tumour. Although the use of SMAD4 immunohistochemistry as a molecular marker has been studied in multiple studies, this has mostly been performed in small cohorts and in different subgroups using different survival endpoints. Most studies report an association between SMAD4 loss and a poor prognosis, but this is not consistent. To our knowledge, there has never been a comprehensive study combining all these results to truly establish the predictive value of SMAD4 immunohistochemistry for clinical use. Therefore, we conducted a meta-analysis to correlate the SMAD4 immunohistochemistry expression with survival outcomes.

Methods

Publication selection

Pubmed and Google Scholar were used to search for potentially relevant literature. The search entry used was: ('colorectal OR large intestine' OR 'large bowel' OR 'colon' OR 'colonic' OR 'rectal' OR 'rectum') AND ('cancer' OR 'carcinoma' OR 'tumor' OR 'tumour' OR 'neoplasm' OR 'cancers') AND ('SMAD4' OR 'DPC4') AND ('marker' OR 'signature molecule' OR 'molecular marker' OR 'markers' OR 'biomarkers' OR 'biomarker' OR 'marker' OR 'prognosis' OR 'predictive' OR 'survival'). Additionally, reference lists of the studies found and of systematic

Study	Year	Country	Stage	# patients	Inclusion period	AB	Dilution
Alazzouzi[15]	2005	Finland	III	86	1993-1997	Santa Cruz	1:1000
Alhopuro[5]	2005	Finland	III	75	1994-1998	Santa Cruz	1:1000
Isaksson[18]	2006	Sweden	I-III	86	1987-1994	Santa Cruz	1:50
Bacman[17]	2007	Germany	II-III	305	1991-2001	Santa Cruz	1:50
Mesker[19]	2009	Netherlands	I-II	118	1980-2001	Santa Cruz	1:400
Gulubova[20]	2010	Bulgary	I-IV	138	1997-2006	Santa Cruz	1:50
Li[21]	2011	China	I-IV	147	2003-2004	Zhongshan Biotechnology	1:150
Baraniskin[22]	2011	Germany	IV	190	NA	Santa Cruz	1:100
Ahn[23]	2011	South Korea	I-IV	429	1991-2000	Santa Cruz	1:200
Isaksson[24]	2012	Sweden	I-IV	441	1995-2003	Santa Cruz	1:100
Lampropoulos[25]	2012	Greece	I-IV	195	2005-2006	Santa Cruz	1:100
Roth[26]	2012	Switzerland	II-III	1381	NA	Santa Cruz	0,2 mg/mL
Voorneveld[16]	2013	Netherlands	I-IV	209	1983-2004	Santa Cruz	1:400

Study	Site	Age (y)	Follow up	Outcome	Cytoplasm Nuclear	Preserved SMAD4 (%)	Adj. therapy
Alazzouzi[15]	Colon/rectum	mean 70,1	at least 6 years	DFS and OS	NA	26	No
Alhopuro[5]	Colon/rectum	mean 59	mean 8,7 years	DFS and OS	NA	86,7	Yes
Isaksson[18]	Colon/rectum	NA	NA	CSS	Nuclear	90,7	No
Bacman[17]	Colon	median 64	median 91 months	CSS	Nuclear	85,6	Yes
Mesker[19]	Colon	mean 68,2	up to 25 years	DFS and OS	Nuclear	76,5	No
Gulubova[20]	Colon/rectum	median 65	median 37,6 months	OS	Nuclear	88,4	No
Li[21]	Colon	NA	up to 5 years	DFS and OS	Nuclear	74,1	No
Baraniskin[22]	Colon/rectum	mean 64,4	NA	OS	Nuclear	65,8	Yes
Ahn[23]	Colon/rectum	mean 57	median 56 months	DFS	NA	47,3	Yes
Isaksson[24]	Colon/rectum	NA	NA	CSS	Nuclear	80,3	Yes
Lampropoulos[25]	Colon/rectum	mean 68,6	median 56 months	CSS	Both	61,5	No
Roth[26]	Colon	median 60	median 69 months	OS and DFS	NA	78,8	Yes
Voorneveld[16]	Colon	mean 68,9	median 65 months	CSS	Nuclear	60	No

reviews were also checked for potential articles. The search was performed in September 2014.

First, the abstracts were checked for relevance and full articles were retrieved when potentially eligible. To be included in the analysis studies had to have been performed in resected colorectal carcinomas and immunohistochemistry for SMAD4 had to have been performed. One or more of the following endpoints had to be described; overall survival (OS), disease-free survival (DFS) or cancer-specific survival (CSS). Both univariate and multivariate analyses were taken into account, although separately analysed. All selected studies were checked according to a 20-point quality control system developed previously (Table S1). [10-12]

Statistical analysis

Data was extracted by two independent researchers. When not reported, the

logHazard Ratio (HR) and Confidence Interval (CI) were extracted using previously published methods. [13-14] The meta-analysis was performed in STATA12 using the *metan* package. A pooled HR and 95% CI was calculated for each endpoint (OS, DFS and CSS) and presented in a forest plot. An HR 1> implies a worse prognosis for SMAD4 negative CRCs. A p-value of <0.05 was considered significantly different. Heterogeneity was calculated and presented as X^2 and I^2. We chose to perform random effect models based on the fact that the studies differed in size, country and most importantly in the method of scoring and cut off values. The $\log_e$ standard error of the HR and $\log_e$HR of each study were plotted in a funnel plot to assess potential publication bias.

Results

Search results

The search resulted in 137 studies. On the basis of the abstracts 19 studies were considered eligible of which 6 were eventually excluded based on the fact the HR and CI could not be extracted. The 13 eligible studies consisted of a total of 3800 patients, ranging from 86-1381 per study. The main characteristics of the studies can be found in table 1. We included overall survival (OS), cancer-specific survival (CSS) and disease-free survival (DFS) in this study, but analysed the different endpoints separately. We also made a distinction between univariate and multivariate analyses. Five studies only included colon cancer, the other eight studies included both colon and rectal cancers. Six studies reported that a portion or all of the patients received adjuvant chemotherapy after resection. Three studies did not report the mean or median age and three studies did not report the mean or median follow-up period. Five studies reported CSS, five studies reported DFS and seven studies reported OS. Eight studies described specifically that only nuclear staining of SMAD4 was considered positive. One study considered both cytoplasmic and nuclear staining as positive and four studies did not describe their scoring methods. All studies used SMAD4 antibodies produced by Santa Cruz Biotechnology Inc. (Dallas, TX, USA) except for Li et al who used an antibody from Zhongshan Biotechnology Inc. All studies except Isaksson 2012 had dichotomised the scoring. For Isaksson 2012 we have compared the categories depicted as $SMAD4^{high}$ versus $SMAD4^{loss}$ and we have not considered the category

SMAD4^{moderate}. The mean and median positive SMAD4 score is 70,9% and 76,5% respectively with a range of 26-90,7%.

Cancer specific survival

Three studies included a univariate CSS with a total of 600 patients (range 86-305). The pooled HR is 1,75, which is not significantly different (95%CI: 0,93–3,32; z=1,69; p=0,09). Four studies included a multivariate CSS with a total of 931 patients (range 86-441). The pooled HR is 2,17, which is significantly different (95%CI: 1,56–3,01; z=4,65; p=0,000), indicating that SMAD4 loss is associated with a worse cancer specific survival in this pooled multivariate analysis.

Disease free survival

Six studies included a univariate DFS with a total of 2236 patients (range 75-1381) and three studies included a multivariate DFS with a total of 1646 patients (range 118-1381). The pooled HR for the univariate DFS is 2,11 (95%CI: 1,36–3,28; z=3,32; p=0,001) and the pooled HR for the multivariate DFS is 2,15 (95%CI: 1,56–3,01; z=4,65; p=0,000), which are both significantly different. The univariate and the multivariate DFS meta-analyses confirm that SMAD4 loss is associated with a poor disease free survival.

Overall survival

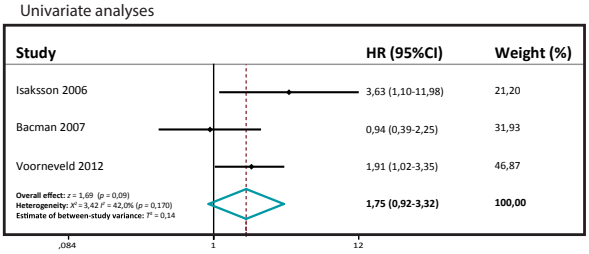
Seven studies included a univariate OS with a total of 2135 patients (range 75-1381) and four studies included a multivariate OS with a total of 1836 patients (range 118-1381). The pooled HR for the univariate OS is 2,30 (95%CI: 1,41–3,73; z=3,36; p=0,001) and the pooled HR for the multivariate OS is 2,28 (95%CI: 1,30–4,00; z=2,89; p=0,004). Both the univariate and the multivariate OS meta-analyses showed that SMAD4 loss is associated with a poor overall survival.

Publication bias

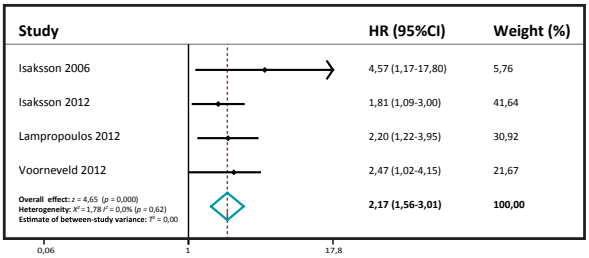
The funnel plots for the assessment of potential publication bias show one considerable outlier in the univariate and multivariate OS and the univariate DFS. Although in the univariate OS this does not result in a significant Egger test, we chose to exclude the outliers in all the meta-analyses and recalculate the pooled HRs. Exclusion of the outlier reduces the pooled HR of the unvariate OS to 1,673

Cancer specific survival

Univariate analyses

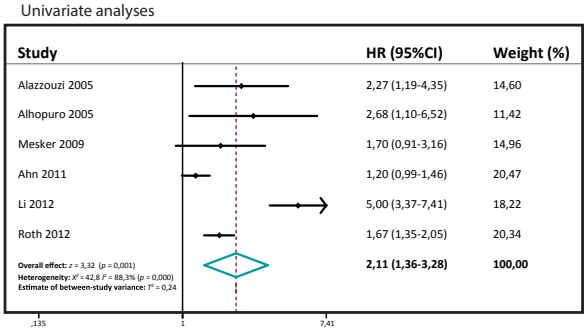


Multivariate analyses

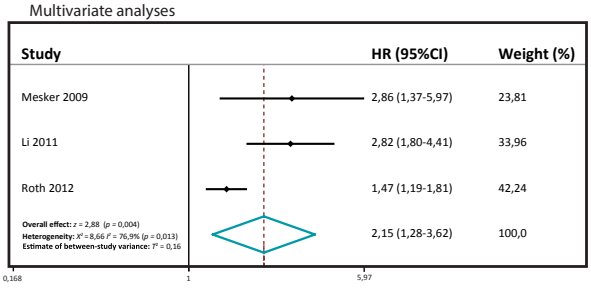


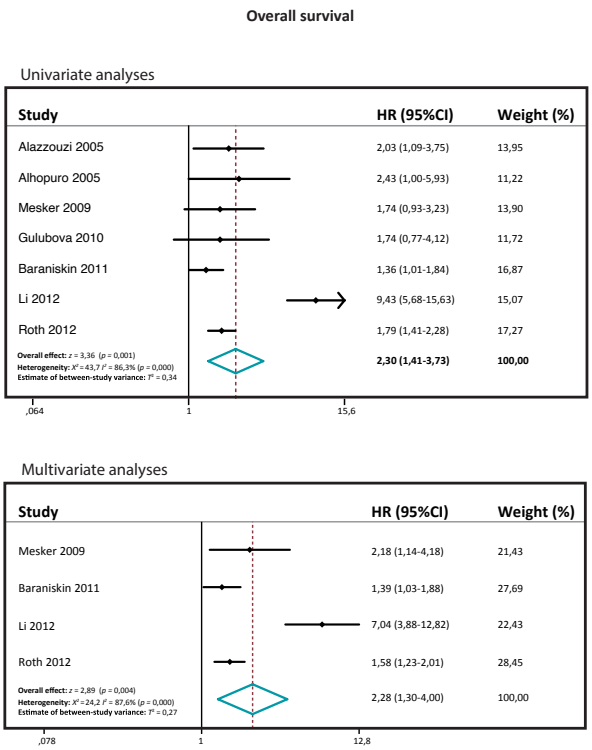
Disease free survival

Univariate analyses



Multivariate analyses



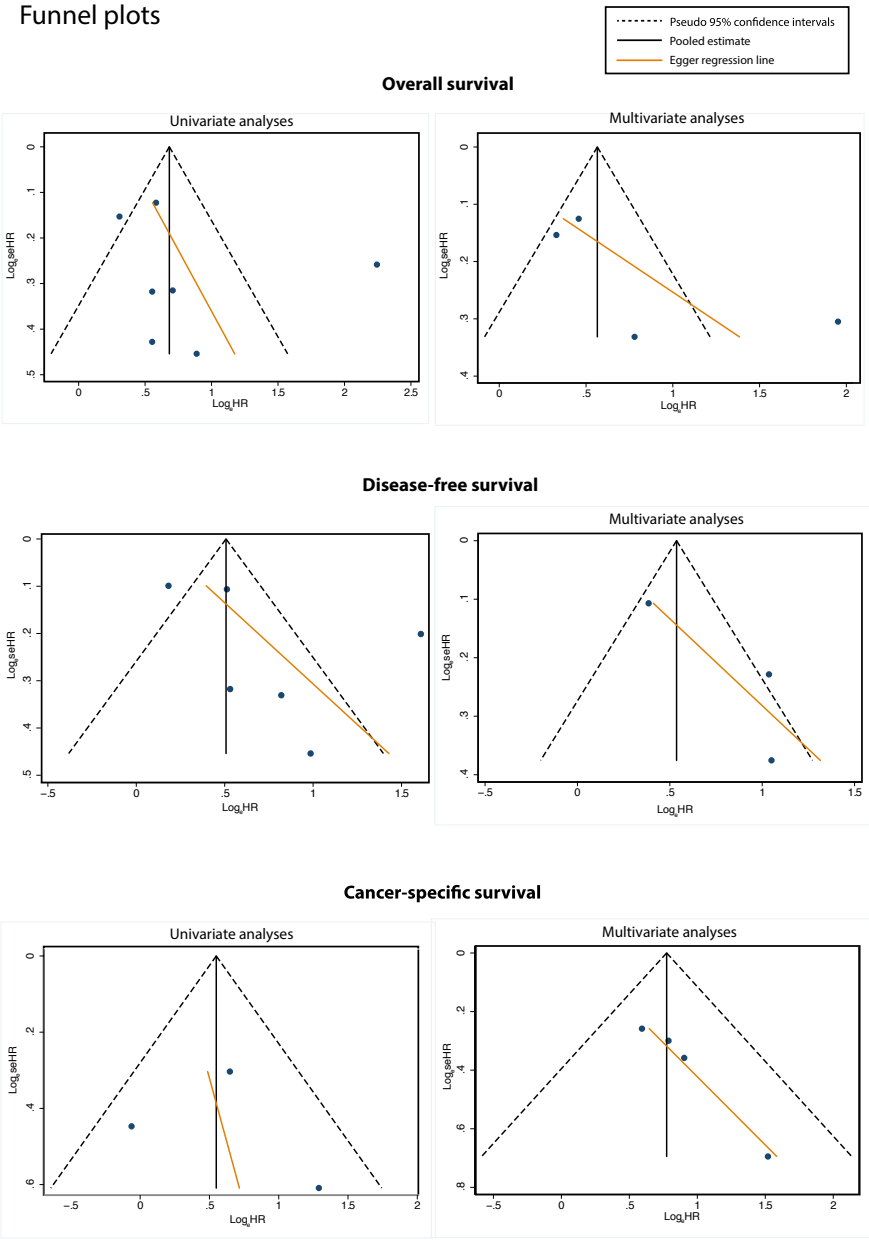


(95%CI: 1,42–1,96; *z*=6,08; *p*=0,000). The pooled HR of the multivariate OS is reduced to 1,55 (95%CI: 1,29–1,86; *z*=4,68; *p*=0,000) and the pooled HR of the univariate DFS is 1,6 (95%CI: 1,23–2,08; *z*=3,53; *p*=0,000).

Discussion

Molecular profiling of individual tumours potentially allows a personalized approach to cancer treatment. Estimation of prognosis plays an important role in decisions about treatment, and this is currently almost entirely dependent on histopathological staging. *SMAD4*, located on chromosome 18q21, has frequently been reported to be a useful prognostic marker. Several studies have reported the prognostic value of SMAD4 expression using immunohistochemistry[15-16],

Funnel plots



although this has not been entirely consistent[17]. To our knowledge, this is the first comprehensive meta-analysis of the predictive value of SMAD4 expression using immunohistochemistry.

We included three different endpoints, namely OS, CSS and DFS and both univariate and multivariate analyses. All the meta-analyses, except the univariate CSS, showed a significant difference in HR implying that loss of SMAD4 expression as measured by immunohistochemistry is associated with a poor prognosis. 13 studies have investigated the prognostic value of SMAD4 loss using different types of endpoints resulting in relative few studies per single endpoint. The analyses of the univariate OS and DFS included the largest numbers of studies (seven each) which makes these the most reliable results. To investigate the heterogeneity, I^2 was calculated which was more than 50% in most of the cases, except for the univariate and multivariate analysis of the CSS. Conventionally, when the I^2 is more than 50% and the distribution is significantly heterogeneous (p<0,05) the fixed model cannot be used and the random effects model has to be applied, but prior to conducting the analysis we had already decided to use the random effects model for all the meta-analyses because of the variation in the methodology of the studies included. One aspect that varies between the studies is the percentage of SMAD4 preservation, which is dependent on the staining and scoring method used. SMAD4 is the common mediator of the BMP/TGFβ signalling pathways and complexes with phosphorylated R-SMADs, which then enter the nucleus to modulate gene transcription. Only nuclear localisation shows active functional SMAD4. Not all studies used only nuclear staining and four studies did not report what they considered as positive. Another source of heterogeneity is the population that was used in each of the studies. Seven studies included all the stages and the other six included only one or two stages. Six studies included patients that had received chemotherapy, while in seven studies no adjuvant therapy was applied. These differences can affect the outcome of the individual studies and can increase the heterogeneity in the meta-analysis.

Despite the fact that the pooled studies of univariate cancer specific survival did not show a significant difference, we conclude, based on the other 5 meta-analyses all showing statistically significant associations, that immunohistochemical analysis of SMAD4 expression is a useful prognostic marker in colorectal cancer.

Immunohistochemistry is a relative easy technique to perform and is readily available in most hospital pathology departments. International recommendations to standardise SMAD4 scoring methodology are required.

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	Isaksson 2006	Bacman 2007	Li 2011	Isaksson 2012	Lampropoulos 2012	Roth 2012	Voorneveld 2013	Alazzouzi 2005	Alhopuro 2005	Baraniskin 20	Mesker 2009	Gulubova 2010	Ahn 2009
study group													
population described													
Gender/age	1	1	1	1	1	1	1	1	1	1	1	1	1
Histology	1	1	1	1	1	1	1	1	1	1	1	1	1
period of recruitment	1	1	1	1	1	0	1	1	1	0	1	1	1
Inclusion/exclusion	1	1	0	1	1	0	1	1	1	1	1	1	1
Study attrition													
>90% of identified included in final analysis	0	1	1	1	1	1	1	1	1	0	1	0	1
Reasons for loss to follow up given	1	1	0	1	1	1	1	1	1	1	1	0	0
Peri-operative mortality details	1	1	0	0	1	0	0	0	0	1	1	0	0
Scientific methodology													
IHC methodology outlined													
Details of 1ste/2nd Abs used	1	1	1	1	1	1	1	1	1	1	1	1	1
Concentration	1	1	1	1	1	1	1	1	1	1	1	1	1
Positive negative control	1	1	0	1	1	0	1	0	0	0	1	1	1
Description scoring system													
>1 independent scorer	1	0	1	1	1	1	1	1	1	1	0	1	1
Blind	0	0	1	1	1	1	1	1	1	1	0	0	0
Criteria for positive outlined													
Distribution (membr vs nuclear)	0	0	1	1	0	0	1	0	0	1	1	1	0
% pos for classification stated	1	0	0	1	1	0	1	0	0	1	1	1	1
Confounding factors considered													
Adjuvant therapy details provided	1	1	0	0	1	1	1	1	1	1	1	1	1
Histology breakdown according to IHC staining	1	1	1	1	1	1	1	1	1	0	0	0	1
Statistical analysis													
HR (CI) provided	1	1	1	1	1	1	1	0	0	0	0	0	0
Exact p-value	1	1	1	1	1	1	1	1	1	1	1	1	1
number at risk for Kaplan Meier	1	1	1	1	0	1	1	1	1	1	1	1	1
Number of censored cases	1	0	0	0	0	1	1	1	1	0	0	0	1
total	17	15	13	17	17	14	19	15	15	14	15	13	15

Chapter 7

Evaluation of the prognostic value of pSMAD immunohistochemistry in colorectal cancer

Eur J Cancer Prev. 2013 Sep;22(5):420-4

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SMAD4 mutations and recent Genome-Wide Association Studies (GWAS) show the importance of bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) signalling in the development of colorectal cancer (CRC). Loss of SMAD4 has been implicated as a predictive marker in CRC. As activation of the BMP and TGF-β pathways leads to phosphorylation of SMAD1,5,8 and SMAD2,3, respectively, and both need SMAD4 for translocation to the nucleus, we aimed to investigate whether nuclear staining of pSMAD1,5,8 and pSMAD2, 3 can be used as predictive markers in CRC. A Tissue Microarray (TMA) was constructed of tissue from 209 patients diagnosed with CRC. TMA was stained and scored for the nuclear presence of SMAD4, pSMAD2,3 and pSMAD1,5,8. Loss of SMAD4, pSMAD2,3 and pSMAD1,5,8 was observed in 40, 38 and 73% of the cases, respectively. The incidence of SMAD4 loss was significantly higher in the advanced stages. There was a correlation between loss of SMAD4 and loss of pSMAD1,5,8, but not between loss of SMAD4 and loss of pSMAD2,3. Loss of SMAD4 correlated with a poorer survival. Loss of one of the pSMADs did not correlate with a poorer outcome. Combining different SMAD stainings did not improve the prediction. SMAD4 expression is a prognostic marker in CRC. Nuclear expressions of pSMAD1,5,8 and pSMAD2,3 are not useful prognostic markers in CRC.

Introduction

Survival and disease recurrence of colorectal cancer (CRC) have improved significantly over the last decade mainly because of early diagnosis and new treatment options. Despite this progress, CRC remains one of the leading causes of cancer-related deaths in the western world (Jemal et al., 2011). Improved molecular understanding of CRC has made detailed molecular profiling of individual tumours possible to potentially allow personalized therapy. Estimation of prognosis, which is currently almost entirely dependent on histopathological staging, is essential to decision making. In the search for simple molecular prognostic markers KRAS and p53 mutations and loss of 18q have been the most intensively investigated as possible prognostic factors (Zlobec and Lugli, 2008; Walther et al., 2009; Pritchard and Grady, 2011). SMAD4, a key signal transduction element of the bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) tumour suppressor pathways, is located in chromosome 18q21 and is believed to be targeted by the deletion of 18q in CRC (Thiagalingam et al., 1996). Moreover, frequent germ line mutations in SMAD4 in patients with Juvenile Polyposis and somatic alterations in SMAD4 in CRC underscore the importance of SMAD4 in CRC (Howe et al., 2001). Patients with Dukes C CRCs expressing high SMAD4 levels by immunohistochemical analysis have significantly better survival (Alazzouzi et al., 2005). Recent Genome-Wide Association Studies (GWAS) in CRC have identified genetic alterations within multiple members of the BMP pathway as being associated with an increased risk of CRC, namely, BMP2, BMP4, SMAD4, BMPR1a, Gremlin1 and SMAD7 (Alazzouzi et al., 2005; Tomlinson et al., 2011). Therefore, both the results of prognostic studies and GWAS indicate the importance of the BMP/TGF-β signalling pathways in the development of CRC.
Activation of BMP signalling leads to phosphorylation of SMAD1,5 or 8 (pSMAD1,5,8), whereas TGF-β signalling results in phosphorylation of SMAD2,3 (pSMAD2,3). Both pSMAD1,5,8 and pSMAD2,3 complexes need SMAD4 to translocate to the nucleus for the regulation of gene transcription. Therefore, nuclear localization of pSMAD1,5,8 or pSMAD2,3 should indicate activation of the BMP or TGF-β pathways, respectively. We have previously shown loss of nuclear pSMAD1,5,8 expression in 70% of patients with CRC, indicating loss of BMP pathway activity (Kodach et al. 2008b). In this study, we aimed to investigate

whether nuclear pSMAD1,5,8 or pSMAD2,3 expression, biomarkers for the activity of the BMP and TGF-β pathways, can be used as prognostic markers in CRC, and to compare this with the prognostic value of SMAD4, which has been shown to have prognostic significance in CRC (Alazzouzi et al., 2005; Isaksson-Mettavainio et al., 2006; Kouvidou et al., 2006).

Methods

Formalin-fixed, paraffin-embedded tissues from 209 CRC cases between the years of 1983 and 2004 were used for the compilation of a Tissue Microarray (TMA). Blocks were selected from the archives of the Pathology Department at the Academic Medical Centre, Amsterdam and Leiden University Medical Center, Leiden. Patients with rectal cancer who had received preoperative radiotherapy were excluded. The study was approved by the Institutional Review Boards of the investigators. Our study included 115 men (55%) and 94 women (45%), their ages ranging from 30 to 91 years, with a mean of 68,9 years (±12 SD) and a median of 70 years. Dukes stage was known for all tumours and our study included six patients with Dukes A (3%), 136 patients with Dukes B (65%), 55 patients with Dukes C (26%) and 12 patients with Dukes D (6%). One patient with a Dukes D stage tumour with liver metastases was operated with the intention to treat with additional chemotherapy. Tumour grade was known for 120 of the 209 patients. Three cores of tissue from each cancer specimen were taken and one normal core as a control. The TMA was sectioned and stained for SMAD4 (Santa Cruz Biotechnology, Santa Cruz, California, USA), pSMAD1,5,8 and pSMAD2,3 (both Cell Signalling, Boston, Massachusetts, USA) according to previously described methods (Kodach et al., 2008a). Stainings were scored blindly and independently

Table 1: Scoring system for SMAD4, p-SMAD1,5,8 and p-SMAD2,3

	Percentage of cells stained			
Intensity	**<10%**	**10-30%**	**30-50%**	**>50%**
No staining	0	0	0	0
Weak staining	0	0	1	1
Moderate staining	0	1	2	3
Strong staining	1	2	3	3

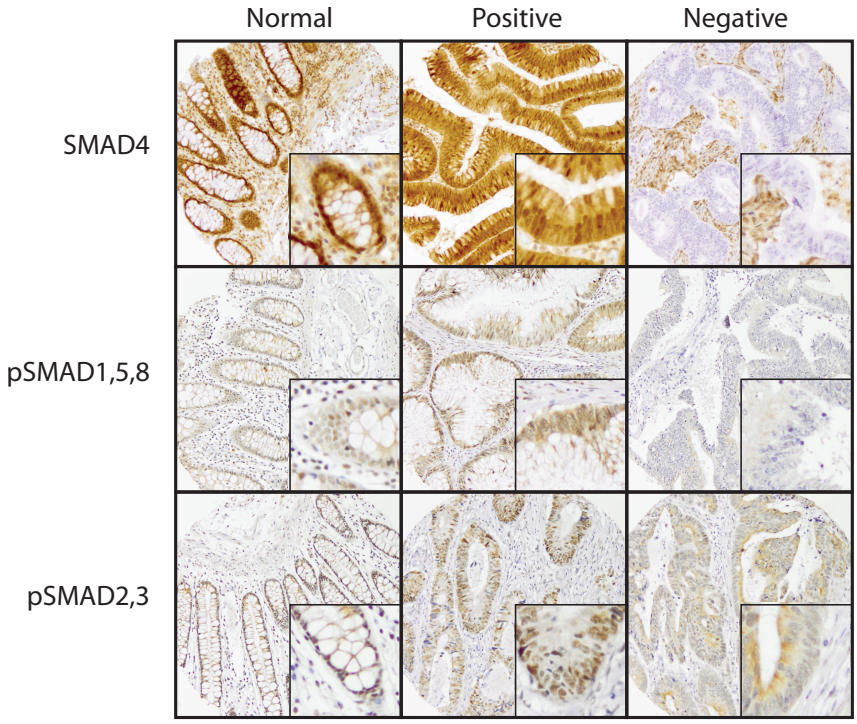


Figure 1: Representative SMAD4, pSMAD1,5,8 and pSMAD2,3 stainings of Colorectal Cancer samples from patients

by two researchers on the basis of the number of cells stained and the intensity of the staining as described in ***Table 1***, counting only the cells with a nuclear staining. A mean score of 0 or 1 point was considered negative and a mean score of 2 or 3 points was considered positive. ***Figure 1*** shows examples of positive and negative stainings of the SMADs.

Statistical analyses were carried out using the statistical package of social science, version 17.0.2 for Windows (SPSS Inc., Cary, North Carolina, USA). The mean follow-up period was 71 months, the median was 65 months and the range was 1.5–257 months. One hundred and twenty-five (59.5%) patients were alive after the follow-up period, 40 (19%) died of a CRC-related cause and 44 (22%) died of a cause other than CRC. Cox proportional hazard regression was used for univariate and multivariate survival analyses. Log-rank tests were used for survival analysis in the Venn diagram. P-value less 0.05 was considered a significant difference.

Fisher's exact test and $\chi 2$-tests were used as appropriate. All statistical tests were carried out two sided.

Results

To examine the association between nuclear SMAD4, pSMAD1,5,8 and pSMAD2,3 expression in tumours and patient survival, we divided the patients into two groups depending on the intensity of the stainings as described in the Methods section. In our study, 84 patients (40%) showed negative nuclear SMAD4 expression, 151 patients (72%) showed negative expression of nuclear pSMAD1,5,8 and 79 patients (38%) scored negative for nuclear pSMAD2,3 in the tumour tissue (***Table 2***). We observed significantly more negative SMAD4 staining in more advanced CRCs with Dukes C+D stages (50,7% negative) versus early Dukes stages A+B (35,2% negative) (P=0.036). The distribution of the pSMAD2,3 staining scores in the different Dukes stages was significantly different (P=0.04), showing relatively more negative stainings in the Dukes D stage (67% negative) compared with Dukes A–C (33, 40 and 25% negative, respectively) (***Table 2***). We did not find significant differences in pSMAD1,5,8 stainings between cancers with different Dukes stages.

As SMAD4 is considered necessary for the nuclear translocation of the phosphorylated R-SMAD complexes (pSMAD1,5,8 or pSMAD2,3), a high correlation between the absence of nuclear pSMAD1,5,8 or pSMAD2,3 stainings

Table 2: SMAD expression levels based on Dukes stages

		Dukes				
	total	**A**	**B**	**C**	**D**	**p-value**
SMAD4						
negative	84 (40%)	2 (33%)	48 (35%)	29 (53%)	5 (42%)	0.17
positive	125 (60%)	4 (67%)	88 (65%)	26 (47%)	7 (58%)	
pSMAD1,5,8						
negative	151 (72%)	4 (67%)	102 (75%)	38 (69%)	7 (58%)	0.56
positive	58 (28%)	2 (33%)	34 (25%)	17 (31%)	5 (42%)	
pSMAD2,3						
negative	79 (38%)	2 (33%)	55 (40%)	14 (25%)	8 (67%)	0.04
positive	130 (62%)	4 (67%)	81 (60%)	41 (75%)	4 (33%)	

Table 3: Association of SMAD4 loss with expression of pSMADs

	SMAD4		
	negative ***n=84*** **(%)**	positive ***n=125*** **(%)**	***p***
pSMAD1,5,8			
positive	15 (18)	43 (34)	0.01
negative	69 (82)	82 (66)	
pSMAD2,3			
positive	54 (64)	76 (61)	0.66
negative	30 (36)	49 (39)	

and negative SMAD4 staining might be expected. Indeed, we observed a significant correlation between nuclear SMAD4 and nuclear SMAD1,5,8 (P=0.015), although 17% of SMAD4-negative CRCs still showed positive nuclear pSMAD1,5,8 staining. However, there was no correlation between nuclear SMAD4 and nuclear pSMAD2,3, and a majority of SMAD4-negative cancers showed positive staining for nuclear pSMAD2,3 (64%) (***Table 3***). Interestingly, 66% of SMAD4-positive CRC showed a negative expression of nuclear pSMAD1,5,8 and 39% of SMAD4-positive CRC were negative for nuclear pSMAD2,3 (***Table 3***). This could be because of mutations at the receptor level or methylation of R-SMADs as alternative mechanisms leading to inactivation of the BMP or the TGF-β pathway (Markowitz et al., 1995; Kouvidou et al., 2006; Kodach et al., 2008b). Finally, we analysed the effect of the nuclear expression status of SMAD4, SMAD1,5,8 and SMAD2,3 on cancer-associated patient survival. In a univariate analysis, the 'age of onset', sex and tumour grade were not associated with a significant difference in survival. The multivariate inclusion condition was set to P equal to 0.1 and as a result 'age of onset' and sex were not included in the multivariate analysis. In our study population, loss of nuclear SMAD4 correlated with a poorer survival, independently (P=0.043) as well as in a Cox proportional hazard regression (P=0.047). This confirms that SMAD4 is a valuable prognostic marker for CRC not only in patients with Dukes C CRC but also in an unselected group with different Dukes stages (***Table 4***). In contrast, pSMAD1,5,8 or pSMAD2,3 immunohistochemical nuclear status did not predict a poorer survival. Also, within each of the Dukes stages, no difference in survival was found for the pSMAD stainings (data not shown). As SMAD4 loss means the inactivation of both pathways simultaneously, we investigated whether

Table 4: Univariate and multivariate analysis

	Univariate			Multivariate		
	HR	**95% CI (low-high)**	**p-value**	**HR**	**95% CI (low-high)**	**p-value**
Age	1,017	(0,990-1,045)	0.222	-	-	-
Gender (male/female)	1,024	(0,547-1,917)	0.941	-	-	-
Tumour grade	1,554	(0,920-2,624)	0.099	2,468	(1,343-4,535)	0.004
Dukes stage	4,735	(3,096-7,241)	<0.0001	4,703	(2,839-7,790)	<0.0001
SMAD4	1,905	(1,022-3,553)	0.043	2,468	(1,023-4,148)	0.047
pSMAD1,5,8	1,022	(0,511-2,046)	0.951	2,048	(0,908-4,766)	0.084
pSMAD2,3	1,148	(0,559-2,199)	0.678	0,869	(0,430-1,756)	0.696

HR = Hazard ratio, CI = Confidence Interval

simultaneous pSMAD1,5,8 and pSMAD2,3 loss, which should also result in the loss of both signalling pathways simultaneously, was predictive of survival. We found no significant association between coexistent pSMAD1,5,8 and pSMAD2,3 loss and cancer-associated survival (***Figure 2***).

We next investigated whether there was any possible added value of combining pSMAD1,5,8 or pSMAD2,3 stainings to the prognostic value of nuclear SMAD4 status. We found no improvement over the prognostic value of SMAD4 staining (***Figure 2***).

Discussion

In this study, we observed a significant correlation between loss of nuclear SMAD4 staining and poorer survival of patients with CRC. These data confirm the significance of SMAD4 loss. We observed loss of SMAD4 in 40% of cases, which is in concordance with previous studies proving the reproducibility of the SMAD4 staining (Alazzouzi et al., 2005; Isaksson-Mettavainio et al., 2006).

Nuclear expression of pSMAD1,5,8 and pSMAD2,3 can be used to monitor the activity of the BMP and the TGF-β pathways (Itoh et al., 2000). We therefore evaluated whether the nuclear expression of pSMAD1,5,8 and pSMAD2,3 can be used to predict CRC outcome and whether it can be combined with SMAD4 expression. Although loss of BMP signalling, as judged by nuclear pSMAD1,5,8 expression, was observed in 72% of the cases, this was not associated with a worse survival. The same applies for nuclear pSMAD2,3, which was lost in 38% of the cases. These results are perhaps surprising as the importance of BMP and TGF-β signalling and their role as tumour suppressors in CRC is well documented in the literature (Itoh et al., 2000; Ten et al., 2002). From this, it might be expected that

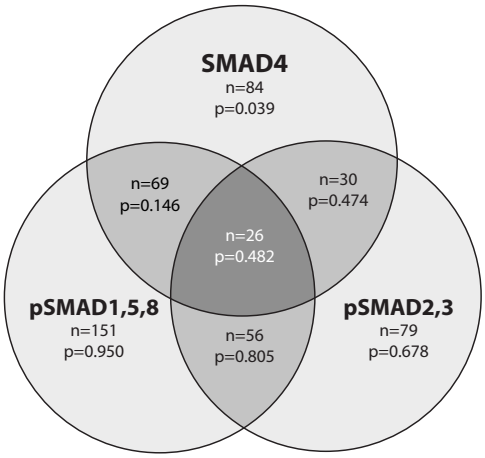


Figure 2: Survival prediction of SMAD expression patterns

loss of BMP or TGF-β pathway activity would be associated with worse patient outcome. We found, however, that only SMAD4 loss and not pSMAD1,5,8 or pSMAD2,3 loss predicted a poorer outcome. One possible explanation could be that loss of SMAD4, which is a key signal transduction molecule of all TGF-β family pathways, leads to the simultaneous inactivation of BMP, TGF-β and activin pathways, whereas loss of one of the arms at the level of the R-SMADs (either pSMAD1,5,8 or pSMAD2,3) is not sufficient to lead to a significant difference. Moreover, loss of SMAD4 does not necessarily lead to loss of nuclear pSMAD2,3 expression as shown in Table 3. pSMAD2,3 is able to shuttle between the nucleus and the cytoplasm without SMAD4, even though it cannot modulate the TGF-β pathway target genes without SMAD4. Furthermore, in the TGF-β pathway, SMAD4 also plays a regulatory role rather than just a trafficking role (Liu et al., 1997; Lampropoulos et al., 2012b). Nuclear pSMAD2,3 may thus be an imperfect marker for the activation of the TGF-β pathway, possibly explaining why its absence does not correlate with a poorer survival in this study (Ten et al., 2002). Although we observed a concordance between nuclear SMAD4 loss and nuclear pSMAD1,5,8 loss, as we have shown previously (Kodach et al., 2008b), we did observe nuclear pSMAD1,5,8 in SMAD4-negative cancers, which suggests that pSMAD1,5,8 may be able to translocate to the nucleus without SMAD4

contrary to the current dogma. It is possible that despite the care we have taken in developing reliable immunohistochemistry protocols for the antibodies we have used, our results may have been affected by the reliability and reproducibility of immunohistochemistry using phospho-specific antibodies that are more sensitive to tissue processing and fixation than other antibodies (Pinhel et al., 2010).

To our knowledge, this is the first study in which the prognostic value of pSMAD1,5,8 has been evaluated, whereas two studies have shown pSMAD2,3 expression to have prognostic value (Xie et al., 2003; Lampropoulos et al., 2012a). These two studies showed a 6.2 and 28.7% loss of pSMAD2, respectively, compared with our 38%. The differences may be because of the use of different antibodies and scoring systems. In summary, our study shows that nuclear SMAD4 expression is a prognostic marker in CRC. Conversely, nuclear expressions of pSMAD1,5,8 and pSMAD2,3 are not useful prognostic markers in CRC.

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Chapter 8

Reduced expression of Bone Morphogenetic Protein Receptor 1A in pancreatic cancer is associated with a poor prognosis

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Aim

The expression of SMAD4, the central component of the transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signalling pathways, is lost in 50% of pancreatic cancers and is associated with a poor survival. Although the TGF-β pathway has been extensively studied and characterised in pancreatic cancer, there is very limited data on BMP signalling, a well-known tumour-suppressor pathway. BMP signalling can be lost not only at the level of SMAD4 but also at the level of BMP receptors (BMPRs), as has been described in colorectal cancer.

Methods

We performed immunohistochemical analysis of the expression levels of BMP signalling components in pancreatic cancer and correlated these with survival. We also manipulated the activity of BMP signalling in vitro.

Results

Reduced expression of BMPR1A is associated with a significantly worse survival, primarily in a subset of SMAD4-positive cancers. In vitro inactivation of SMAD4-dependent BMP signalling increases proliferation and invasion of pancreatic cancer cells, whereas inactivation of BMP signalling in SMAD4-negative cells does not change the proliferation and invasion or leads to an opposite effect.

Conclusion

Our data suggest that BMPR1A expression is a good prognostic marker and that the BMP pathway is a potential target for future therapeutic interventions in pancreatic cancer.

Introduction

Pancreatic cancer is a particularly lethal disease with a 5-year survival of only 4% (Jemal et al, 2011). The poor prognosis is partly because early stage pancreatic cancers typically do not lead to clinical symptoms and partly because of the poor response to chemotherapy (Vincent et al, 2011). At the time of diagnosis, the tumour has usually invaded surrounding organs or metastasised, resulting in 80–85% of the tumours being inoperable. There is thus an urgent need for better understanding of the molecular mechanisms involved in order to develop alternative treatment options and better predict prognosis.

Mutations in SMAD4, also known as depleted in pancreatic cancer locus 4 (DPC4), occur in >50% of pancreatic cancers (Hahn et al, 1996; Rozenblum et al, 1997; Tascilar et al, 2001). The inactivation of SMAD4 occurs relatively late in the adenomato-carcinoma sequence with loss of expression first seen at the Pan-IN3 stage and being associated with a poorer prognosis (Wilentz et al, 2000; Tascilar et al, 2001). The relevance of SMAD4 loss has been shown by restoration of SMAD4 in SMAD4-depleted pancreatic cancer cell lines, which leads to reduced growth (Duda et al, 2003).

SMAD4 is a central and critical component of both the transforming growth factor-β (TGF-β) and the bone morphogenetic protein (BMP) signalling pathways. 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 (Massague et al, 2000; Jazag et al, 2005; Schniewind et al, 2007; Romero et al, 2008). TGF-β receptor II mutations are found in 4–7% of pancreatic cancers further supporting the importance of TGF-β signalling in pancreatic cancer tumourigenesis (Massague, 1998; Hansel et al, 2003).

The role of the BMP signalling pathway in pancreatic cancer is much less clear. BMP ligands bind to a complex of transmembrane serine threonine kinase receptors consisting of type I (BMPR1A or BMPR1B) and type II (BMPR2) receptors. On binding of the BMPs to the receptor complex, the type I receptor (BMPR1A or BMPR1B) is phosphorylated by BMPR2, which leads to downstream phosphorylation and activation of the BMP-specific SMADs, SMAD1, 5 and 8.

Phosphorylated SMAD1, 5 or 8 then complexes with the co-SMAD, SMAD4. SMAD4 is crucial for the modulation of gene transcription as it facilitates the translocation of pSMAD1/5/8 to the nucleus (Massague, 1998). Germline mutations of BMPR1A and SMAD4 are found in juvenile polyposis, a rare autosomal dominant polyposis syndrome with a high lifetime risk of colorectal cancer (Howe et al, 1998, 2001), and BMPR2 expression loss is found in colorectal cancers with microsatellite instability (Kodach et al, 2008b). This further suggests that the BMP pathway is important in maintaining epithelial cell homeostasis. However, little is known about the expression levels of the different BMP signalling components in pancreatic cancer.

Therefore, we set out to assess expression levels of the BMP receptors (BMPRs) in pancreatic cancer tissue and relate this to patient survival data. We then tested the relevance of our findings by investigating the influence of BMPR expression on pancreatic cancer cell proliferation and invasion in vitro.

Methods

Immunohistochemistry

Pancreatic ductal adenocarcinoma (PDAC) tissue was stained according to previously described methods (Kodach et al, 2008a). A list of antibodies with the concentrations used is provided in the ***Supplementary Methods***.

Tissue microarray

A tissue microarray (TMA) containing PDAC tissue was constructed from formalin-fixed, paraffin-embedded tissue from 41 patients with PDAC who underwent surgery in the Leiden University Medical Center. All cases were reported by a single GI pathologist (HM). Survival data of the 41 patients with PDAC included in the TMA were collected. The mean survival time from the date of surgery was 373±s.d. 268 days (range 37–1367 days). All patients died within the follow-up period.

TMA analysis

Analysis was performed in a blinded manner by two investigators independently. SMAD4 expression was scored according to previously described methods (Kodach et al, 2008a). Immunohistochemical staining for the receptors was scored according to a scoring system we have developed and validated for colorectal cancer, as shown in ***Supplementary Methods***. Further division of expression was made into negative (scores 0 and 1) and positive (scores 2 and 3). The final score of agreement for all stainings was k>0.7.

Immunoblotting

Western blot analysis was performed according to previously described methods (Kodach et al, 2008b). A list of antibodies with the concentrations used is provided in the ***Supplementary Methods***.

PCR

PCR was performed according to previously described methods (Kodach et al, 2008b). Primer sequences and protocols can be provided on request. GAPDH was used as a loading control.

Cell lines

PANC-1, MIA PaCa-2 and Bx-PC3 human PDAC cell lines were obtained from the ATCC (Manassas, VA, USA) and were grown in Dulbecco's modified Eagle's medium 4.5 g l^{-1} glucose and L-glutamine (Invitrogen, Breda, The Netherlands), supplemented with 50Uml^{-1} penicillin and 50 mgml^{-1} streptomycin and 10% foetal calf serum (Invitrogen). Cells were grown in monolayers at 37 °C in a humidified atmosphere containing 5% CO_2.

Invasion assay

In all, 8 mM pore size HTS FluoroBlok Cell Culture Inserts (BD Falcon, Breda, The Netherlands) were coated with 100 ml of a 1 : 1 mix of serum-free media and Matrigel. Inserts were placed in 24-well plates (Corning Incorporated, Corning, NY, USA). Cells were labelled with 5 mM CellTracker Green CMFDA (Invitrogen) according to the manufacturer's instructions after which they were detached using trypsin with 2mM EDTA. Cells were transferred in medium containing 1% FCS to the upper well of a transwell invasion system. A volume of 600 ml of culture

medium containing 10% FCS was used as an attractant and placed in the lower chamber. Fluorescence of the invaded cells at the lower side of the transwell was measured every 2 h using the BioTek Flx800 (BioTek, Winooski, VT, USA). Data were corrected for background fluorescence and migration start points were set to zero.

MTT assay

Cells were seeded in 96-wells plates. For viability measurement, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added for 2 h at 37 °C (0.5 mg ml–1) after which the absorbance of the samples at 562 nm was measured.

BMP ligand and BMP inhibitor

Stock solution of recombinant human BMP-2 (R&D systems, Oxon, UK) was prepared in PBS and subsequently dissolved in culture medium for treatment (100 ng ml^{-1}). Stock solutions of the BMPR1A inhibitor LDN-193189 (AxonMedchem BV, Groningen, The Netherlands) were prepared in dimethyl sulfoxide and subsequently dissolved in culture medium for treatment.

shRNA against SMAD4

Lentiviral constructs expressing shRNAs targeting SMAD4 (TRCN0000040028) and a non-targeting control construct (SHC002) were obtained from the Sigma MISSION shRNA library (Sigma-Aldrich, St Louis, MO, USA). Production of lentiviruses by transfection into 293T cells has been described earlier (Carlotti et al, 2004). Cells were selected using puromycin.

siRNA knockdown

For siRNA knockdown of BMPR1A, cells were transfected with either siRNA against BMPR1A (ID: s280) or scrambled siRNA (Invitrogen). Lipofectamine 2000 (Invitrogen) was used for all transfections according to the manufacturer's instructions.

Luciferase reporter assay

For quantitative measurement of BMP, reporter assays were used. Cells were

transfected with BRE-luc constructs (kindly provided by P ten Dijke). Transfection with a pcDNA3.1 vector expressing Renilla luciferase was used as a transfection control. Luciferase activity was measured using a Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. Lipofectamine 2000 (Invitrogen) was used for all transient transfections according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed with Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA USA) using the Student's t-test and with PASW Statistics 18 for Windows (SPSS, Inc., IBM, NY, USA) using Kaplan–Meier analysis, Fisher's exact test, X^2 test, log-rank test and Cohen's kappa as appropriate.

Results

Expression of BMPR1A protein is frequently reduced in PDAC.

In order to investigate the expression levels of the BMP signalling components, we selected the 41 cases of PDAC from our TMA and performed immunohistochemistry for SMAD4, BMPR1A, BMPR1B and BMPR2. Examples of the stainings can be found in ***Supplementary Figure 1***. SMAD4 is lost in half (53.7%) of the PDAC cases, which is in concordance with the literature (Tascilar et al, 2001). BMPR1A, BMPR1B and BMPR2 expression is reduced in 53.7%, 9.8% and 29.3% of PDAC, respectively (***Table 1***). We then related the BMPR expression levels to the SMAD4 status and observed no correlation, thus SMAD4 and the BMPRs are lost independently in PDAC. Loss of SMAD4 expression has previously been shown to be associated with a poor prognosis and we could confirm this in our dataset (p=0.04; ***Figure 1A***). Reduced BMPR1A expression is associated with poorer survival (p=0.008; ***Figure 1B)***. Interestingly, when stratifying for the SMAD4 status a significant difference can be observed within the SMAD4-positive group, but not within the SMAD4-negative group (***Figure 1C and D***). This suggests that changes in BMPR1A expression only have major consequences when SMAD4 expression is still intact. SMAD4 is a central component of the BMP signalling pathway and when lost there is gross impairment of canonical BMP signalling. From this, it would be expected that reduction of BMPR1A expression

Table 1: Expression of BMP signalling components in PDAC: The correlation between SMAD4 loss and the level of BMP receptor expression as determined by immunohistochemical analysis. There is no significant difference in BMP receptor expression between SMAD4 expressing and non-expressing cancers.

Scoring, n (%)		**all PDAC (n=41)**		**SMAD4 positive (n=19)**		**SMAD4 negative (n=22)**		**p-value**
BMPRIA	**0**	2	(4.9)	0	(0)	2	(9.1)	0.277
	1	20	(48.8)	10	(52.6)	10	(45.5)	
	2	17	(41.5)	9	(47.4)	8	(36.4)	
	3	2	(4.9)	0	(0)	2	(9,1)	
BMPRIB	0	0	(0)	0	(0)	0	(0)	0.147
	1	4	(9.8)	0	(0)	4	(18.2)	
	2	31	(75.6)	16	(84.2)	15	(68.2)	
	3	6	(14.6)	3	(15.8)	3	(13.6)	
BMPRII	**0**	0	(0)	0	(0)	0	(0)	0.560
	1	12	(29.3)	5	(26.3)	7	(31.8)	
	2	26	(63.4)	13	(68.4)	12	(54.5)	
	3	3	(7.3)	1	(5.3)	3	(13.6)	

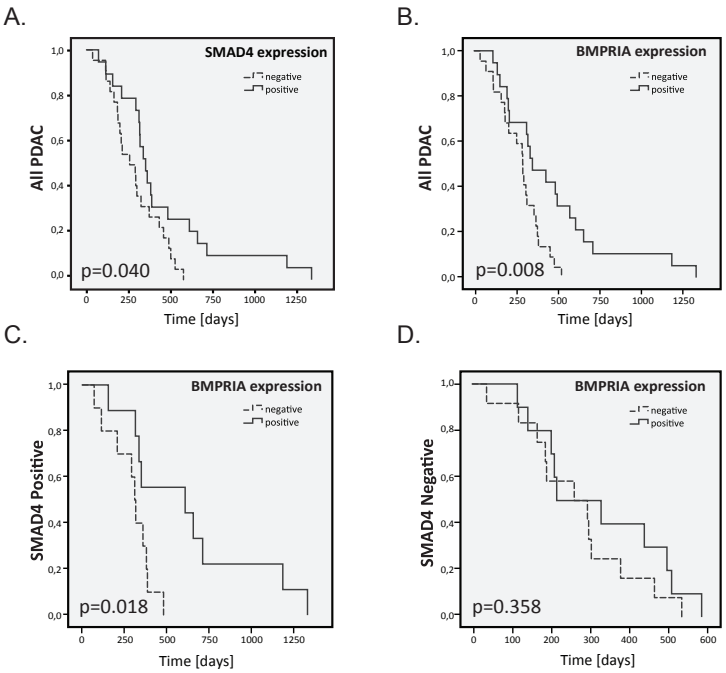


Figure 1. Kaplan–Meier graphs representing the survival of 41 patients after surgical resection of PDAC based on SMAD4 and BMPRIA expression in a TMA. (A) SMAD4 positive vs SMAD4 negative (P=0.040). (B) BMPRIA positive vs BMPRIA negative (P=0.008). (C) BMPRIA positive vs BMPRIA negative within SMAD4-positive PDAC (P=0.018). (D) BMPRIA positive vs BMPRIA negative within SMAD4-negative PDAC (P=0.358). P-values are based on log-rank tests.

in SMAD4-negative cells would have little additional impact on canonical BMP signalling, because it is already lost as a consequence of the SMAD4 loss. Reduced expression of BMPR1B or BMPR2 does not lead to a difference in survival, even when stratifying for SMAD4 expression (***Supplementary Figure 2***).

BMPR1A reduction leads to an increase in proliferation and invasion.

In order to investigate the functional role of the BMP pathway in PDAC in vitro, we first investigated the expression of BMPR1A and SMAD4 in three PDAC cell lines (PANC-1, MIA PaCa-2 and Bx-PC3). RT–PCR using primers specific for exon 13 of BMPR1A suggests that BMPR1A RNA is expressed in all three PDAC cell lines (***Figure 2A***). MIA PaCa-2 expresses lower levels of BMPR1A compared with PANC-1 and Bx-PC3 as shown by western blot analysis (***Figure 2B***). PANC-1 and MIA PaCa-2 both express SMAD4, whereas Bx-PC3 does not. The colorectal cancer cell lines HCT116 and SW480 were used as controls. HCT116 expresses SMAD4, but low levels of BMPRs and SW480 expresses no SMAD4 and high levels of BMPRs. In a luciferase reporter assay for BMP pathway signalling activity (BRE-luc), treatment of the cell lines with BMP-2 for 24 h leads to a significant increase in luciferase activity in PANC-1 and a less significant increase in MIA PaCa-2, but no increase in Bx-PC3 (***Figure 2C)*** (Korchynskyi and Ten, 2002; Kodach et al, 2008b). We conclude that PANC-1 and MIA PaCa-2 have the ability to activate BMP signalling, whereas Bx-PC3 has not.

We knocked down BMPR1A expression using siRNA and subsequently performed viability/proliferation and matrigel invasion assays. From the human data shown in ***Figure 1B—D***, we would predict that knockdown of BMPR1A expression in the SMAD4-positive cell lines PANC-1 and MIA PaCa-2 will have functional effects, but knockdown of BMPR1A in the SMAD4-negative cell line Bx-PC3 should have no functional effect because BMP signalling is already lost because of SMAD4 loss. ***Figure 2D*** shows the effect of BMPR1A knockdown after 48 h on cell viability/proliferation. A significant increase in viability/proliferation can be seen in the SMAD4-positive cell lines after BMPR1A knockdown. SMAD4-negative Bx-PC3 cells do not show an effect, as expected. Next, we performed a matrigel invasion assay in which we knocked down BMPR1A and measured fluorescently labelled cells that have passed through the matrigel in a transwell

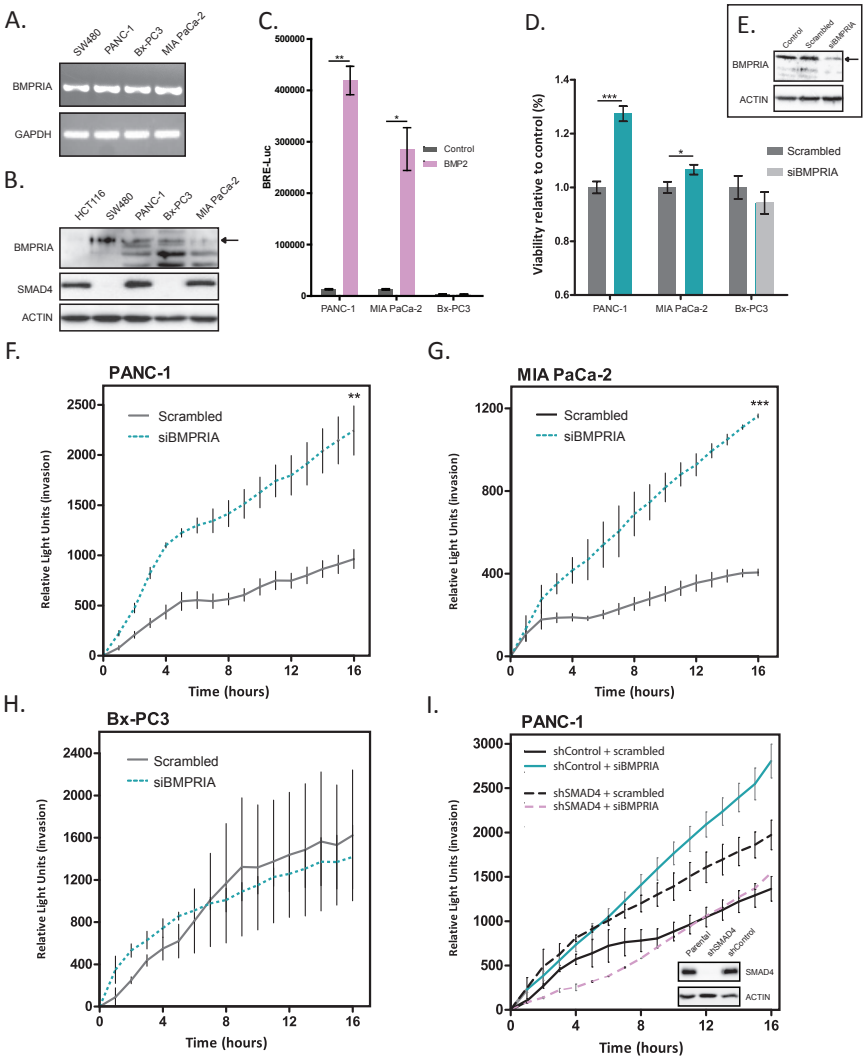


Figure 2. BMPRIA knockdown increases proliferation and invasion in SMAD4-positive PDAC cell line cells. (A) RT–PCR showing RNA expression of BMPRIA in the PDAC cell lines PANC-1, MIA PaCa-2 and Bx-PC3. GAPDH is used as a loading control. (B) Western blot analysis showing SMAD4 (61 kD) and BMPRIA (~66 kD) expression. The colorectal cancer cell lines HCT116 and SW480 were used as controls. Actin was used as a loading control. (C) The ability of BMP ligands to activate BMP signalling in PDAC cell lines PANC-1, MIA PaCa-2 and Bx-PC3. BMP signalling activation is shown by BRE-luciferase activity after treatment with 100 ng ml−1 BMP-2 dissolved in PBS for 24 h. (D) The effect of BMPRIA knockdown for 48 h on viability/proliferation in PANC-1, MIA PaCa-2 and Bx-PC3. Transfection with siRNA against BMPRIA was used for BMPRIA

system. In PANC-1 as well as the MIA PaCa-2 cells, an increase in invasion can be observed after BMPR1A knockdown. BMPR1A reduction in Bx-PC3 does not lead to a significant difference in invasion (***Figure 2F–H***).

The effects of the BMP pathway seem to be dependent on the SMAD4 status. To investigate the function of SMAD4 in PDAC cells, we made a cell line in which SMAD4 was stably knocked down by performing lentiviral transduction with shRNA against SMAD4 in SMAD4-positive PANC-1 cells, as well as a control cell line using a control construct. We then silenced BMPR1A and performed an invasion assay. Knockdown of SMAD4 leads to increased invasion (***Figure 2I***). The use of siBMPR1A in the shControl (SMAD4 positive) cells results in more invasion. We expected that silencing of BMPR1A in the shSMAD4 clone would not have any effect, but instead it lowers the invasion rate.

BMPR1A knockdown changes the expression of angiogenesis, mesenchymal, cancer stem cell and matrix modifier markers.

To further elaborate the cell changes resulting from BMPR1A loss in SMAD4-positive PDAC cells, we knocked down BMPR1A in PANC-1 cells and measured the mRNA levels of a set of markers associated with angiogenesis, epithelial-to-mesenchymal transition (EMT), cancer stemness and the modification of extracellular matrix (***Figure 3***). BMPR1A knockdown results in an increase in *VEGF*, *TSP1* and *ANGPT1* associated with angiogenesis. We also measured several markers associated with EMT and saw a downregulation of *CDH1* (encodes for E-cadherin) and a significant upregulation of VIMENTIN, NES and

knockdown. Transfection with scrambled siRNA was used as a control. (E) Western blot analysis showing BMPRIA knockdown (~66 kD) using transfection of siRNA against BMPRIA in PANC-1. siRNA scrambled was used as a control. Actin was used as a loading control. (F–H) The effect of BMPRIA knockdown on invasion in PANC-1 (F), MIA PaCa-2 (G) and Bx-PC3 (H). Matrigel in Fluoblock filters were used in a transwell system. Cells were labelled with Celltracker and invasion through matrigel was calculated using fluorescent measurements in the bottom wells every hour. The measurements were corrected for background fluorescence and set to zero at starting point. Transfection with siRNA against BMPRIA was used for BMPRIA knockdown. Transfection with scrambled siRNA was used as a control. (I) SMAD4 was knocked down in PANC-1 cells using shRNA. The non-targeting control construct SHC002 was used to make a control cell line. Western blot analysis shows SMAD4 (61 kD) knockdown. Actin is used as a loading control. Subsequently BMPRIA was knocked down using siRNA. Scrambled siRNA was used as a control. Next, a matrigel invasion array was performed. (All experiments were performed in triplicate. Mean±s.e.m. is shown, *P<0.05, **P<0.005, ***P<0.0005).

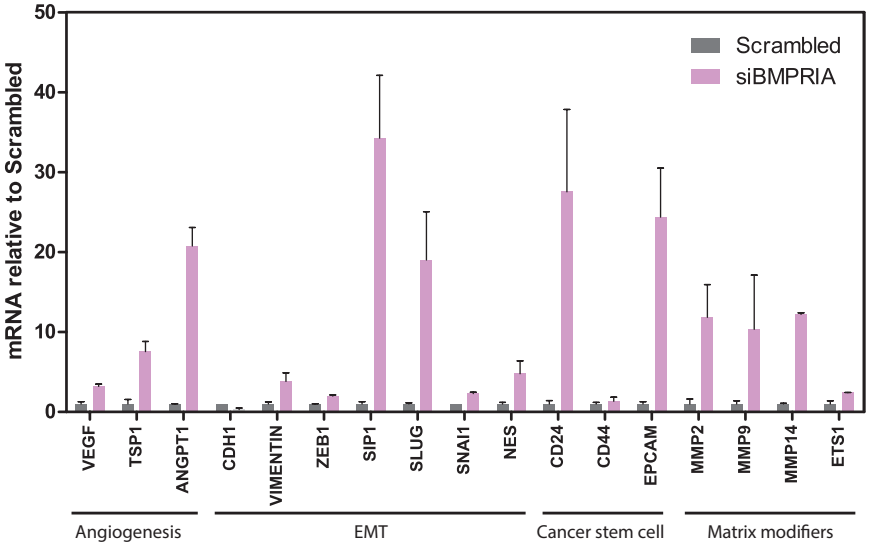


Figure 3. BMPRIA knockdown leads to an increase in markers associated with angiogenesis, EMT, cancer stemness and matrix modification in PANC-1 cells. A significant increase is seen in VEGF, TSP1, ANGPT1, VIMENTIN, SIP1, SLUG, NES, CD24, EPCAM, MMP2, MMP14 and ETS1.

the transcription factors SIP1 and *SLUG*. These changes suggest a change towards a more mesenchymal type of cell. No significant changes in the transcription factors *ZEB1* and *SNAI1* were observed. BMPR1a knockdown also resulted in the upregulation of the stem cell markers *CD24* and *EPCAM*, but not in changes in *CD44*. The expression of matrix metalloproteinases 2 and 14 (MMPs) is also significantly increased after BMPR1a knockdown. These MMPs are involved in the breakdown of extracellular matrix and tissue remodelling, which is associated with tumour progression (Ellenrieder et al, 2000).

The overall results of changes in SMAD4 and BMPR1A expression are schematically presented in ***Figure 4***.

The BMP pathway as a treatment target.

We further investigated whether the BMP pathway could be used as a treatment target. We treated the three cell lines (PANC-1, MIA PaCa-2 and Bx-PC3) with BMP-2 and the BMPR1A inhibitor LDN-193189 for 48 h and investigated the effect on cell viability/proliferation. Treatment with 100 ng ml^{-1} BMP-2 resulted

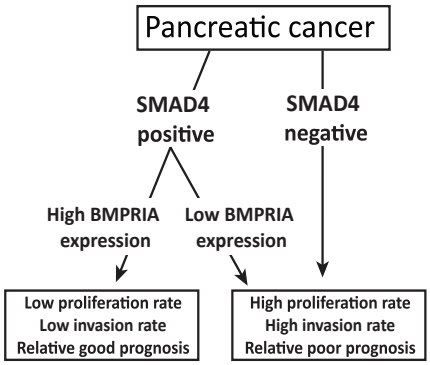


Figure 4. A scheme presenting the effects of SMAD4 and/or BMPRIA loss in PDAC.

in a decrease in viability in the SMAD4-expressing cell lines PANC-1 and MIA PaCa-2, but in an increase in the SMAD4-negative Bx-PC3 (***Figure 5A***). We also treated the cells with different concentrations of the BMPR1A inhibitor LDN-193189 (***Figure 5B***). Up to 50 nM an increase in viability/proliferation can be seen in the SMAD4-positive cells. A further increase in concentration results in a reduction in viability/proliferation, probably because of toxic effects. In Bx-PC3, a dose-dependent decrease in viability/proliferation can be observed.

We repeated these experiments using the shSMAD4 PANC-1 cells and observed that BMP2 treatment of the SMAD4-expressing cells (shControl) results in a decrease in viability, but not in the shSMAD4 cells (***Figure 5C***). BMP inhibition results in a decrease in cell viability in the shSMAD4 cells but not in the shControl cells (***Figure 5D***). We conclude that the BMP signalling pathway can induce tumour promotion or suppression dependent on the SMAD4 status (***Figure 5E***).

Discussion

We set out to investigate the expression levels of BMP signalling components in PDAC and link this to patient's survival data. We found that in 53.7% of the cases BMPR1A expression is reduced, and that this is associated with a poorer survival (p=0.008). When we stratified for SMAD4 expression it could be observed that reduced BMPR1A expression only makes a difference in SMAD4-positive cancers.

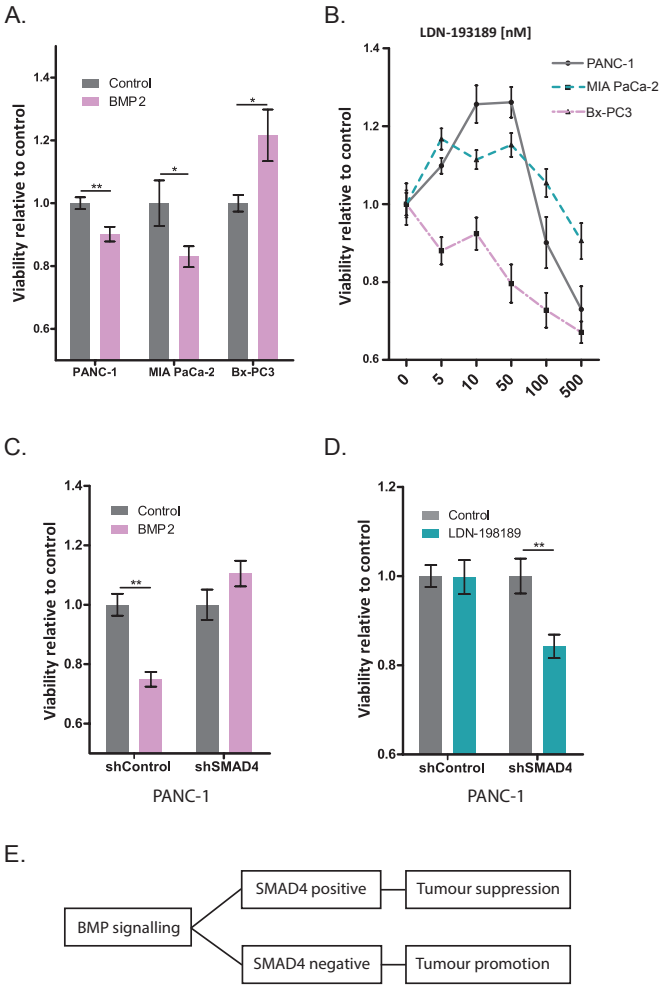


Figure 5. Treatment of (PDAC) cell lines PANC-1, MIA PaCa-2 and Bx-PC3 with BMP-2 and BMPR1 inhibitor LDN-193189. (A) PDAC cell lines were treated with 100 ng ml−1 of BMP-2 for 48 h. Cell proliferation/viability was subsequently assessed using an MTT assay was performed. (B) PDAC cancer cell lines were treated with different concentrations of LDN-193189 for 48 h. Subsequently an MTT assay was performed. (C and D) PANC-1 cells were transduced with either shRNA against SMAD4 or an empty control vector. The cell lines were treated with 100 ng ml−1 of BMP-2 for 48 h or 5 nM of LDN-193189 and proliferation/viability assessed using an MTT assay. The cell proliferation/viability is compared with the control condition, which is set to 1. (E) Proposed effect of BMP signalling based on SMAD4 status

SMAD4 is necessary for the canonical BMP pathway and loss of SMAD4 results in a defect in canonical BMP signalling. Subsequent reduction of BMPR1A expression in SMAD4-negative cancer does not reduce the canonical BMP signalling activity further and probably has little additional impact. Reduction of either BMPR1B or BMPR2 does not lead to a difference in survival and does not occur as frequently as loss of BMPR1A expression (9.8% and 29.3%).

Reduction in the expression of one or more BMPRs has been found in several other tumour types. Aberrant BMPR2 expression based on immunohistochemistry has been found in prostate cancer (Kim et al, 2004) and in renal cell carcinomas (Kim et al, 2003). Our group has previously identified mutations in the 3'UTR of BMPR2 in microsatellite instable colorectal cancer, which resulted in reduced expression levels (Kodach et al, 2008b). Although BMPR1A protein expression in PDAC has not previously been published, BMPR1A expression in PDAC has been assessed as part of the human protein atlas (www.proteinatlas.org) using a different antibody (CAB019398; Strategic Diagnostics Inc., Newark, DE, USA). This also showed absent BMPR1A expression in 56% of the cancers and strong expression in normal pancreatic tissue.

To further explore the effects of reduced BMPR1A expression in PDAC, we investigated three PDAC cell lines (PANC-1, MIA PaCA-2 and Bx-PC3). It has previously been published that BMPR1A is not expressed in MIA PaCa-2 cells at mRNA level using northern blot (Kleeff et al, 1999). We found that MIA PaCa-2 expresses lower levels of BMPR1A protein compared with PANC-1 and Bx-PC3, but equal levels of mRNA by RT–PCR using primers specific for exon 13. We found no PDAC cell lines with complete loss of BMPR1A. This is consistent with our immunohistochemistry staining results where we found that, although 53.7% had reduced expression levels of BMPR1A and were thereby regarded as 'negative' in our scoring system, only a small portion (4.9%) of the PDAC had completely lost BMPR1A expression. The underlying reason for the reduced BMPR1A protein expression we observe remains unclear. Large-scale mutational studies of large cohorts of PDAC (n¼100) have not revealed mutations in BMPR1A (Biankin et al, 2012) and studies that specifically mention analysis of BMPR1A in pancreatic cancer have also not revealed mutations (n=31; Jones et al, 2008; Kan et al, 2010). Although none of these studies has focussed specifically on BMPR1A, it seems unlikely that mutations in the coding region of BMPR1A can explain

the altered protein expression at the frequency that we observe. As MIA PaCa-2 seems to have normal mRNA levels in our analyses, but reduced protein levels, the cause will most likely be post-transcriptional such as miRNA inhibition of translation. For our protein expression studies using immunohistochemistry and immunoblotting, we used a polyclonal antibody raised against amino acids 24–83 of BMPR1A as used by several other groups (Deng et al, 2007; Barros et al, 2008; Medici et al, 2010; Du et al, 2011). On immunoblots using this antibody, we detect a major band at the predicted height of around 60 kD with other bands at approximately 50 and 45 kD as shown in the antibody product datasheet and as also seen with polyclonal BMPR1A antibodies from other manufacturers (e.g., ab38560 from Abcam (Cambridge, UK)) in cell lysates of various origins. The specificity of these antibodies is confirmed by the virtual disappearance of all these bands when PANC-1 cells are treated with BMPR1A-specific siRNA (***Figure 2E***). The reduction of BMPR1A expression in SMAD4-positive PDAC cells resulted in an increase in cell viability, matrigel invasion and the upregulation of several markers associated with angiogenesis, EMT, cancer stemness and matrix modification. These processes can lead to a more aggressive cancer. The markers were measured using qPCR only, and therefore the conclusions must be considered preliminary, but this might be the reason that reduced BMPR1A expression is associated with a poorer prognosis. These results have to be further confirmed at protein level and using functional assays.

Finally, we investigated the possibility of using BMP pathway modulation as a novel treatment. Treatment with BMP-2 results a decrease in viability in SMAD4-positive PDAC cells, but an increase in viability in SMAD4-negative cells. Treatment with LDN-193189, a small molecule BMPRI inhibitor, results in an increase in viability in SMAD4-positive cells, but a decrease in viability SMAD4-negative cells. It may be that BMP signalling has a dual role dependent on SMAD4 expression. It could be that when SMAD4 is present BMP acts as a tumour suppressor, whereas when SMAD4 is lost BMP acts as a tumour promoter. This phenomena, of a contradictory effect dependent on the expression status of a particular protein, has previously been reported in colorectal cancer with the TGF-β signalling pathway and SMAD4 expression (Zhang et al, 2010) where the SMAD4 expression status determined the metastatic effects of TGF-b. A second

example is the TGF-β switch from anti-proliferative into pro-metastatic dependent on the p53 mutational status (Adorno et al, 2009). In the absence of SMAD4, BMPs cannot signal through the canonical SMAD4-dependent signalling route. That BMP ligands still have important biological effects in the absence of SMAD4, as we and others have shown, is evidence for important non canonical SMAD4-independent signalling. There is not much data on non-canonical BMP signalling, but it is known that SMAD independent BMP signalling can activate p38MAPK, ERK and JNK in colorectal cancer (Grijelmo et al, 2007). p38, ERK and JNK are mitogen-activated protein kinases associated with tumour progression, which could explain the increase in viability observed after activating non-canonical BMP signalling.

This data would suggest that modulating the BMP pathway as part of a combined treatment strategy, as has been suggested in other cancers, will have to be targeted to specific tumours based on their SMAD4 expression status. Theoretically, BMP treatment could be used for SMAD4-positive cancers and BMPR1A inhibitors might be possible candidates for treatment of SMAD4 mutated PDACs, analogous to the use of TGF-βRIA inhibitors (Ge et al, 2006).

Although activation or inhibition of the same pathway for treatment of the same cancer type is perhaps conceptually confusing, at the very least these data would suggest caution in treating PDAC with BMP-modulating agents as they may potentially have adverse effects. The tumour stroma has a major role in the development and progression of PDAC, also affecting cancer therapy. In these studies, we have made use of well validated and frequently used in vitro models consisting of the tumour epithelial cells only. Further research in more complex in vitro and in vivo models will be needed to investigate the effects of BMP manipulation on stromal cells and their interaction with cancer cells.

In summary, we have studied the expression levels of BMPRs in PDAC tissue based on immunohistochemistry and linked this to patient's survival data. We show that a reduction in the expression of BMPR1A is associated with a poorer prognosis. Stratifying for SMAD4 reveals that this is mainly in a sub-population of SMAD4-positive cancers. BMPR1A expression might be useful as a prognostic bio-marker, especially when combined with SMAD4. By manipulating BMPR1A in vitro, we show that BMPR1A has tumour-suppressive effects, which are dependent on the

SMAD4 status. The small molecule BMPR1A inhibitor LDN-193189 has anti-tumour activity in SMAD4-negative PDAC cells.

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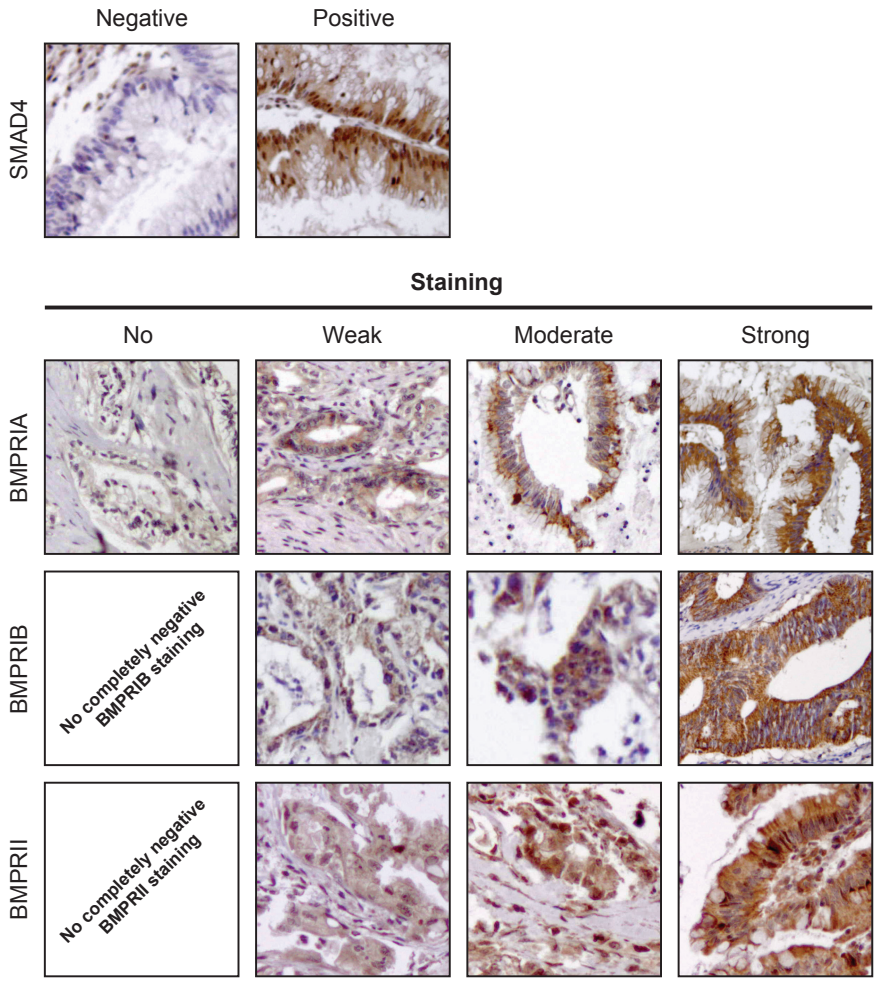
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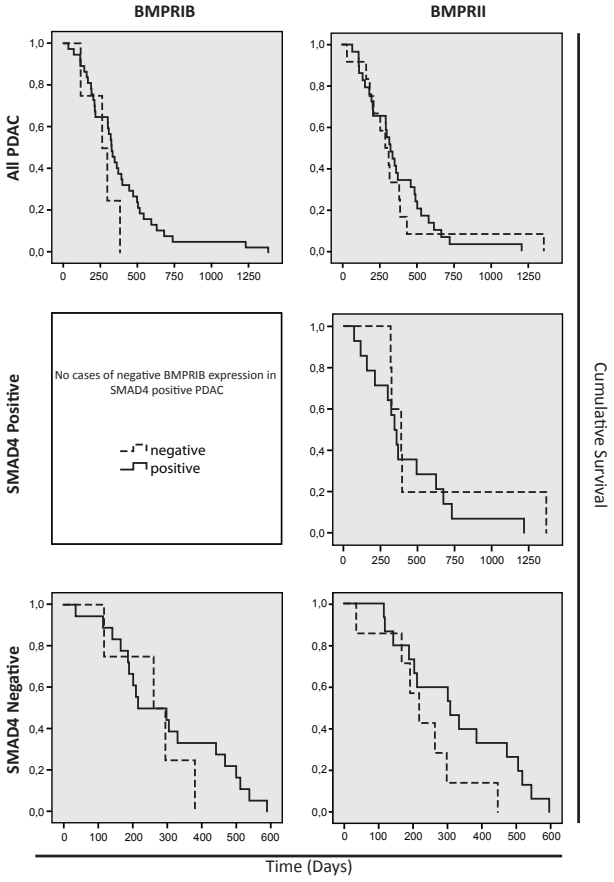
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Supplementary figure 1: SMAD4 and BMPReceptor staining examples



Supplementary figure 2: Survival based on BMPRIB and BMPRII expression levels



Chapter 9

Statin Use After Diagnosis of Colon Cancer and Patient Survival

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Aims

Statin use has been associated with a reduced incidence of colorectal cancer, and might also affect survival of patients diagnosed with colon cancer. Statins are believed to inhibit Ras signaling, and also activate the bone morphogenetic protein (BMP) signaling pathway in colorectal cancer cells. We investigated the effects of statins on overall survival of patients with a diagnosis of colon cancer, and whether their effects were associated with changes in KRAS or the BMP signaling pathways.

Methods

Data were derived from the PHARMO database network (Netherlands) and linked to patients diagnosed with colon cancer from 2002 through 2007, listed in the Eindhoven Cancer Registry. We obtained information on causes of death from statistics Netherlands. We constructed a tissue microarray of 999 colon cancer specimens from patients who underwent surgical resection from 2002 through 2008. Survival was analyzed with statin user status after diagnosis as a time-dependent covariate. Multivariable Poisson regression survival models and Cox analyses were used to study the effect of statins on survival. Tumor tissues were analyzed by immunohistochemistry for levels of SMAD4, BMPR1A, BMPR1B, and BMPR2 proteins. Tumor tissues were considered to have intact BMP signaling if they contained SMAD4 plus BMPR1A, BMPR1B, or BMPR2. DNA was isolated from tumor tissues and analyzed by quantitative PCR to detect mutations in KRAS. The primary outcome measures were overall mortality and cancer-specific mortality.

Results

In this cohort, 21.0% of the patients (210/999) were defined as statin users after colon cancer diagnosis. Statin use after diagnosis was significantly associated with reduced risk of death from any cause (adjusted relative risk [RR], 0.67; 95% CI, 0.51–0.87; P=.003) and death from cancer (adjusted RR, 0.66; 95% CI, 0.49-0.89; P=.007). Statin use after diagnosis was associated with reduced risk of death from any cause or from cancer for patients whose tumors had intact BMP signaling (adjusted RR, 0.39; 95% CI, 0.22–0.68; P=.001), but not for patients whose tumors did not have BMP signaling (adjusted RR, 0.81; 95% CI, 0.55–1.21; P=0.106; P<.0001 for the interaction). Statin use after diagnosis was not associated with reduced risk of death from any cause or from cancer for patients whose tumors did not contain KRAS

mutations (adjusted RR, 0.81; 95% CI, 0.56–1.18; P=.273) or whose tumors did have KRAS mutations (adjusted RR, 0.59; 95% CI 0.35–1.03; P=.062; P for the interaction=0.90).

Conclusions

In an analysis of 999 patients with a diagnosis of colon cancer, we associated statin with reduced risk of death from any cause or from cancer. The benefit of statin use is greater for patients whose tumors have intact BMP signaling, independent of KRAS mutation status. Randomized controlled trials are required to confirm these results.

Introduction

Although colorectal cancer survival has doubled in the last 40 years, 5 year survival remains low at only 65%.[1] Current chemotherapy for colorectal cancer results in significant toxicity limiting its use in early stage disease, in the elderly, and in patients with comorbidity so that there is a clear unmet clinical need for new less toxic treatment options. Previously, aspirin has been shown to increase survival when used after diagnosis, thus providing a potential new minimally toxic adjuvant treatment option for colorectal cancer. [2,3] Statins may represent such a treatment option as well, either alone or in combination with aspirin. Aside from their proven efficacy in primary and secondary prevention of cardiovascular morbidity and mortality[4], statins have been shown, in several but not all studies, to reduce the risk of developing colorectal cancer[5]. *In vitro* and *in vivo* studies indicate that statins inhibit proliferation and induce apoptosis in colorectal cancer cells[6,7]. However, the exact molecular mechanism by which statins influence colorectal cancer remains debated. Cancer therapy is increasingly focused on personalized therapy via pharmacological modulation of specific molecular pathways targeted to sensitive tumors identified by molecular subtyping; statins could potentially be more effective in a specific subgroup of cancers.

There are several potential molecular mechanisms of action to explain the therapeutic effects of statins in colorectal cancer. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase; an enzyme that plays an essential role in mevalonate synthesis. Inhibition of the mevalonate pathway does

not only disrupt cholesterol synthesis, but also farnesyl pyrophosphate synthesis, which is essential for the prenylation of GTPases like *KRAS*[8]. It is therefore thought that statins might act on colorectal cancer by inhibiting *KRAS*. *KRAS* mutations are prevalent in 40% of colorectal cancers and result in constitutively active form of *KRAS*.[9]

Another theory hypothesizes that statins act through activation of the Bone Morphogenetic Protein Signaling (BMP) pathway. Statins activate the BMP pathway in bone[10] and we have previously shown that they also do this in colorectal cancer.[11] Interestingly statins are only effective in colorectal cancer cells where BMP signaling is intact[7]. BMP signaling is frequently disrupted in colorectal cancer through loss of SMAD4, the central component of the signaling cascade, or reduced BMP receptor expression.[12,13]

The aim of our study was to evaluate whether statins might be effective as adjuvant therapy in colon cancer by correlating post-diagnosis statin use with patient survival in a cohort in which we have previously observed a survival benefit with aspirin use after diagnosis. Secondly, we tried to uncover the molecular background in which statins are able to execute their tumor suppressive function, thereby considering the *KRAS* mutational status and the BMP signaling pathway functionality in relation to statin use and patient survival.

Methods

Retrospective study cohort

All patients diagnosed with colon cancer between 2002 and 2007 were selected from the Eindhoven Cancer Registry. This southern region of the Netherlands is served by 10 hospitals each serving a population between 150 000 and 250 000 people. Data on statin use (simvastatin, pravastatin, cerivastatin, fluvastatin, atorvastatin and rosuvastatin) was derived from the PHARMO database network (PHARMO, Netherlands). The central patient database of the PHARMO record linkage system has recently been linked to the Eindhoven Cancer Registry database; this is described in detail by Van Herk-Sukel et al[14]. Information about the cause of death was obtained from Statistics Netherlands (CBS). Formalin-fixed paraffin-embedded (FFPE) tissue blocks were retrieved from 1026 colon cancer

patients who had a surgical resection between 2002 and 2008.[15] Rectal cancers were not included, as many of these tissue specimens will have been exposed to preoperative radiotherapy, which may influence tumor molecular characteristics. Twenty-seven patients with more than one colon tumor at the time of diagnosis were excluded for this study; thus the total cohort consisted of 999 patients.

TMA production

Three 1.0 mm diameter cores were obtained from FFPE tumor blocks and transferred into a receiver paraffin block using the TMA Master (3D Histech, Budapest, Hungary) as described in detail before.[15] Representative tumor sites were identified by two independent researchers using haematoxylin and eosin stained sections (with a qualified pathologist confirming the identification of the tumor).

Immunohistochemistry & TMA scoring system

Determination of microsatellite stability status by immunohistochemical analysis has been previously described.[15] SMAD4 and BMP receptors were stained according to previously described methods.[16] Examples of tumor core stainings are shown in ***Supplementary Figure 1***. Scoring was performed in a blinded fashion by two investigators (P.V. and J.H.) independently according to previously described methods. [16] Three cores per tumor were analyzed and an average score per tumor was calculated. The concordance between investigators was 87% (κ = 0.70, 95% CI 0.604 - 0.796). Final scoring was reached by consensus. The tumors were divided in 'intact BMP signaling' and 'non-intact BMP signaling' based on the expression of SMAD4, BMPR1a, BMPR1b and BMPR2. If either SMAD4 or one of the BMP receptors scored negative the tumor was designated as having 'non-intact BMP signaling'.

KRAS mutation analysis

DNA was extracted from 2.0 mm diameter cores taken randomly from 663 of the 999 blocks, as previously described.[15] For determination of *KRAS* mutations status, hydrolysis probe assays were performed for the major known mutations (hotspots) in codon 12 and 13 for *KRAS*; c.34G>A; p.G12S, c.34G>C; p.G12R, c.34G>T; p.G12C, c.35G>A; p.G12D, c.35G>C; p.G12A, c.35G>T; p.G12V,

c.38G>A; p.G13D and c.37G>T; p.G13C, as previously described.[15] Hydrolysis probe assays were analyzed using qPCR analysis software (CFX manager version 3/0, Bio-Rad). Mutation detection was performed blindly by two independent observers (M.R and R.E).

Statistics

Definition of statin user

Statistical analyses were performed using the statistical packages SPSS (version 20.0 for Windows, IBM SPSS statistics) and STATA/SE (version 12 for windows, StataCorp LP). A *p*-value of <0.05 was considered statistically significant. The vital status of patients (alive/dead) was identified via medical records or through linking the Eindhoven Cancer registry data with the municipal population registries that have information on the vital status (alive or deceased). Follow-up started 30 days from diagnosis of colon cancer (T0), and was ended January 2012 or at the date of death. Patients who died within 30 days after diagnosis were excluded from the survival analyses (2.4%). Statin non-users were classified as those who never had a prescription for statin or had a prescription for less than 14 consecutive days after diagnosis of colon cancer. Statin users were defined as those who had been given a prescription for statins for 14 days or more after a colon cancer diagnosis.

Time dependent survival analyses

A time-dependent exposure survival analysis for Overall Survival (OS) and Cancer Specific Survival (CSS) was performed, in which patients were defined as non-users from T0 to the first use and users from first use to the end of the follow-up. A parametric survival model with an exponential (Poisson) distribution was used. Secondly, a Cox proportional hazard model was used with statin use as a time varying covariate to confirm the analyses. Adjustments for potential confounders were made for sex, age (continuous), stage (pathological stage or clinical stage when pathological stage was unknown), adjuvant chemotherapy (yes/no), grade, year of incidence, microsatellite status and comorbidity (yes/no). Comorbidities included respiratory disease, cardiovascular disease, digestive disease, musculoskeletal disease, neurologic disease or endocrine disease. Subgroup analyses were performed for sex, age, stage, grade, chemotherapy, aspirin use after

diagnosis, and frequent use of statin (≥3, ≥5 or ≥7 prescriptions). Frequent users were defined as patients who had a given number (or more) consecutive repeat prescriptions for statins. User time started after the completion of the 3, 5 or 7 prescriptions. Survival time of patients who had less than 3, 5 or 7 refills and time prior to the prescription requirements of the frequent users was classified as non-user time in this analysis.

Sensitivity analyses for ACE inhibitors and benzodiazepines

A sensitivity analyses was performed to assess the association of two other groups of medicine, benzodiazepines and ACE inhibitors, and survival. For this, the same methodology as for statin use was followed and a time-dependent parametric survival model with an exponential (Poisson) distribution was used, in which patients were defined as non-users from T0 to the first use and users from first use to the end of the follow-up. Adjustments for potential confounders were made for sex, age (continuous), stage (pathological stage or clinical stage when pathological stage was unknown), adjuvant chemotherapy (yes/no), grade, year of incidence, microsatellite status and comorbidity (yes/no). An extra analysis was performed excluding statin users from the group of post-diagnosis ACE inhibitors users.

A lag of 6 months

In another analysis a lag of 6 months after diagnosis was introduced to reduce any bias in prescribing that may arise due to impending death, although our definition of a statin user was specifically chosen to minimize this sort of bias due to disease progression.

Molecular subtypes

Finally, differential associations of statin use with CSS by tumor molecular subtype were investigated by subgroup analyses for intact BMP signaling status and *KRAS* mutation status followed by an interaction analysis.

Results

Statin use after colon cancer diagnosis and survival

In total, 999 colon cancer patients were included in this study. ***Table 1*** shows the characteristics of the population according to statin use or non-use after diagnosis and ***Figure 1*** shows a flow diagram of the patient inclusion in the various analyses. In this cohort, 21.0% (210/999) were defined as statin users after diagnosis. During follow-up until January 2012, 465 deaths were recorded of whom 69 were statin users (32.9% of statin users) and 396 were nonusers (50.2% of nonusers). Statin users were predominately male, older of age and had more comorbidities. Furthermore, tumors found in statin users have a lower stage than tumors found

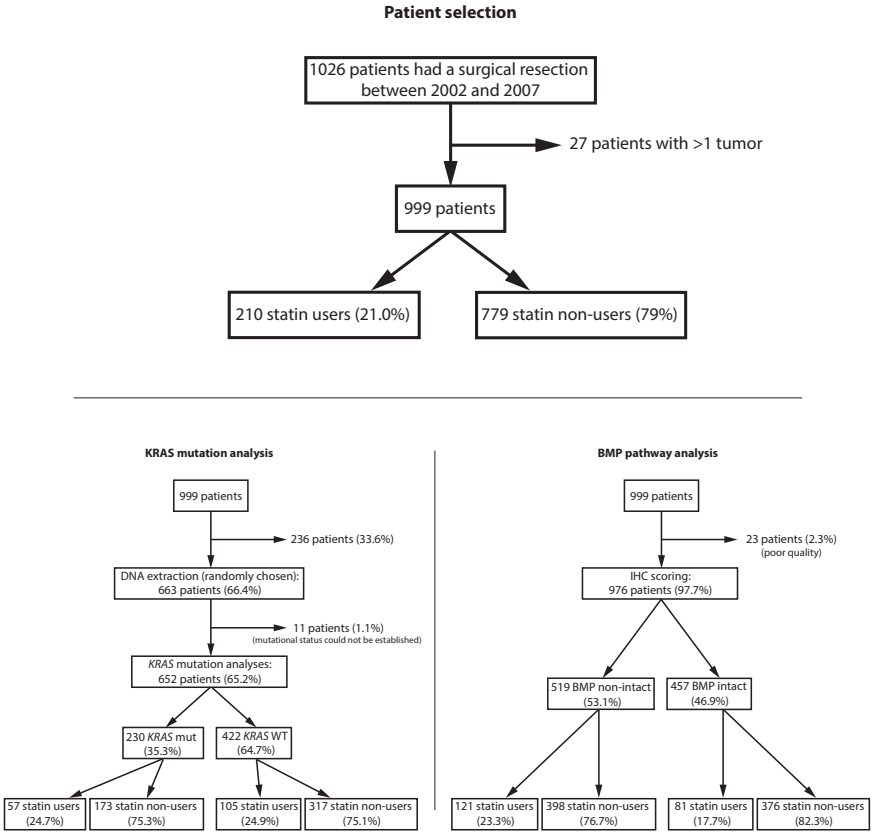


Figure 1.

in non-users (*p=0.005*). The mean and median duration of prescriptions was 76.6 and 90 days respectively and the mean number of prescriptions per patient was 23 (range 1- 215). Overall, 51.7% of the patients showed no discontinuation over the study period and 36.6% stopped for 30 days or less, which is not considered as discontinuation. Overall, 11.7% of the patients discontinued statin use for more than 30 days, 7% more than 90 days. Median follow-up of the cohort was 3.3 years (range 0.01-8.27), with a median follow-up of 4.1 years (range 0.001-7.94) for patients who were alive during the study period. Median first start of post-diagnosis statin use was at 1.9 years (range 0.005-6.31). There were 396 deaths in 789 non-users of statins and 69 deaths in 210 patients who used statins.

The 5-year overall survival (OS) for non-users was 54.6% (95%CI 50.8-58.1) and

Table 1 Baseline Characteristics of the Colon Cancer Patients according to Use of Statin after Diagnosis

	All patients (N=999)	**Statin non-users (N=789)**	**Statin users (N=210)**	***p*-value**
Sex				
Male	505 (50.6)	379 (48.0)	126 (60.0)	0.002
Female	494 (49.4)	410 (52.0)	84 (40.0)	
Age				
<65	342 (34.2)	282 (35.7)	60 (28.6)	<0.001
66-74	304 (30.4)	211 (26.7)	93 (44.3)	
75 and older	353 (35.4)	296 (37.5)	57 (27.1)	
Year of diagnosis				
2002-2004	451 (45.2)	354 (44.9)	97 (46.2)	0.732
2005-2007	548 (54.8)	435 (55.1)	113 (53.8)	
Disease stage				
I	138 (13.8)	99 (12.6)	39 (18.6)	0.005
II	402 (40.2)	308 (39.0)	94 (44.8)	
III	287 (28.7)	231 (29.3)	56 (26.7)	
IV	169 (16.9)	149 (18.9)	20 (9.5)	
Unknown	3 (0.3)	2 (0.3)	1 (0.5)	
Comorbidity				
No	443 (44.3)	401 (50.8)	42 (20.0)	<0.001
Yes	556 (55.7)	388 (49.2)	168 (80.0)	
Microsatellite status				
MSI	90 (9.0)	70 (8.9)	20 (9.5)	0.727
MSS	870 (87.1)	690 (87.5)	180 (85.7)	
Unknown	39 (3.9)	29 (3.7)	10 (4.8)	
Chemotherapy				
No	691 (69.2)	542 (68.7)	149 (71.0)	0.529
Yes	308 (30.8)	247 (31.3)	61 (29.0)	

65.7% (95%CI 57.8-72.4) for statin users. Statin use after diagnosis was significantly associated with a reduced risk of death from any cause with a Rate Ratio (RR) of 0.65 (95% CI 0.50-0.84, *P*=.001) and death from cancer; RR of 0.64 (95% CI 0.47-0.85, *p=0.002*). When adjusted for potential confounders this effect remained with an adjusted RR of 0.67 for OS (95% CI 0.51-0.87, *P*=0.003) and an adjusted RR of 0.66 for CSS (95%CI 0.49-0.89, *p=0.007*) (Table 2). ***Supplementary Table 1*** shows the Rate Ratios for variables used in the multivariable analyses other than statin use. Subgroup analyses by stage at diagnosis showed no association for mortality in stage I (adjusted RR 1.42 (95%CI 0.50-4.02, *p=0.90*) and stage III (adjusted RR 1.10 (95%CI 0.65-1.88, *p=0.71*), but showed an association between statin use and mortality for stage II (adjusted RR 0.45 (95%CI 0.24-0.87, *p=0.02*) and stage IV (adjusted RR 0.43 (95%CI 0.25-0.76, *p=0.004*). The interaction test for mortality

Table 2 Rate Ratio for Death (Time-Dependent Analysis Overall Mortality & Cancer Specific Mortality), According to Use or Nonuse of Statin after Diagnosis

Statin (all prescriptions)	**Statin non-users**	**Statin users****	**p-value**
Patients	789	210	
Deaths	396	69	
Deaths from cancer	311	53	
Rate Ratio OS (95%CI)	1.0 (reference)	0.65 (0.50-0.84)	0.001
Adjusted Rate Ratio OS (95%CI)*	1.0 (reference)	0.67 (0.51-0.87)	0.003
Rate Ratio CSS (95%CI)	1.0 (reference)	0.64 (0.47-0.85)	0.002
Adjusted Rate Ratio CSS (95%CI)*	1.0 (reference)	0.66 (0.49-0.89)	0.007
Stage subgroups (CSS)			
Stage I	1.0 (reference)	1.42 (0.50-4.02)	0.51
Stage II	1.0 (reference)	0.45 (0.24-0.87)	0.02
Stage III	1.0 (reference)	1.10 (0.65-1.88)	0.71
Stage IV	1.0 (reference)	0.43 (0.25-0.76)	0.004
Statin frequent use ≥3 prescriptions			
Patients	802	197	
Deaths from cancer	313	51	
Rate Ratio CSS (95%CI)	1.0 (reference)	0.63 (0.47-0.85)	0.002
Adjusted Rate Ratio CSS (95%CI)*	1.0 (reference)	0.72 (0.52-1.00)	0.049
Statin frequent use (≥5 prescriptions)			
Patients	815	184	
Deaths from cancer	319	45	
Rate Ratio CSS (95%CI)	1.0 (reference)	0.57 (0.42-0.78)	<0.001
Adjusted Rate Ratio CSS (95%CI)*	1.0 (reference)	0.65 (0.46-0.91)	0.012
Statin frequent use (≥7 prescriptions)			
Patients	826	173	
Deaths from cancer	322	42	
Rate Ratio CSS (95%CI)	1.0 (reference)	0.56 (0.41-0.78)	<0.001
Adjusted Rate Ratio CSS (95%CI)*	1.0 (reference)	0.63 (0.44-0.89)	0.009

*Adjusted for sex, age, comorbidity, year of incidence, histological grade, stage, microsatellite status, chemotherapy and aspirin use, ** Statin use after diagnosis

and stage is *p<0.01*. Interestingly, frequent use of statins, especially more than 7 refills further reduced risk of death with an adjusted RR of 0.63 (95% CI 0.44-0.89, *p=0.009*).

We have previously shown that low-dose aspirin use after diagnosis was associated with a survival benefit in this cohort[15]. We therefore performed a further analysis in patients that only used statins or only used aspirin or a combination of both (***Table 3***). Compared to patients who used neither aspirin or statins after diagnosis, isolated statin use and isolated aspirin use were both significantly associated with a redcued risk of death from cancer with an adjusted RR of 0.48 (95% CI 0.31-0.73) for isolated statin use and 0.54 (95% CI 0.35-0.85) for isolated aspirin use. A combination of both statin and aspirin use was not significantly associated with mortality; adjusted RR of 0.69 (95% CI 0.46-1.03). The notion that statins act independently of aspirin use is further confirmed by the fact that statin use was significantly associated with a reduced risk of death in patients who did not use aspirin after diagnosis (adjusted RR 0.51, 95% CI 0.33-0.79, *p=0.003*).

Including a lag of 6 months was associated with a reduced risk of death from cancer with a RR of 0.64 (95%CI 0.47-0.85, *p=0.002*) and an adjusted RR of 0.66 (95%CI 0.48-0.89, *p=0.007*).

ACE inhibitors and benzodiazepines

We performed sensitivity analyses with post-diagnosis ACE inhibitor, a group of cardiovascular medicines not associated with colon cancer survival, and observed a significant RR of 0.75 (95%CI 0.59-0.96), *p=0.024*), but a non-significant adjusted RR of 0.81 (95%CI 0.62-1.05, *p=0.114*). When excluding statin users, ACE inhibitor use has a RR of 0.98 (95%CI 0.70-1.38, *p=0.93*). We also analyzed a completely different class of drugs; benzodiazepines. These were not associated

Table 3 Rate Ratio for cancer specific mortality (time-dependent analysis), according to aspirin or statin use

	N	Deaths from cancer	Adjusted Rate Ratio (95%CI)	p-value
Aspirin or statin use				
No aspirin or statin use	711	288	1.0 (reference)	0.0002
Statin use, no aspirin	106	23	0.48 (0.31-0.73)	
Aspirin use, no statin	78	23	0.54 (0.35-0.85)	
Both aspirin and statin use	104	30	0.69 (0.46-1.03)	
Aspirin non-users				
Statin non-users	711	288	1.0 (reference)	0.003
Statin users	106	23	0.51 (0.33-0.79)	

with an improved survival (*p=0.03*).

Statin use, survival and KRAS mutations

KRAS mutation status (wild-type/mutation) was established in 98% (652/663) of the tumor cores that were randomly taken from the 999 original tumor cores. There were no differences in baseline characteristics between patients of whom

Table 4 Rate Ratio for cancer specific mortality (time-dependent analysis), according to Tumor KRAS mutation status, BMP signaling pathway status and use or non-use of statin after diagnosis

	Statin non-users	Statin users	p-value
All cancers			
Patients	789	210	
Deaths from cancer	311	53	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.66 (0.49-0.89)	0.007
Adjusted Hazard Ratio (95%CI)* ‡	1.0 (reference)	0.71 (0.53-0.97)	0.032
KRAS Wild-type cancers			
Patients	317	105	
Deaths from cancer	122	31	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.82 (0.54-1.25)	0.35
Adjusted Hazard Ratio (95%CI)* ‡	1.0 (reference)	0.86 (0.56-1.31)	0.48
KRAS Mutant cancers			
Patients	173	57	
Deaths from cancer	74	15	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.59 (0.33-1.08)	0.086
Adjusted Hazard Ratio (95%CI)* ‡	1.0 (reference)	0.64 (0.35-1.17)	0.148
Intact BMP-signaling			
Patients	376	81	
Deaths from cancer	124	18	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.39 (0.22-0.68)	0.001
Adjusted Hazard Ratio (95%CI)* ‡	1.0 (reference)	0.42 (0.24-0.74)	0.003
Non-intact BMP-signaling			
Patients	398	121	
Deaths from cancer	179	33	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.81 (0.55-1.21)	0.31
Adjusted Hazard Ratio (95%CI)*‡	1.0 (reference)	0.89 (0.60-1.33)	0.58
Intact BMP-signaling and wild-type cancers			
Patients	150	44	
Deaths from cancer	43	12	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.81 (0.40-1.62)	0.55
Intact BMP-signaling and mutant cancers			
Patients	89	18	
Deaths from cancer	34	6	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.59 (0.22-1.57)	0.29

*Adjusted for sex, age, comorbidity, year of incidence, histological grade, stage, microsatellite status and chemotherapy, ** Statin use after diagnosis

† Poisson model ‡ Cox model

§ KRAS analyses: 663 of 999 patiënts analysed; 652 of 663 samples included

BMP analyses: 976 of 999 samples included

the DNA was extracted and the KRAS mutation status was established and those in which this was not successful. A *KRAS* mutation was found in 35.3% (230/652) of the samples, which shows a prevalence that is in accord with other studies.[17] ***Supplementary Table 2*** summarizes the clinical characteristics of the patients based on the KRAS mutation status and statin use. Statin use was not significantly different in patients with *KRAS* wild-type tumors (24.9%) and *KRAS* mutated tumors (24.8%). The effect of *KRAS* mutation status on the survival benefit associated with statin use after diagnosis was analyzed. Statin use after diagnosis was not associated with a reduced risk of death from cancer in *KRAS* wild-type tumors (adjusted RR 0.82 (95% CI 0.54-1.25, *p=0.35*) or *KRAS* mutated-tumors (adjusted RR 0.59 95% CI 0.33-1.08, *p=0.086*); test for interaction *p=0.4566* (***Table 4***). A Cox proportional hazard model confirmed the results of the Poisson model (adjusted RR for KRAS mutant cancers; 0.64, 95% CI 0.35-1.17, *p=0.148*). An interaction analysis showed no statistical significant difference between the two molecular tumor subtypes (*p-value* for interaction = 0.457). ***Supplementary Table 4*** shows the results based on overall mortality.

Statin use, survival and BMP signaling

Analysis of BMP signaling pathway components was performed on 976 tumor sections (97.7% of total). Loss of 2.3% of the tumor sections in this analysis was a consequence of staining artifacts and loss of material during the staining procedure. The tumors were categorized as having either 'intact BMP signaling' or 'non-intact BMP signaling' based on the expression levels of SMAD4 and the BMP receptors (see methods and materials for a full description of the scoring system). We presumed that the BMP signaling pathway was intact when all components were expressed and that the BMP signaling pathway was 'non-intact' when there was absence of expression of one of the components. In this cohort, 519 patients (53.2%) showed non-intact BMP signaling in their tumor sections and 457 patients (46.8%) intact BMP signaling. ***Supplementary Table 3*** summarizes the clinical characteristics of the patients based on BMP signaling status and statin use. 23.3% of patients in whom we observed non-intact BMP signaling in the tumor sections were statin users and 17.7% of patients with intact BMP signaling were statin users. In both groups statin users had more comorbidities. Within the 'intact BMP signaling' group, low stage tumors (*p=0.012*) were more prevalent in

statin users than in statin non-users.

To test our hypothesis that statins act via the BMP pathway in colon cancer prevention, we analyzed the effect of BMP signaling pathway status on statin associated patient survival (***Table 4***). The significant reduced risk of death associated with statin use after diagnosis was more prominent in the group of tumors that exhibited intact BMP component expression with an adjusted RR of 0.39 (95% CI 0.22-0.68, *p=0.001*) than for patients with tumors in which we observed a non-intact BMP signaling pathway (adjusted RR 0.81, 95% CI 0.40-1.62, *p=0.55*). A Cox proportional hazard model confirmed the results of the Poisson model (adjusted RR 0.42, 95% CI 0.24-0.74, *p=0.003*). An interaction analysis showed a statistically significant difference between the two molecular tumor subtypes (*p-value* for interaction <0.001). ***Supplementary Table 4*** shows the results based on overall mortality.

Discussion

In this large observational study, we show in our cohort that statin use initiated or continued after colon cancer diagnosis is associated with a significant reduced risk of death from any cause with an RR of 0.65 and death from cancer with an RR of 0.64. To test for associations between the molecular tumor subtype and the effect of statins on colon cancer survival, we analyzed both the *KRAS* mutation status and the protein expression of multiple elements of the BMP signaling pathway in the tumor samples. We have previously performed similar analyses of the BMP pathway in both pancreatic ductal adenocarcinoma and colorectal cancer.[16,18] We found that the association between statins and survival did not differ *KRAS* mutant versus KRAS wildtype tumor, but the survival benefit associated with statin use was stronger in tumors with an intact BMP signaling pathway. This is in accordance with findings from our previous *in vitro* and rodent studies showing that statins are only effective anti-tumor agents in tumors where the BMP signaling pathway is functional. [7,11]

Statins may be an attractive candidate for use as adjuvant therapy in colorectal cancer as they are already widely used and well tolerated.[19,20] Statins have previously been shown to reduce the incidence of colorectal cancer. A large

retrospective study published in 2005 assessing the association between statin use and colorectal cancer incidence showed a 47% reduction in the risk of developing colorectal cancer.[5] Although several subsequent studies have failed to confirm this effect[21,22], a recent meta-analysis combining 42 large observational studies, case-control studies and randomized control trials showed an overall risk reduction of 10% for the development of colorectal cancer in statin users providing more evidence for the antitumor potential of statins.[23]

Despite the large number of trials that have investigated the association between colorectal cancer incidence and statin use, relatively few studies have investigated the effect of adjuvant statin use on patient survival in colorectal cancer. A study from Scotland including 308 patients with colorectal cancer found a non-significant reduction in colorectal cancer-specific mortality in statin users.[24] An American study of 407 patients with rectal cancer who received chemo-radiotherapy also found a non-significant reduction in cancer-specific mortality in statin users before and after surgery[25]. A further American study in 842 patients with stage III colon cancer did not detect any association between patient-reported statin use after diagnosis and cancer recurrence[26] and a Dutch study found no association between Statin use, KRAS mutation status and metastatic-colorectal cancer progression-free survival after chemotherapy.[27] Some of these studies investigated specific colorectal cancer subgroups[25-27] or had limitations such as relatively small size[24-27], measurement of medication use at one time-point[26], and potential for immortal time bias[24]. In the biggest study to date from the UK a cohort of 7,657 patients with colorectal cancer were analyzed using time-dependent Cox regression models. Statin use post-diagnosis was associated with a significant reduction in overall survival.[28] This is consistent with our findings of a strong association between statin use and overall patient survival. The strengths of our study are the use of registered drug prescriptions to ascertain drug exposure rather than patient questionnaires, a relatively large cohort of 999 patients, molecular analysis of tumor tissue and analysis of the effect of combined exposure to aspirin and Statins. This is particularly interesting in the light of conflicting evidence as to whether a combination of aspirin and Statins may be more effective than one or other alone[29-32].

A recently published study with this cohort showed an association between low-dose aspirin use after diagnosis and improved survival.[15] Interestingly, isolated use

of either statins or aspirin after diagnosis are both associated with an improved survival in patients, indicating that statins and aspirin improve patient outcome independently. Although there is considerable *in vitro* evidence that a combined therapy of statins and aspirin could be beneficial[33], we could not find a synergistic or additive effect of statin combined with aspirin use. Separating this cohort based on statin and/or aspirin use results in a relative small number of patients per group; a larger cohort will be needed to assess the additive effect of combination therapy.

The molecular mechanisms responsible for the anti-tumor effects of statins on colorectal cancer are not fully understood. As mentioned in our introduction several mechanisms have been proposed two of which we have investigated in this study, namely that statins act on KRAS by influencing its prenylation and that statins act by activating the BMP signaling pathway.

Firstly, we investigated the influence of *KRAS* mutation status on the association between statin use and patient survival. There is evidence that statins act through the inhibition of the Ras/Raf pathway from studies performed in cancer cell line cells and xenograft mouse models. For example, it has been shown that Lovastatin inhibits the Raf/MEK/ERK pathway in leukemia cells resulting in apoptosis[34] and that Atorvastatin can disrupt *KRAS*/Raf complexes leading to inhibition of AKT and ERK in non-small cell lung cells.[35] The most clinically relevant data came from a study showing that Simvastatin could overcome cetuximab resistance in colon cancer cell line cells harboring *KRAS* mutations and not in cells with *BRAF* mutations, implying that only *KRAS* mutant cancers would benefit from statin treatment.[36] In our cohort we did not see a difference in the association between statin and survival comparing *KRAS* WT cancers and *KRAS* mutant cancers. There was no significant reduced risk of death associated with statin use in either group, although the results in *KRAS* mutant tumors nearly reached significance. The reason for the loss of a significant protective effect of statins when separating the cancers based on the *KRAS* mutation status is probably the low number of cancers in each group. A larger cohort is therefore necessary to adequately assess the influence of *KRAS* mutation status on the survival benefit associated with statin use. Our data is consistent with two studies that have found no association between the *KRAS* mutation status, statin use and colorectal cancer survival[26,27]. However, in these studies statin use after diagnosis was not associated with improved survival in colorectal cancer overall, in contrast to our study, making

the studies difficult to compare.

Secondly we investigated the influence of tumor expression of elements of the BMP signaling pathway on the association between statin use and patient survival. The BMP signaling pathway functions as a tumor-suppressor in colorectal cancer, inducing cell differentiation and apoptosis of colonic epithelial cells and negatively regulating WNT signaling.[37,38] In a screen of 30000 compounds Lovastatin and Simvastatin were the two most potent enhancers of BMP2 expression in bone.[10] We have subsequently shown that statins inhibit colorectal cancer cell proliferation and induce apoptosis through increasing BMP2 expression, but only when the BMP pathway is fully functional.[7] BMP 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 (BMPR1a and BMPR1b), which phosphorylate the receptor-associated SMADS (SMAD1,5 and 8) that subsequently complex with SMAD4 and translocate to the nucleus to regulate gene transcription. When expression of either SMAD4 or any one of the BMP receptors is lost, the canonical BMP signaling pathway cannot be activated. We assessed the expression levels of SMAD4, BMPR1a, BMPR1b and BMPR2 using immunohistochemistry as we have performed previously. In this cohort, statins are associated with a reduced risk of death in cancers that express both SMAD4 and BMP receptors (described in the results as intact BMP signaling), suggesting that the anticancer benefit of statin may be BMP dependent. Since this is the first study to investigate the role of the BMP signaling pathway in the association between statins and colon cancer mortality, comparison with other studies is not possible. These results require confirmation in further cohorts.

There are several limitations to the study. This is a retrospective study in which randomization was not possible. Immortal time bias was avoided by using a time dependent model, but confounding by disease progression may still occur. Next to a parametric survival model with exponential (Poisson) distribution, a Cox proportional hazard model was used with statin use as a time varying covariate to confirm the analyses. Our methodology for defining user and non-user time will minimize confounding by disease progression. This can arise where poor prognosis or advanced disease influences statin usage with statins being stopped in these patients potentially leading to a spuriously lower number of deaths in

statin users. This depends on how 'statin user' is defined and how statin user time is accrued. In our study user time is accrued after the first prescription, even if a patient subsequently stopped using statins, effectively avoiding this bias.
Reverse causation was further addressed by applying a lag of exposure of 6 months which also resulted in a significant difference.
Because reduced risk of death could potentially be explained by reductions in cardiovascular events in statin users we also analyzed risk of death from any cause. Statin use can also be a sign of compliance. Consistent statin use could therefore be a surrogate marker for health consciousness, which can cause a 'healthy user bias' in studies like ours. We observed similar effects of aspirin and statins on colon cancer mortality implying that both medications are protective or that a hidden factor connecting aspirin and statins is responsible. However, analyses with ACE inhibitors and benzodiazepines proved that this was not the case.
There were differences in baseline characteristics between statin users and statin non-users that could have confounded our results; statin users were more often male, were older, had more comorbidity, and had earlier stage tumors. However, when adjusting for these confounders, a clear association between statin use and a better prognosis remained. However, residual confounding by unknown factors could still influence the results. Lastly, the assessment of active BMP signaling is not perfect. The fact that all the components of BMP signaling are present or the fact one or more components are missing does not necessarily mean that there is or isn't active BMP signaling. Because of the complex matrix of downstream signaling targets, that can be activated or inhibited by canonical and/or non canonical BMP signaling, it is difficult to find a suitable (surrogate marker) for BMP signaling activity. Phospho-SMAD1,5 or 8 are commonly used *in vitro* to determine BMP signaling activity, but phospho-proteins are very sensitive to tissue processing and fixation, making the assessment of expression levels in human tissue inaccurate.[40]
In another study using human colon cancer tissue we found nuclear p-SMAD1,5 or 8 without the presence of SMAD4; which is thought to be necessary for nuclear translocation of p-SMAD1,5, or 8. We can't evaluate whether this is the result of tissue processing or SMAD4 independent non canonical BMP signaling. We do know that loss of SMAD4 in colon cancer is associated with a poor patient survival and negative nuclear p-SMAD1,5, or 8 staining is not.[39]

In conclusion, in our cohort statin use after diagnosis is associated with a survival benefit in patients with colon cancer. More importantly, this survival benefit was most pronounced in patients whose tumors retain expression of BMP signaling pathway components. In the future, BMP signaling functionality may serve as a predictive biomarker to select patients for adjuvant statin therapy. However, our data are preliminary and other studies, preferably randomized clinical trials, are needed to confirm the beneficial effects of statins on colon cancer survival and BMP signaling as a predictive biomarker.

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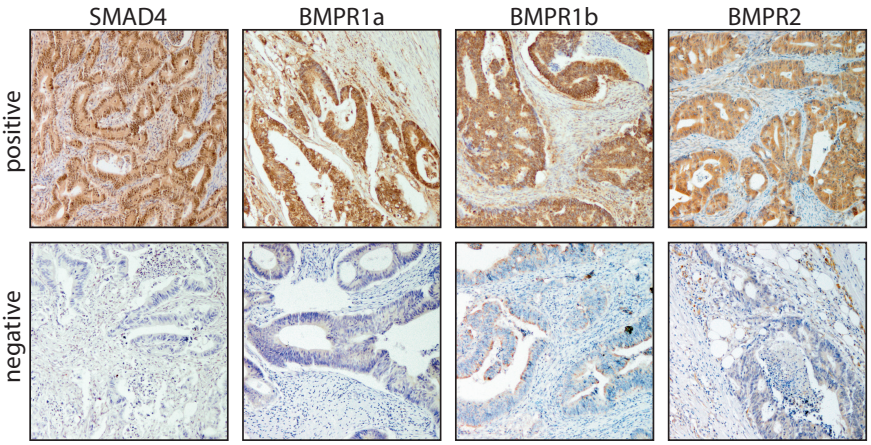
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Supplementary table 1. Rate Ratio for cancer specific mortality (time-dependent analysis) for variables other that statin use.

Multivariable model	Reference category	Rate Ratio	p-value
Sex	1.0 (male)	0.84 (0.67-1.04)	0.10
Age	1.0 (continuous)	1.04 (1.03-1.06)	<0.001
Comorbidity	1.0 (no comorbidity)	1.39 (1.10-1.77)	0.007
Incidence year	1.0 (continuous)	1.02 (0.95-1.08)	0.60
Grade	1.0 (grade I)	1.21 (0.80-1.84) grade II	<0.001
		2.29 (1.47-3.57) grade III	
		1.21 (0.70-2.08) unknown grade	
Stage	1.0 (stage I)	1.53 (0.94-2.51) stage II	<0.001
		3.80 (2.31-6.27) stage III	
		22.93 (13.93-37.74) stage IV	
		3.15 (0.42-23.89) unknown stage	
Microsatellite status	1.0 (negative)	0.93 (0.63-1.37)	0.71
Chemotherapy	1.0 (no)	0.69 (0.52-0.90)	0.007
Aspirin use	1.0 (no)	0.69 (0.50-0.95)	0.02

Supplementary figure 1



Supplementary Table 2. Baseline Characteristics of the Colon Cancer Patients according to *KRAS* and Use of Statin after Diagnosis
Overall statin use: 210 patients (21.0%).

	All patients (N=999)		***KRAS* Wild-Type (N=422)**			***KRAS* Mutation (N=230)**	
		No statin	Statin	P-value	No statin	Statin	P-value
Sex							
Male	505 (50.6)	151 (47.6)	67 (63.8)	0.004	95 (54.9)	39 (68.4)	0.073
Female	494 (49.4)	166 (52.4)	38 (36.2)		78 (45.1)	18 (31.6)	
Age							
<65	342 (34.2)	100 (31.6)	20 (19.1)	<0.001	51 (29.5)	20 (35.1)	0.190
66-74	304 (30.4)	82 (25.9)	53 (50.5)		47 (27.2)	20 (35.1)	
75 and older	353 (35.4)	135 (42.6)	32 (30.5)		75 (43.4)	17 (29.8)	
Year of diagnosis							
2002-2004	451 (45.2)	159 (50.2)	49 (46.7)	0.535	80 (46.2)	25 (43.9)	0.754
2005-2007	548 (54.8)	158 (49.8)	56 (53.3)		93 (53.8)	32 (56.1)	
Disease stage							
I	138 (13.8)	41 (12.9)	25 (23.8)	0.05	26 (15.0)	10 (17.5)	0.291
II	402 (40.2)	132 (41.6)	44 (41.9)		63 (36.4)	22 (38.6)	
III	287 (28.7)	93 (29.3)	22 (21.0)		54 (31.2)	21 (36.8)	
IV	169 (16.9)	50 (15.8)	13 (12.4)		30 (17.3)	4 (7.0)	
Unknown	3 (0.3)	1 (0.3)	1 (1.0)				
Comorbidity							
No	443 (44.3)	153 (48.3)	16 (15.2)	<0·001	82 (47.4)	16 (28.1)	0.010
Yes	556 (55.7)	164 (51.7)	89 (84.8)		91 (52.6)	41 (71.9)	
Microsatellite status							
MSI	90 (9.0)	34 (10.7)	14 (13.3)	0.750	11 (6.4)	4 (7.0)	0.682
MSS	870 (87.1)	269 (84.9)	86 (81.9)		157 (90.8)	50 (87.7)	
Unknown	39 (3.9)	14 (4.4)	5 (4.8)		5 (2.9)	3 (5.3)	

Supplementary Table 3 Baseline Characteristics of the Colon Cancer Patients according to intact BMP signaling and Use of Statin after Diagnosis

	All patients (N=999)	**Non-intact BMP signaling (N=519)**			**Intact BMP signaling (N=457)**		
		No statin	Statin	P-value	No statin	Statin	P-value
Sex							
Male	505 (50.6)	195 (49.0)	73 (60.3)	0.029	178 (47.3)	48 (59.3)	0.052
Female	494 (49.4)	203 (51.0)	48 (39.7)		198 (52.7)	33 (40.7)	
Age							
<65	342 (34.2)	137 (34.4)	41 (33.9)	0.001	141 (37.5)	18 (22.2)	<0.001
66-74	304 (30.4)	106 (29.6)	51 (42.2)		100 (26.6)	40 (49.4)	
75 and older	353 (35.4)	155 (38.9)	29 (24.0)		135 (35.9)	23 (28.4)	
Year of diagnosis							
2002-2004	451 (45.2)	188 (47.2)	56 (46.3)	0.854	155 (41.2)	36 (44.4)	0.594
2005-2007	548 (54.8)	210 (52.8)	65 (53.7)		221 (58.8)	45 (55.6)	
Disease stage							
I	138 (13.8)	44 (11.1)	20 (16.5)	0.055	52 (13.8)	17 (21.0)	0.012
II	402 (40.2)	138 (34.7)	53 (43.8)		168 (44.7)	36 (44.4)	
III	287 (28.7)	128 (32.2)	32 (26.5)		96 (25.5)	23 (28.4)	
IV	169 (16.9)	86 (21.6)	16 (13.2)		60 (16.0)	4 (4.9)	
Unknown	3 (0.3)	2 (0.5)				1 (1.2)	
Comorbidity							
No	443 (44.3)	205 (51.5)	26 (21.5)	<0.001	188 (50.0)	16 (19.8)	<0.001
Yes	556 (55.7)	193 (48.5)	95 (78.5)		188 (50.0)	65 (80.3)	
Microsatellite status							
MSI	90 (9.0)	34 (8.5)	10 (8.3)	0.425	35 (9.3)	10 (12.4)	0.628
MSS	870 (87.1)	356 (89.5)	106 (87.6)		333 (88.6)	70 (86.4)	
Unknown	39 (3.9)	8 (2.0)	5 (4.1)		8 (2.1)	1 (1.2)	

Supplementary table 4 Time-Dependent Analysis Overall Mortality, according to Tumor KRAS mutation status, BMP signaling pathway status, and Use or Non-use of Statin after Diagnosis

	Statin non-users	Statin users**	*p*-value
All Cancers			
Patients	789	210	
Deaths	396	69	
Adjusted Rate Ratio (95%CI)*†	1.0 (reference)	0.67 (0.51-0.87)	**0.003**
Adjusted Hazard Ratio (95%CI)*§	1.0 (reference)	0.68 (0.53-0.90)	**0.007**
KRAS Wild-type cancers			
Patients	317	105	
Deaths	162	40	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.81 (0.56-1.18)	0.273
Adjusted Hazard Ratio (95%CI)*§	1.0 (reference)	0.85 (0.59-1.23)	0.390
KRAS Mutant cancers			
Patients	173	57	
Deaths	89	18	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.59 (0.35-1.03)	0.062
Adjusted Hazard Ratio (95%CI)*§	1.0 (reference)	0.59 (0.34-1.03)	0.065
Intact BMP signaling			
Patients	376	81	
Deaths	158	24	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.46 (0.29-0.74)	**0.001**
Adjusted Hazard Ratio (95%CI)*§	1.0 (reference)	0.49 (0.31-0.79)	**0.003**
Non-intact BMP signaling			
Patients	398	121	
Deaths	229	42	
Adjusted Rate Ratio (95%CI)* †	1.0 (reference)	0.75 (0.53-1.06)	0.106
Adjusted Hazard Ratio (95%CI)*§	1.0 (reference)	0.78 (0.55-1.11)	0.169

*Adjusted for sex, age, comorbidity, year of incidence, histological grade, stage, microsatellite status and chemotherapy, ** Statin use after diagnosis

† Poisson model

§ Cox model

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.

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Nederlandse samenvatting

Van een goede balans naar kanker

De darm is een groot orgaan en bestaat uit de dunne darm en de dikke darm. De dunne darm is ongeveer 6-7 meter lang en is onder andere belangrijk voor het verteren en opnemen van voedsel. Om dit mogelijk te maken bestaat de dunne darm uit allerlei vouwen om het oppervlak te vergroten. Daarnaast is de binnenkant bekleed met villi, een soort vingerachtige uitsteeksels, waardoor het totale contactoppervlak tussen de 150-200 m^2 ligt. De dikke darm (colon) heeft deze villi niet en is voornamelijk verantwoordelijk voor absorptie van water. De binnenbekleding van de darm, ook wel intestinaal epitheel genoemd, vernieuwt zich in een rap tempo. Zoals eerdergenoemd bestaat het intestinaal epitheel in de dunne darm uit villi. Tussen de villi zitten crypten; dit zijn een soort kommetjes waar stamcellen in zitten. Deze stamcellen delen zich en de nieuwe cellen worden omhooggeduwd naar de transit amplifying zone. Dit is een zone onderaan de villi waar veel celdelingen plaatsvinden. De nieuwe cellen gaan omhoog langs de villi en zullen uiteindelijk in apoptose (geprogrammeerde celdood) gaan zodra ze op de top van de villi zijn. Dit is een continu proces wat zorgt voor snelle vernieuwing van het hele epitheel. De reis van een nieuwe cel in de crypt tot aan apoptose boven aan de villi duurt ongeveer 5 dagen. Het colon heeft geen villi, maar het principe van een stamcelcompartiment onderin de crypt waar nieuwe cellen ontstaan met migratie naar boven is hetzelfde.

Stamcellen kunnen in principe worden wat ze willen. Maar hoe weet een cel dat het een stamcel moet blijven, of dat het een ander soort cel, een gedifferentieerde cel, moet worden? Hoe weten de cellen of ze onderin de crypt moeten blijven of moeten migreren naar boven? En hoe weten de cellen dat ze bovenin het programma 'celdood' aan moeten zetten, afscheid moeten nemen van het darmepitheel en zo uiteindelijk in de ontlasting terecht komen? We weten nog lang niet alles van dit proces, en we zullen hier nog veel ontdekkingen doen de komende jaren, maar we hebben een basaal inzicht in het systeem. Cellen staan onder invloed van signaaltransductie paden. Deze signaaltransductie paden kunnen allerlei effecten hebben; bijvoorbeeld verandering in een ander type cel (differentiatie), celdeling (proliferatie), verplaatsing van de cel (migratie) en celdood (apoptose). Dit gebeurt via morphogenen. Een morphogen is een signaal molecuul wat ergens door een bron geproduceerd wordt en vervolgens door het omgevende weefsel diffundeert. Op deze manier ontstaat er een gradiënt, waarbij de concentratie dicht bij de bron

hoog is en langzaam afneemt naar mate de afstand tot de bron groter wordt. Op basis van de concentratie zijn er bepaalde drempels waardoor er een effect op de cel optreedt.

Signaaltransductie is een methode van het lichaam om een heleboel systemen in het lichaam te regelen. Zo 'weten' je botten bijvoorbeeld wanneer ze moeten stoppen met groeien. Er zijn veel verschillende signaaltransductie paden, maar voor deze thesis wil ik er twee uitlichten. Dit zijn het WNT-signaal pad en het BMP (Bot Morfogenetisch Proteïne) signaal pad. WNT-signalering is actief onderin de crypt en zorgt voor celdeling. Daarnaast zorgt WNT ervoor dat de stamcellen niet differentiëren en gewoon stamcellen blijven. De nieuwe cellen gaan vervolgens omhoog en komen onder invloed van het BMP-signaal pad. BMP zorgt eerst voor differentiatie en later, als de cellen bovenaan de villus zijn, voor apoptose. De WNT en BMP-signaalpaden zijn in balans en hebben invloed op elkaar. Zo zorgt WNT-signalering voor onderdrukking van BMP, en BMP zorgt voor onderdrukking van WNT. Zolang deze twee signaal paden in balans (homeostase) zijn, is er geen probleem, maar het is goed voor te stellen dat als BMP ineens niet meer goed werkt, de cellen niet meer doodgaan en zich ophopen. Hetzelfde geldt voor WNT; als deze signalering te hard werkt ontstaan er te veel nieuwe cellen. Op deze manier kan er uiteindelijk kanker ontstaan. Er kunnen op allerlei niveaus fouten ontstaan in dit systeem. Zo kan het onder andere misgaan in de productie van de morphogenen, de ontvangers van de signalen (receptoren) en het signaaltransductie pad in de cel. Het signaaltransductie pad in de cel is een cascade van eiwitten die het signaal doorgeven vanaf de ontvangst (receptor) tot aan het DNA, alwaar vervolgens bepaalde genen tot expressie komen. Fouten in signaalpaden zijn een van de mogelijke oorzaken van kanker. In een orgaan als de darm waar het epitheel zo snel wordt vernieuwd is er sprake van een hoge mate van celdeling, en waar cellen delen worden ook fouten gemaakt. Zo kunnen fouten ontstaan in het BMP en WNT pad, maar ook in veel andere processen. Het is niet voor niets dat darmkanker een veel voorkomende ziekte is. In 2018 zijn er in Nederland ongeveer 14.100 nieuwe patiënten met dikke darmkanker gediagnosticeerd (www.volksgezondheidenzorg.info).

In deze thesis hebben we geprobeerd meer inzicht te krijgen in hoe het BMP-signaal pad werkt en wat de rol is in het ontstaan en de progressie van darmkanker. We

weten dat bepaalde erfelijke vormen van darmkanker kiembaan mutaties hebben in onderdelen van het BMP-signaal pad. Dit suggereert dat BMP inderdaad belangrijk is in het behoud van een normale darm.

Hoofdstuk 2: BMP-signaalpad in de normale darm

We hebben gekeken naar de effecten van een te hardwerkend BMP-signaalpad op de crypten en villi in de darm. Er was al eerder gekeken naar wat er zou gebeuren als het BMP-signaalpad niet meer goed werkt. In verschillende muisstudies werd BMP geïnactiveerd door bijvoorbeeld een BMP-receptor te verwijderen of door een stofje (Noggin) te introduceren dat BMP onderdrukt. Als gevolg hiervan ontstonden poliepen in de darmen van deze muizen. Poliepen zijn een soort uitstulpingen in de darm welke kunnen ontaarden in kanker. Wij deden het tegenovergestelde in een muismodel, namelijk overactivatie van BMP door middel van een constant geactiveerde BMPreceptor. Hierbij zagen we forse veranderingen in het epitheel. Het leek totaal niet meer op de bekende crypt-villus structuur. Opvallend was dat de darm zich 6 maanden later volledig had hersteld. We snappen deze effecten nog niet volledig. Direct na inductie (=activatie) zien we verlies van stamcellen; deze conclusie hebben we getrokken doordat er geen expressie meer is van OLFM4 (een stamcel marker). Het ontbreken van stamcellen zou moeten betekenen dat er geen nieuwe cellen zouden kunnen ontstaan. Vijf dagen later is OLFM4 er echter ineens weer. We hebben hiervoor verschillende theorieën. Inductie, en dus activatie van BMP, is niet in alle cellen gebeurd, waardoor een zeer klein aantal cellen gezorgd heeft voor volledige repopulatie. Dit lijkt gezien het drastische beeld onwaarschijnlijk, maar is niet onmogelijk. Een andere optie is dat sommige gedifferentieerde cellen weer stamcellen worden en zo opnieuw beginnen met het creëren van crypten en villi; ze de-differentiëren. Verder onderzoek is nodig om dit beeld te kunnen verklaren. We zijn nog aan het onderzoeken hoe we activatie van BMP kunnen gebruiken om poliepvorming tegen te gaan. Dit doen we door het overactivatiemodel te combineren met verschillende darmkanker modellen in muizen. Mogelijk dat de 'reset' die lijkt te ontstaan door BMP-activatie een gunstig effect heeft.

Hoofdstuk 3: Een alternatief BMP-signaalpad

Het BMP signaalpad wordt beschouwd als een signaalpad dat beschermd tegen

kanker doordat het zorgt voor differentiatie van cellen en voor apoptose. Het wordt gezien als tumor suppressor, in tegenstelling tot tumor promotors, die juist zorgen voor kanker progressie. Het BMP signaalpad wordt geactiveerd door BMPs die zich hechten aan BMP-receptoren. Vervolgens worden de BMP-receptoren geactiveerd via phosphorylatie (het binden van een fosfaatgroep). Daarna wordt het signaal doorgegeven aan SMAD 1,5,8 wat ook wordt geactiveerd door phosphorylatie. SMAD1,5,8 vormt een complex met SMAD4 en kan zo naar de celkern gaan voor transcriptie van specifieke genen. Transcriptie is het proces waarbij DNA wordt vertaald in mRNA, een tussenstap in de uiteindelijke productie van eiwitten. Het signaal pad via SMAD4 is het normale signaal pad van BMP, hetgeen we het canonical (standaard) BMP-pad of 'SMAD4 afhankelijk BMP-pad' noemen.

In 40-60% van de sporadische darmkankers wordt verlies van SMAD4 gevonden. Verlies van SMAD4 in darmkanker is geassocieerd met een slechtere overleving. Er werd gedacht dat dit kwam door het verlies van de tumor onderdrukkende functies van BMP. Wij hebben ontdekt dat bij het verlies van SMAD4, BMP switched van een tumor suppressor naar een tumor promotor. Activatie van BMP zonder SMAD4 hebben we het non-canonical BMP-pad of 'SMAD4 onafhankelijke BMP pad' genoemd. Dit SMAD4 onafhankelijke BMP-pad heeft invloed op de tumor progressie en zorgt voor toename van metastasering.

We hebben gevonden dat ROCK hierbij een belangrijke rol speelt. Het ROCK-pad heeft invloed op de opmaak van het skelet van een cel. Tevens kan ROCK het cell skelet veranderen. Door deze verandering kan een cel bijvoorbeeld bewegen. Activatie van ROCK resulteert in een toename van beweging van (kanker)cellen waardoor deze het omgevende weefsel kunnen invaseren om uiteindelijk te kunnen metastaseren. We hebben deze processen bekeken in kankercellijnen en een metastase muismodel en zien dat activatie van het SMAD4 onafhankelijke BMP-pad zorgt voor een toename van metastasen. We zien ook dat dit effect opgeheven kan worden door toevoeging van een ROCK-inhibitor (Fasudil). ROCK zou een potentieel doel voor behandeling kunnen zijn. ROCK-inhibitoren worden reeds gebruikt bij pulmonale hypertensie en cerebrale vasospasme. Als kanttekening moet vermeld worden dat ROCK-inhibitie bij één studie heeft geleid tot ontwikkelingsdefecten in muisembryo's.

We hebben ook naar de rol van het SMAD4 onafhankelijk BMP-pad in mensen gekeken. De aanwezigheid van BMP-receptoren in SMAD4 negatieve kankers

is in ons cohort van humane colorectaal kankers geassocieerd met een slechtere overleving. Als zowel BMP-receptoren negatief zijn als SMAD4, dan is de overleving gelijk aan SMAD4 positieve kankers.

Hoofdstuk 4: De ß-catenine paradox

In dit hoofdstuk onderzoeken we de interactie tussen WNT en BMP-signalering. Een opvallende observatie is een verhoogd WNT-signaal aan de rand van de tumor met tegelijk een laag WNT-signaal in het centrum. Mutaties in het WNT-signaal zijn veel voorkomend in darmkanker; bijvoorbeeld door een APC of beta-catenine mutatie. Deze mutaties zijn vaak gelijk in alle tumorcellen terwijl er dus wel een verschillend niveau van activiteit is. Er zijn dus mogelijk andere factoren die de WNT-activiteit bepalen. We hebben eerst gekeken naar andere factoren in de kankercellen zelf. Hiervoor hebben we gekozen voor SMAD4 en p53. SMAD4 vanwege de eerdergenoemde interactie tussen BMP en WNT, en vanwege het vele voorkomen van SMAD4 verlies in darmkanker. P53 werd gekozen vanwege een eerder gepubliceerd effect van p53 mutaties op WNT-activiteit. Om dit te onderzoeken hebben we naar WNT-activiteit, in de vorm beta-catenine expressie, en naar p53 en SMAD4 expressie gekeken in humaan weefsel van darmkanker. We hebben ingezoomd op het invasieve front; de plek waar de kanker door het omliggende weefsel groeit. Hierbij zagen we dat zowel verlies van SMAD4 als afwijkende p53 expressie geassocieerd is met hoge WNT-activiteit in het invasieve front. SMAD4 onafhankelijke BMP-signalering is dus belangrijk voor invasie en het ontstaan van metastasen, zoals we zagen in hoofdstuk 3, en moduleert WNT-activiteit in het invasieve front. Onze volgende vraag was in hoeverre factoren in het stroma, dit zijn de omliggende celstructuren, invloed hebben op de kankercellen. Zorgt mogelijk niet alleen de kanker zelf voor progressie, maar speelt de omgeving ook een rol? En wat is de functie van BMP-signalering in deze mogelijk tumor-stroma interactie.

Hoofdstuk 5: De invloed van stroma

In dit hoofdstuk vragen we ons af of het stroma een bron kan zijn van BMPs waardoor SMAD4 onafhankelijke BMP-signalering onder andere aan het invasieve front wordt geactiveerd. BMPs zijn morphogenen die BMP-signalering kunnen activeren. Eerdere studies lieten zien dat de aanwezigheid van een grote

hoeveelheid stroma zorgt voor een slechtere overleving. Opvallend was hierbij dat dit voornamelijk het geval was bij tumoren waarbij SMAD4 niet aanwezig was. Wij vroegen ons af waarom dit zo is.

Stroma bestaat uit allerlei verschillende celtypen. Het meest voorkomende celtype in het stroma is de fibroblast. Daarom hebben we gekeken naar de interactie tussen darmkanker cellen en fibroblasten. Door gebruik te maken van *in vitro* invasie assays vonden we dat SMAD4 negatieve kankercellen sneller naar fibroblasten toe migreren. Mogelijk produceren fibroblasten iets wat voor migratie van kankercellen zorgt. Dit werd ondersteund doordat we dit experiment herhaald hebben met geconditioneerd medium van fibroblasten; dat is oud medium waarin de fibroblasten enige tijd hebben gezeten. Hierbij zagen we hetzelfde effect; SMAD4 negatieve kankercellen migreren naar het geconditioneerde medium van fibroblasten. Vervolgens hebben we het effect van kankercellen op fibroblasten onderzocht. Wanneer fibroblasten in contact komen met SMAD4 negatieve kankercellen dan produceren ze meer BMP2. BMP2 is een van de BMPs die de signalering kan activeren. Onze theorie is dat fibroblasten uit het stroma BMP2 produceren wanneer ze in de buurt zijn van SMAD4 negatieve kankercellen. BMP2 activeert vervolgens het SMAD4 onafhankelijk BMP-pad in deze kankercellen, wat zorgt voor meer migratie, invasie en uiteindelijk metastasen van de kankercellen. Dit hebben we vertaalt naar de kliniek door te bewijzen dat de aanwezigheid van BMP2 in het stroma van colorectaal kanker is geassocieerd met een slechtere overleving in SMAD4 negatieve kankers.

Hoofdstuk 6 en 7: Het voorspellen van overleving

Naast *in vitro* en *in vivo* studies hebben we ons ook bezig gehouden met het vinden van prognostische markers gebruik makend van het BMP-signaalpad. Voorspelling van patiënt overleving speelt een belangrijke rol in behandel beslissingen. De voorspellende waarde van het verlies van SMAD4 verschilt echter per studie. In hoofdstuk 6 hebben we deze studies samengenomen in een meta-analyse waarbij we zien dat de mate van SMAD4 expressie een goede voorspeller van overleving is. Opvallend was dat elke studie een eigen scorings methode had om te bepalen wat positief en negatief was. In hoofdstuk 7 hebben we gekeken of geactiveerd SMAD1,5,8 en SMAD2,3 ook een voorspellende waarde hebben. We hebben phospho-SMADs (geactiveerde SMADs) gekleurd in humaan

colorectaal weefsel. Nucleaire expressie van pSMAD1,5,8 en pSMAD2,3 is een teken van respectievelijk geactiveerde BMP-signalering en geactiveerde TGFbeta signalering. We vonden echter geen associatie tussen nucleaire pSMAD expressie in darmkanker en de overleving van patiënten. Waarom het verlies van SMAD4 wel geassocieerd is met een slechtere overleving en verlies van nucleair p-SMAD niet, kan verschillende redenen hebben. SMAD4 verlies zorgt voor uitschakeling van zowel BMP als TGFbeta. Nucleair phospho-SMADs betekent activatie van de canonical signaalpaden. Mogelijk heeft SMAD4 verlies nog invloed op andere paden. Daarnaast is de phospho kleuring niet zo robuust; het kunnen dus op technisch vlak geen ideale markers zijn.

Hoofdstuk 8: Een uitstapje naar alvleesklierkanker

Alvleesklierkanker heeft een zeer matige overleving met een 5 jaarsoverleving van ongeveer 4% (afhankelijk naar welke studie je kijkt). Dit komt deels doordat ten tijde van de diagnose er al lokale invasie of metastasen op afstand zijn waardoor een operatie vaak niet mogelijk is. Verder is de respons op chemotherapie beperkt. Ook in alvleesklierkanker komt inactivatie van SMAD4 voor en is dit geassocieerd met een slechtere overleving. Om die reden hebben we een exploratieve studie gedaan naar de rol van het BMP-pad in alvleesklierkanker. We zagen dat in 53,7% van de gevallen er een verminderde expressie is van BMP-receptor 1A en dat geassocieerd is met een slechtere overleving in patiënten waarbij SMAD4 nog intact is. Vervolgens hebben we BMPR-receptor 1A expressie *in vitro* gemanipuleerd waarbij vermindering van de expressie zorgde voor meer celdeling (proliferatie) en meer invasie. In een volgende studie willen we kijken hoe we het BMP kunnen gebruiken als doel van behandeling van alvleesklierkanker (niet in deze thesis).

Hoofdstuk 9: Uit de oude doos, statines!

In hoofdstuk 9 presenteren we onze laatste studie van de thesis. Hier hebben we niet gekeken naar directe manipulatie van het BMP-signaalpad, maar naar statines. In eerdere studies hebben we darmkanker cellen behandeld met statines en zagen toen activatie van het BMP signaalpad. Aangezien statines al jarenlang relatief veilig worden gebruikt voor vermindering van cardiovasculair risico, is het een ideaal middel. Het voordeel is dat het bijwerkingspatroon uitgebreid bekend is. Een ander voordeel is dat vanwege wijdverspreid gebruik er retrospectieve

studies mogelijk zijn. Er zijn al veel studies geweest naar het gebruik van statines als chemopreventie (het voorkomen van kanker) met zeer wisselende resultaten. Er is veel minder onderzoek gedaan naar het gebruik van statines als behandeling voor kanker.
Wij hebben een grote observationele studie gedaan waarbij we hebben gekeken naar statine gebruik na de diagnose darmkanker en hierbij zagen we een associatie met een verminderd risico op overlijden. Als we kijken naar onze *in vitro* data zien we dat statines BMP activeren, maar alleen als SMAD4 aanwezig is. Statines zouden dus theoretisch alleen hun werk kunnen doen in SMAD4 positieve kankers. Dit is ook wat wij vonden in ons cohort. Als de patiënten werden verdeeld in SMAD4 positief en SMAD4 negatief, dan hadden statines alleen een positief effect op de overleving bij patiënten met SMAD4 positieve darmkanker. Opgemerkt moet worden dat er gerandomiseerde studies nodig zijn om dit te bevestigen.

Concluderend, met deze thesis zijn we iets meer te weten gekomen over de rol van BMP-signalering in de darm en in de ontwikkeling van darmkanker. Verder hebben we gepoogd nieuwe prognostische markers te vinden of bekende markers te optimaliseren. Als laatste hebben we gekeken naar mogelijkheden om het BMP-pad te gebruiken als doel van kankerbehandeling in de vorm van BMP-inhibitie, BMP-activatie, ROCK-inhibitie en statine behandeling,

List of abbreviations

5-FU	Fluorouracil
AKT (PKB)	Protein Kinase B
APC	Adenomatous Polyposis Coli
ATP	Adenosine Triphosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate p-toluide salt
BMP	Bone Morphogenetic Protein
BMPR	Bone Morphogenetic Protein Receptor
BRDU	5-BRomo-2'-DeoxyUridine
BRE	BMP Responsive Element
BSA	Bovine Serum Albumin
CAG	Chicken Beta-actin Promotor
CAII	Carbonic Anhydrase II
CD1	Cluster of Differentation 1
CDH1	gene coding for Ecadherin
CIMP	CpG Island Methylator Phenotype
CMFDA	5-ChloroMethylFluorescein DiAcetate
CRC	Colorectal Cancer
CSC	Cancer Stem Cells
CTNNB1	gene coding for β-catenin
CYP	Cytochrome P450
DAB	Diaminobenzidine
DCC	Deleted in Colorectal Cancer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	EthyleneDiamineTetraacetic Acid
EGTA	EthyleneGlycol-bis(2-aminoethylether)-N,N,N',N'-Tetraacetic Acid
EMT	Epithelial-to-Mesenchymal Transition
ERK	Extracellular Signal-Regulated Kinase
FCS	Fetal Calf Serum
FEG	Field Emission Gun
G418	Geneticin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GREM1	Gremlin1
GSK	Glycogen Synthese Kinase
HA-tag	HemAgglutinin Tag
HRP	HorseRadish Peroxidase
IC50	half maximal inhibitory concentration
ip	intraperitoneal injection
IRES	Internal Ribosome Entry Site

ITO	Indium Tin Oxide
JNK	c-Jun N-Terminal kinase
JP	Juvenile Polyposis
LacZ	gene coding for Beta-galactosidase
LIMK	LIM Kinase
LUC	LUCiferase
MAPK	Mitogen-Activated Protein Kinase
MKK	Mitogen-Activated Protein Kinase Kinase
mRNA	messengerRNA
NaCl	NatriumChloride
NBT	Nitro Blue Tetrazolium
OLFM4	OLFactoMedin 4
PBS	Phosphate Buffered Saline
pcDNA	protamine complementary DNA
PCR	Polymerase Chain Reaction
qPCR	quantative PCR
RNA	RiboNucleic Acid
ROCK	Rho-associated Protein Kinase
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
SEM	Standard Error of the Mean
shRNA	short hairpin RNA
SIP1	SMN Interacting Protein 1
siRNA	small interfering RNA
SMAD	Small MOthers against Decapentaplegic4
SNP	Single Nucleotide Polymorphism
SSC	Saline Sodium Citrate
TBS	Tris Buffered Saline
TENG-T	10 mM Tris, 5 mM EDTA, 0.15 mM NaCl, 0.25% gelatin, 0.05% Tween 20
TGFβ	Transforming Growth Factor-β
TMA	Tissue Microarray
VIM	gene coding for Vimentin
ZEB2	Zinc Finger E-Box Binding Homeobox 2

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Poster presentations

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Digestive Disease Week, May 3-6 2014, Chicago, IL, USA; *Fibroblasts promote invasion in SMAD4 negative colorectal cancers by producing BMP-2*

United European Gastroenterology Week, October, 2013, Berlin, Germany; *The Bone Morphogenetic Protein Pathway either enhances or inhibits the Wnt pathway depending on SMAD4 and p53 status in colorectal cancer.*

Digestive Disease Week, May 18-21, 2013, Orlando, Fl, USA; *Loss of Bone Morphogenetic Protein Receptor IA predicts poor survival in patients with pancreatic cancer and is responsible for invasiveness and proliferation of cancer cells.*

United European Gastroenterology Week, October 20-24, 2012, Amsterdam, the Netherlands; *Loss of Bone Morphogenetic Protein Receptor 1a predicts poor survival in patients with pancreatic cancer and is responsible for invasiveness and proliferation of cancer cells.*

Digestive Disease Week, May 7-10, 2011, Chicago, IL, USA; *Non-Canonical Bone Morphogenetic Protein Signaling induces Epithelial to Mesenchymal transition in SMAD4 deficient colorectal cancers.*

BMP congress, September 15-18 2010, Leuven, Belgium; *The Role of Bone Morphogenetic Protein Signaling and Transforming Growth Factor β Signaling and their Components in Colorectal Cancer.*

Oral presentations

Digestive Disease Week, May 3-6, 2014, Chicago, IL, USA; *Conditional activation of BMP signalling leads to loss of the stem cell compartment.*

Spring meeting Dutch Gastroenterology Society (NVGE), March 2014, Veldhoven, the Netherlands; *Fibroblasts promote invasion in SMAD4 negative colorectal cancers by producing BMP-2*

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Spring meeting Dutch Gastroenterology Society (NVGE), March 17, 2011, Veldhoven, the Netherlands; *Non-canonical Bone Morphogenetic Protein Signaling induces epithelial to mesenchymal transition in SMAD4 deficient colorectal cancers.*

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Curriculum vitae

Philip Voorneveld was born on October 4th 1984 in Dordrecht, the Netherlands. After graduating from Dalton Lyceum in 2003, he started his medical study at Leiden University. In 2007 he did a scientific internship at the department of gastroenterology; there he developed an interest in both science and gastroenterology. He finished medical school in 2010. He then began his PhD at the laboratory of the department of gastroenterology at the Leiden University Hospital, under the guidance of James Hardwick, working on cell signaling and colon cancer cell behaviour.
In 2015, together with his girlfriend, he set sail for what became a 16 month sailing trip including two Atlantic Crossings. 2016 marked the start of his gastroenterology residency, with the first two years Internal Medicine and 1 year gastroenterology at the Haaglanden Medical Center, the Hague. Currently he is continuing his gastroenterology residency at the Leiden University Hospital.

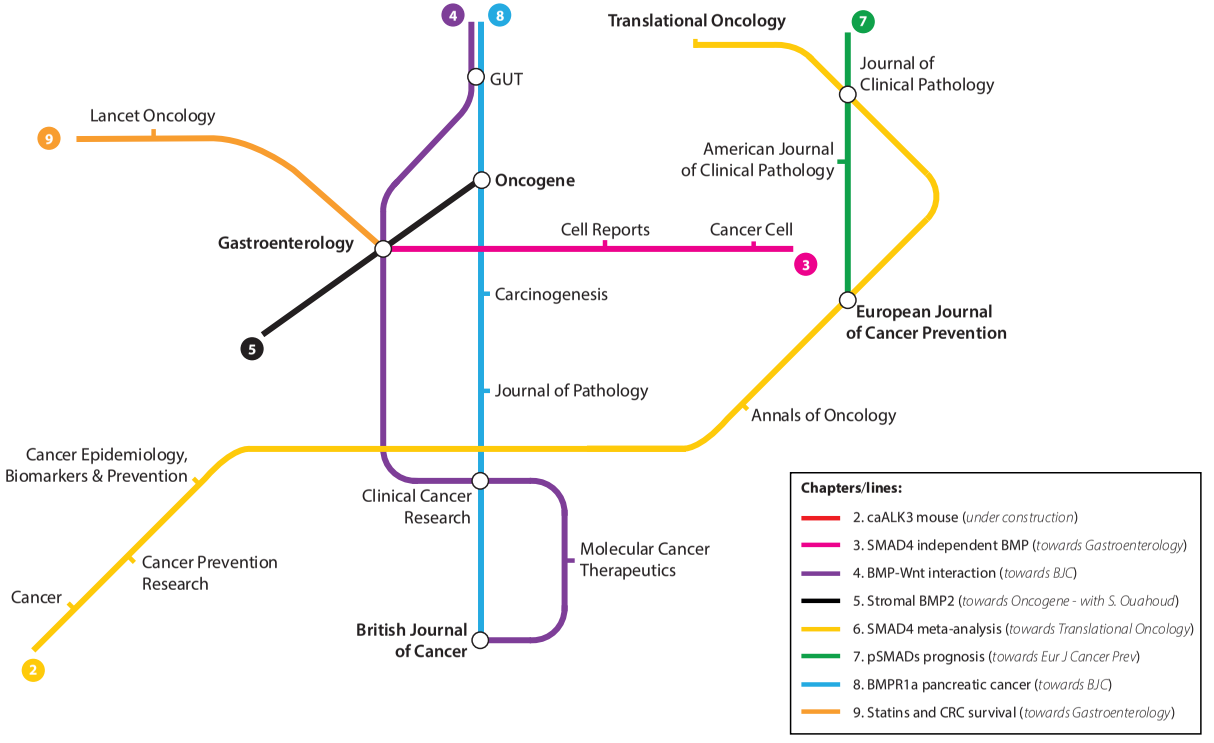
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PhD Map


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