The role of the bone morphogenetic protein pathway in colorectal cancer

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Cover: Kazimir Malevich, ***Black Square*** (1913), Oil on canvas, State Russian Museum, St. Petersburg

This picture of famous Belorussian painter and designer become the turning point in the development of Russian avant-garde. *Black Square* against white background became the symbol, the basic element in the system of the art of suprematism, the step into the new art.

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for my mother

The Role of the Bone Morphogenetic Protein Pathway in Colorectal Cancer

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

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ten overstaan van een door het college voor promoties

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Contents

Chapter 1

General introduction

Chapter 1

Human large bowel epithelial tissue is particularly prone to cancer. At least 5 % of the Western population will develop a colorectal malignancy. As a result colorectal cancer (CRC) is the second leading cause of cancer death in the United States and Europe [1]. The transformation of colon epithelial cells to cancer follows a predictable progression of histological and cytological changes which are the results of genetic and epigenetic alterations occurring during the adenoma-carcinoma sequence of colon cancer formation[2]. Accumulated genetic changes and the selection of advantageous mutations underlie the development of neoplasia and drive the initiation and progression of colon cancer formation.

An understanding of the molecular mechanisms that underlie cancer could offer new prospects for its treatment as currently the treatment of CRC depends on early diagnosis and surgical intervention. It is hoped that through advances in the molecular understanding of cancerogenesis new treatments can be designed, treatments targeted at molecules central to the development of the cancer, so called "biological" treatments.

An alternative approach to reducing mortality from CRC is the long-term use of oral agents that can prevent neoplasms from developing in the colon. Such pharmacologic prevention, known as chemoprevention, is directed at preventing the development of polyps and their subsequent progression to CRC. Analyses of molecular processes leading to initiation and progression of tumor formation, interfering with these molecular events, identification of new potential chemopreventive agents and elucidation of the mechanism of action of potential and currently used medicines, could increase the protective benefit and decrease the potential side effects of the long-term use of chemopreventive agents.

The main focus of the research described in this thesis is gaining more insight into the molecular mechanisms involved in CRC and based on this knowledge, elucidation of new perspectives for chemoprevention.

We focused on the role of the Bone Morphogenetic Protein (BMP) Pathway in CRC as several recent findings suggest the involvement of BMPs in CRC. First, BMP acts as a tumor suppressor promoting apoptosis in mature colonic epithelial cells and therefore perturbations in BMP signaling could lead to increased tumorigenesis[3]. Second, up to 50%

of individuals with Juvenile Polyposis, an inherited syndrome with a high risk of developing CRC, carry germline mutations in either BMPR1a or SMAD4 genes [4,5,6].This is further supported by transgenic mouse models: The villin-noggin mouse in which BMP expression is completely abrogated develops neoplasia[7] and BMPR1a mutant mice develop intestinal polyposis [8].

In the first part of this thesis we investigated the role of the BMP pathway in sporadic colonic adenomas, sporadic CRCs, and in three rare inherited CRC syndromes. Further, we investigated the interaction between the BMP pathway and the main pathway involved in CRC – the Wnt pathway. We also investigated BMP signaling in Helicobacter pylori infected gastric mucosa.

As we know that BMPs exhibit proapoptotic properties, the activation of this pathway could be a valuable target for the development of cancer therapies and chemoprevention. Lovastatin and Simvastatin are the most potent compounds (from 30000 compounds tested) that induce BMP2 gene expression in bone cells[9], thus in the second half of this thesis we investigated the influence of Statin treatment on BMP pathway in CRC cell lines and the mechanism by which statins upregulate BMP2. We also studied the effect of aspirin on the Wnt pathway in CRC as long-term use of NSAIDs results in a decrease in CRC incidence of 40-50% [10].

The main aim of the work presented in this thesis is to improve understanding of the molecular pathways involved in CRC carcinogenesis and then to apply this in the search for compounds to improve its prevention and treatment. Aspirin (**chapter 8**) and Lovastatin (**chapter 6** and **7**) are both naturally occurring compounds found in white willow tree bark (*Salix alba*) and *Aspergillus terreus* (a fungus) respectively. In **chapter 9** we investigate the potential use and molecular mechanism of action of another naturally occurring compound, Violacein, isolated from *Chromobacterium violaceum* found in the Amazon River.

The Bone Morphogenetic Protein (BMP) pathway and Primary Pulmonary Hypertension (PPH)

BMPs belong to TGFβ superfamily of multifunctional cytokines. This superfamily regulates many processes, including cellular proliferation, adhesion, differentiation and

apoptosis. A detailed overview of TGFβ signalling and its role in human diseases is beyond the scope of this introduction and additional information about TGFβ can be found in detailed reviews[11-13].

Bone Morphogenetic Proteins (BMP) were originally identified as molecules that can induce ectopic bone and cartilage formation in rodents[14]. Further investigations revealed that BMPs elicit a wide spectrum of biological responses in different biological contexts. BMPs are key regulators of embryonic development and disruption of the BMP signaling pathway leads to lethality or to severe developmental anomalies[15]. In vertebrates BMPs are involved in the development of nearly all organs and tissues, including the nervous system, lung, kidney, skin, gonads and gut.

Disturbances in the BMP pathway result in primary pulmonary hypertension (PPH). Germline mutations in the BMPR2 gene (2q33-q34) cause familial PPH [16]. PPH is an autosomal dominant disorder characterized by the obliteration of small pulmonary arteries and arterioles, which leads to the persistent elevation of pulmonary vascular resistance, pulmonary hypertention and right heart failure. More then 40 different unique mutations have been unidentified in the BMPR2 gene that causes PPH and haploinsufficiency, loss of only one allele of BMPR2, is sufficient to lead to disease [17].

BMPR2 was first cloned and characterized by Rosenzweig et al[18].The BMPR2 protein is a serine/threonine kinase consisting of an (N-terminal hydrophobic leader aminoacids sequence), extracellular domain (ligand binding), a transmembrane domain, a kinase domain and a long cytoplasmic tail and it is considerably larger (1038 amino acids, 115 kDa) than previously described mammalian serine/threonine kinase receptors due to the presence of its long C-terminal tail with unknown function. BMPR2 mRNA (NM_001204) has a 11449 bp coding sequence (CDS) between 528bp-3644bp and 7kb 3'untrunslated region (3'UTR). The mRNA of BMPR2 is much longer then in other serine threonine kinase receptors. For example, TGFbR2 mRNA (NM_001024847) has 4714 bp, CDS 383bp-2161bp. In **chapter 3** we investigated the expression of BMPR2 in sporadic CRC.

Inherited colorectal cancer syndromes and underlying molecular mechanisms

Approximately 70% of diagnosed CRCs are sporadic and develop due to the accumulation of genetic and epigenetic changes in somatic cells. In 20% there is a familial disposition without a clear Mendelian inheritance pattern. In this group no single causative mutation or epigenetic modification have be found.

Around 5-10% of CRCs are familial with a Mendelian inheritance pattern. Despite their rarity the inherited CRC syndromes have provided many of the key molecular discoveries in CRC as their genetic homogeneity has made it relatively easier to identify common underlying mutations.

Familial Adenomatous Polyposis

Familial Adenomatous Polyposis (FAP) is an autosomal dominantly inherited disease characterised by the development of hundreds to thousands of colorectal tumours (called adenomatous polyps) during the second and third decades of life. Although these benign tumours are not individually lifethreatening, their large numbers guarantee that some will progress to invasive lesions. The affected patients develop cancer in one or more of the polyps at mean age of 44 years, approximately twenty years earlier then the mean age of development of CRC in the general population.

FAP results from germline mutation in the Adenomatous Polyposis Coli (APC) gene situated in chromosome 5q21[19]. When these mutations are accompanied by chromosomal deletion of the residual wild-type allele (Loss of Heterozygosity, LOH) as frequently occurs in the colon, polyps are formed [20]. The APC gene has 15 exons and encodes a large protein (310kD, 2843 amino acids). The vast majority of APC mutations (> 90%) result in premature stop codons and a truncated APC protein[21]. One of the central tumour promoting effects of these mutations is to lead to overactivation of Wnt/β-catenin pathway, which controls the transcription of genes involved in cell adhesion, migration and proliferation. In the absence of Wnt signalling, β-catenin is phosphorylated by Glycogen Synthase Kinase – 3b (GSK-3b) in the so-called β-catenin degradation complex consisting of GSK-3b, APC, axin and β-catenin. The phosphorylation of β-catenin targets it for ubiquitin-mediated proteosomal degradation.

Truncating APC mutations prevent this process from happening. β-catenin therefore accumulates to high level in the cytoplasm, translocates to the nucleus, interacts with other transcription factors like T-cell factor/lymphoid enhancing factor (TCF/Lef) and activates transcription of target genes that favour cell growth and proliferation.

Wnt signaling is required for the establishment of the progenitor compartment in the normal intestinal epithelium [22 23 24]. Wnt proteins also promote the terminal differentiation of Paneth cells, residing at the bottoms of the crypts [25].

Despite the fact that FAP is a rare syndrome accounting for less then 1% of all CRCs, the mutation and dysfunction of the APC with consequent overactivation of Wnt signal transduction pathway has been shown to be critical in the development of sporadic CRCs as well. Around 70% of sporadic CRCs have a somatic mutation of APC gene[26]. In **chapter 4** of this thesis we study the interaction between the proproliferative Wnt pathway and the proapoptotic Bone Morphogenetic protein pathway. In **chapter 8** we study the effect of nonsteroidal anti-inflammatory drugs on this important pathway in CRC.

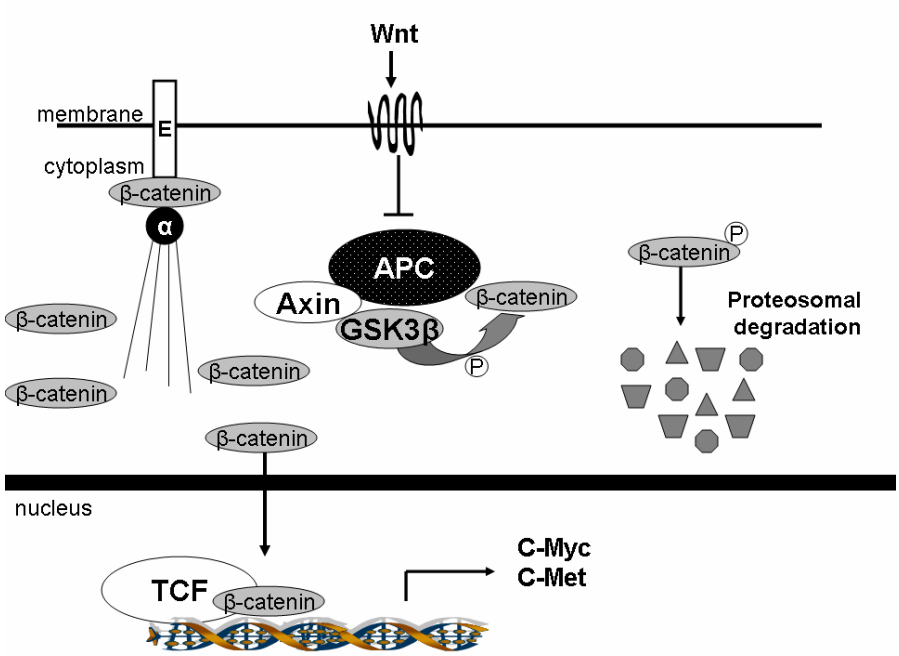

Figure 1. A schematic representation of the Wnt pathway. β-catenin occurs in two locations within the cell: at the cell membrane as a part of the cell cytoskeleton together with E-cadherin (E) and α-catenin (α) and free in the cytoplasm from where it can move into the nucleus and together with TCF, regulate gene transcription. Free cytoplasmic β-catenin levels are regulated by its phosphorylation in the β-catenin reduction complex consisting of APC, Axin and GSK3 β with subsequent proteosomal degradation. Loss of full lenth APC leads to reduced β-catenin phosphorylation and breakdown, thus increasing free cytoplasmic β-catenin levels and allowing more β-catenin to enter the nucleus and activate gene transcription.

Hereditary Nonpolyposis Colorectal Cancer

The Mismatch Repair (MMR) system consists of a complex of proteins that recognize and correct basepair mismatches that occur during DNA replication. Mutations in genes encoding MMR proteins and thus inactivation of the MMR system, leads to the genome wide accumulation of mutations within short repetitive gene sequences (microsatellites) [27]. This condition is named microsatellite instability (MSI). Inactivation of the MMR system due to germline mutations in MMR genes MLH1, MSH2, MSH6 and PMS2 cause Hereditary Nonpolyposis Colorectal Cancer (HNPCC) [28, 29]. HNPCC is an autosomal dominant condition, giving rise to early onset CRCs with a predisposition for proximal colonic involvement. HNPCC is responsible for 3-5% of CRCs.

If a gene possesses a microsatellite and is a tumour suppressor, mutational inactivation of this gene due to MSI leads to a growth advantage for the mutant cells and thus MSI can lead to CRCs. Somatic inactivation of the MMR system is found in approximately 15% of sporadic colon cancers. The main mechanism of inactivation of MMR system in sporadic CRCs is epigenetic silencing of the MLH1 promoter due to aberrant methylation of the promoter region[30, 31].

The classical example of a gene that is affected by MSI is the Transforming Growth Factor Receptor 2 (TGFβR2)[32]. TGFβR2 has a microsatellite region in exon 3 that consists of a 10-basepair polyadenine tract, making it susceptible to mutation when MMR system is insufficient. The mutations in this region are frameshift mutations that result in the deletion of one or two adenines between nucleotides 709-718 in TGFβR2 gene and to truncated TGFβR2 protein which is 129-161 amino acids in length (wild type TGFβR2 is 565 amino acids). As the TGFβ superfamily of multifunctional cytokines induces growth inhibition, apoptosis, and differentiation in intestinal and colonic epithelial cells, the mutational inactivation of TGFβR2 is an event that favours tumour formation[33]. Alterations of TGFbR2 have been identified in up to 30% of CRCs[34].

The TGFβ superfamily consists of the TGFβ, Activin and Bone Morphogenetic Protein subfamilies. The TGFβ superfamily signalling is not exclusively activated by TGFβ but can also be activated via both the Activin and Bone Morphogenetic Protein receptors. Recent work has shown that one of these two potential means of bypassing a TGFβR2 receptor

defect is also dysfunctional in MSI cancers with very high rates of mutation of the Activin Receptor 2 (ACVR2)[35].

It has been shown that BMP signaling is required for apoptosis in colonic epithelial cells similar to TGFβ[3]. Since TGFβR2 and ACVR2 losses are specific to MSI CRC [2,8] and there is nothing known about the BMP pathway in sporadic CRCs, in **chapter 3** of this thesis we examine the role of the BMP pathway in sporadic CRCs and the relationship between the expression of components of the BMP pathway and MSI status of CRCs. In **chapter 2** we aimed to answer the questions whether and where during the adenoma-carcinoma sequence of CRC formation BMP signaling is disrupted.

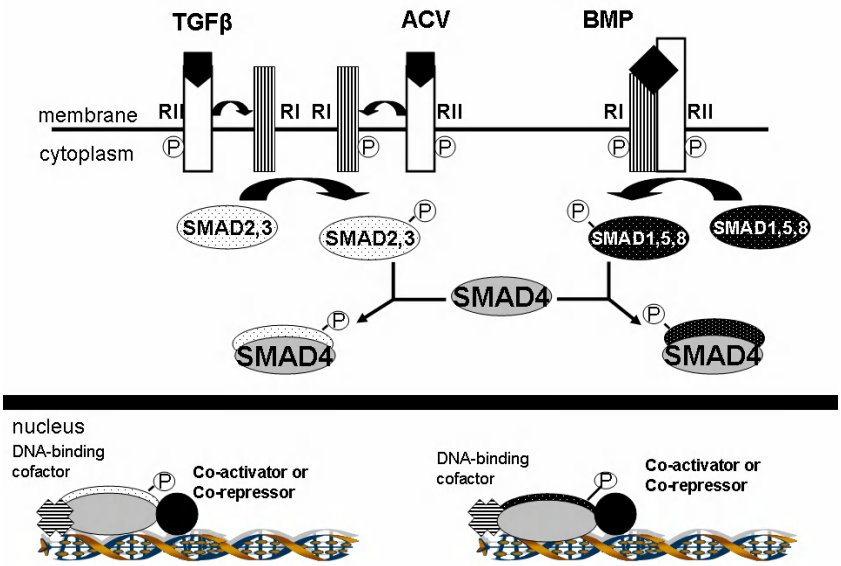

Figure 2. A schematic representation of TGFβ superfamily signalling. Ligands (TGFβs, activins (ACVs) and BMPs) interact in the cell surface with receptors complex. TGFβs and ACVs bind first to a specific type 2 receptor and then form a heterodimeric complex with a specific type 1 receptor. BMPs bind cooperatively to both BMPR2 and BMPR1.The type 1 receptor is phosphorylated by the type 2 receptor kinase and this activated type 1 receptor phosphorylates SMAD2,3 in case of TGFβ and ACV pathways and SMAD1,5,8 in case of BMP pathway. pSMAD2,3 or pSMAD1,5,8 complexes with SMAD4 and these heterodimeric complexes translocate to the nucleus and regulate transcription of genes specific for TGFβ, ACV or BMP pathway.

Juvenile Polyposis

Juvenile Polyposis (JP) is an autosomal dominant disease in which individuals are predisposed to hamartomatous polyps and gastrointestinal cancer [36, 37]. Genetic studies of affected families have revealed germline mutations in SMAD4 and BMPR1a in approximately 20% of patients for each gene [4-6]. While SMAD4 is a central element in the signal transduction pathway of all TGFβ superfamily members, the finding of frequent

BMPR1a mutations in JP is important evidence pointing specifically to a likely role of BMPs in colonic neoplasia.

BMPs initiate signaling by binding cooperatively to transmembrane serine-threonine kinase receptors types 1 and 2, triggering the phosphorylation and activation of the type 1 receptor by the type 2 receptor kinase. The activated type 1 receptor phosphorylates SMADs1,5,8 (receptor-regulated Smads(R-Smads)) and this permits their association with SMAD4 (common-partner Smads (Co-Smads)). This heterodimeric complex then translocates to the nucleus and activates transcription of genes specific for the BMP pathway by interacting with various transcription factors (Runx) and transcriptional co-activators or co-repressors. Id (inhibitor of differentiation or inhibitor of DNA binding) proteins are one of the most important target genes of BMPs[38]. The inhibitory Smads (I-Smads) – Smad6 and Smad7 are induced by BMP signalling, establishing negative feedback loops. I-Smads physically interact with BMPR1 activated by BMPR2, and compete with R-Smads for activation by BMPR1[39]. In addition, I-Smads interact with R-Smads activated by receptors, and prevent complex formation between R-Smads and Co-Smads[40, 41]. Further regulation of intracellular BMP signalling occur via Smurfs (members of the HECT type ligases) which physically associate with and degrade R-Smads[42] and induce ubiquitin-dependent degradation of receptor type 1[43]; via dephosphorylation of receptors by protein phosphatase 1[44]; and via degradation of I-Smads by the Ring type E3 ligase Arkadia[45]. This is a simplified overview of BMP signalling. More detailed information about BMP pathway, its regulation and signalling cross-talk could be found in the specific reviews[46, 47].

In **chapters 2 and 3** we investigated the possible role of BMP signaling in carcinogenesis in the colon and the expression of BMP pathway components in sporadic CRCs. In **chapter 4** we examined the activity of BMP pathway and WNT pathway in a group of patients with FAP, HNPCC and JP.

Epigenetic modification in colorectal cancer

While genetic alterations are a hallmark of human cancer, in many sporadic colorectal carcinomas accumulation of genetic events has been difficult to demonstrate[48]. Recently epigenetic changes in DNA methylation, in association with a repressive chromatin structure, have been identified as critical determinants of tumour progression[49]. Aberrant

DNA methylation of CpG island has been widely observed in human CRCs and is associated with tumour-suppressor gene silencing. This subset of CRCs with an exceptionally high frequency of methylation of "type C" loci, which are defined as loci methylated in cancer, but not in normal tissue, are described as CIMP (CpG island methylator phenotype) cancers[50].

DNA methylation occurs by covalent addition of a methyl group at the 5' carbon of the cytosine ring, resulting in 5-methylcytosine. These methyl groups effectively inhibit transcription. In mammalian DNA, 5-methylcytosine is found primarily at cytosine-guanosine dinucleotides (CpG). Such CpG sites are often found grouped together in stretches of DNA called CpG islands. The accepted definition of a CpG island is a region of DNA greater than 197 bp, with guanine/cytosine content above 0.5 and observed over expected presence of CpG above 0.6. These islands are often found in or near promoter regions of genes, where transcription is initiated. DNA methylation helps to maintain transcriptional silencing in nonexpressed or noncoding regions of the genome. For example, heterohromatin, which is transcriptionally inactive, is heavily methylated at CpG sites and CpG islands in the promoters of genes located on the inactivated X chromosome of females are also methylated. By contrast, CpG islands in germ-line tissue and promoter regions of euchromatin of normal somatic cells remain unmethylated (these regions are in some way protected against methylation), allowing gene expression to occur.

DNA methylation is regulated by DNA methyltransferases (DNMT). DNMTs are enzymes that catalyze the addition of methyl groups to cytosine residues in DNA. In mammalian cells three DNMTs are found – DNMT1, DNMT3a and DNMT3b. DNMT1 appears to be responsible for the maintenance of established patterns of DNA methylation [51], while DNMT3a and DNMT3b seems to mediate establishment of new DNA methylation patterns [52], although recent findings suggest a potential role for DNMT1 in the initiation of promoter CpG island hypermethylation in human cancer cells [53]. The activity of DNMT1, DNMT3a and 3b is elevated in colorectal cancer cells, increasing in a linear fashion with tumour progression [54, 55]. Furthermore, genetic disruption of DNMT activity in mice reduces gastrointestinal tumors in the Min mouse model [56].

Epigenetic alterations do not involve changes in the DNA sequence and are potentially

reversible, therefore demethylating agents could be a valuable option in the development of cancer therapies. Four agents have been employed clinically: 5-azacytidine (azacitidine), 5-aza-2'-deoxycytidine (decitabine), 1-β-D-arabinofuranosyl-5-azacytosine (fazarabine) and dihydro-5-azacytidine (DHAC). DNMTs are depleted by being bound to these agents and are thereby unavailable for the methylation process, resulting in significant demethylation after repeated replication. However, currently known DNMT inhibitors cause significant cytotoxicity [57, 58] as they become incorporate into DNA and RNA and interfere with protein translation [59].

As BMP2 has proapoptotic properties in the colon[3] and the BMP2 promoter is methylated in a subset of gastric cancers[60], in **chapter 7** of this thesis we investigated whether BMP2 is methylated in CRC cell lines and whether Statins could induce demethylation of the BMP2 promoter.

Inhibitors of HMGCoA reductase (Statins)

HMGCoA reductase inhibitors reduce serum cholesterol and decrease the incidence of cardiovascular and cerebrovascular events [61], [62]. The remarkable prevention of cardiovascular disease and the relative safety of statins have led to their widespread use. The Molecular Epidemiology of Colorectal Cancer (MECC) study showed that using Statins for at least 5 years was associated with significant (47%) reduction in the risk for developing CRC[63]. Since this study Statins have attracted even greater attention as possible chemopreventative agents in CRC and several authors have proposed trials of Satins in CRC [64]. However, data from observational studies in humans remains conflicting. The Cancer Prevention Study II Nutrition Cohort reported that current use of cholesterol-lowering drugs was not associated with reduced CRC incidence [65] and a further large case-control study has shown no significant effect of Statins on CRC incidence overall but did find a significantly reduced number of stage IV cancers in users[66] .

Statins prevent formation of mevalonate from HMGCoA by inhibiting the enzyme HMGCoA reductase and thereby inhibit cholesterol synthesis [67]. As well as reducing cholesterol levels, Statins prevent the synthesis of other important isoprenoid intermediates of the mevalonate pathway, such as farnesylpyrophosphate and geranylgeranylpyrophosphate (Figure 3). Posttranslational isoprenylation is important in

determining the membrane localization and function of many cellular proteins including small GTPases like Ras and Rho [68] and thus the major current theory is that Statins prevent CRC through their inhibition of isoprenylation of G-proteins. However the mutation status of these G-proteins has no influence on the sensitivity of cell lines to Statins [69] . Furthermore, adding back geranylgeranylpyrophosphate or inhibition of protein translation with cyclohexamide completely reverse the effects of Statins (both morphological changes and apoptosis) while neither restore the membrane localisation of small GTP-ases such as Rho [70]. Hence it has been suggested that Statins may function through an alternative mechanism involving the production of an unidentified protein[48 70]. Interestingly, Statins can influence RNA stability. For example, Statins induce eNOS (endothelial nitric oxide synthase) expression by posttranscriptional mechanisms[71, 72]. Inhibition of Rho and perhaps other small GTPases leads to increase in eNOS mRNA half-life[64]. The stabilization of eNOS mRNA by simvastatin has been related to an increase in the binding of certain cytoplasmic proteins that recognize a cytidine-rich region within the 3'-untranslated region of eNOS mRNA[73]. Statins reduce the number and size of tumours in various CRC models in mice [74], [75] and they have antitumoural activity in CRC cell lines[76]. In **chapters 6 and 7** we show a novel mechanism of action of Statins in CRC.

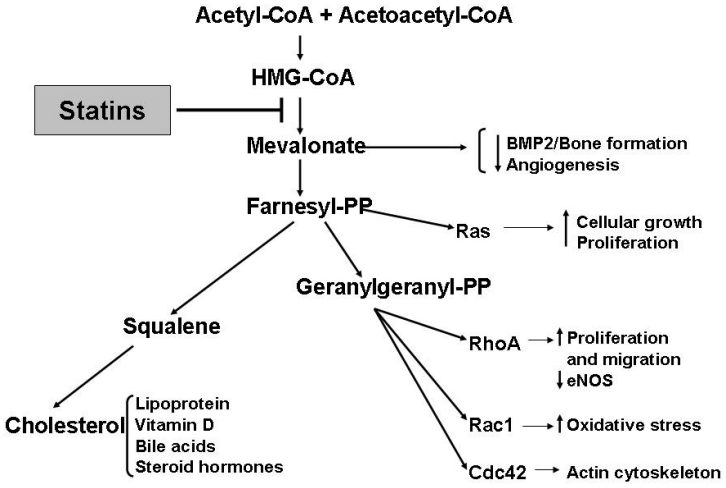

Figure 3. Biological effects of inhibition of HMG-CoA reductase by Statins. Statins inhibit the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate. Decrease in isoprenylation of signaling molecules as Ras, Rho, and Rac leads to modulation of various signaling pathways. Statins also induce BMP2 expression. PP, pyrophosphate; e-NOS, endothelial nitric oxide synthase.

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Chapter 2

The Bone Morphogenetic Protein Pathway is active in human colon adenomas and inactivated in colorectal cancer

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Abstract

Background and Aims:

TGFβ (Transforming Growth Factor β) is important in colorectal cancer (CRC) progression. Bone Morphogenetic Proteins (BMPs), a subgroup within the TGFβ superfamily, have recently also been implicated in CRC but their precise role in CRC has yet to be investigated.

Methods:

We used a tissue microarray and immunohistochemistry of BMP receptors and signal transduction elements in adenomas and CRCs to elucidate the role of BMP signaling in CRC carcinogenesis.

Results:

We find that adenoma specimens express all three BMP receptors (BMPR1a, BMPR1b and BMPR2) and SMAD4, and 20 (90.9%) out of 22 adenomas show active BMP signaling, as judged by nuclear pSMAD1,5,8 expression. In contrast, pSMAD1,5,8 nuclear staining is present in 5 (22.7%) but lost in 17 (77.3%) CRC specimens (cancer vs adenoma, $p<0.0001$). The earliest loss of pSMAD1,5,8 nuclear staining was detected in regions of high grade dysplasia /carcinoma in situ within adenomas. CRCs show frequent loss of BMPR2 ($p<0.0001$) and SMAD4 ($p<0.01$) compared with adenomas. Negative expression of BMPR2 is more frequently observed in earlier-stage cancers (Dukes B) than in advanced cancers (Dukes C) ($p<0.05$).

Conclusions:

Taken together these results indicate that loss of BMP signaling correlates tightly with progression of adenomas to cancer, and occurs relatively early during cancer progression.

Introduction

Colorectal cancer is the second leading cause of cancer death in the United States and Europe[1]. The transformation of colon epithelial cells to cancer follows a predictable progression of histological and cytological changes which are the results of genetic alterations occurring during the adenoma-carcinoma sequence of colorectal cancer (CRC) formation[2]. Accumulated genetic changes and the selection of advantageous mutations underlie the development of neoplasia and drive the initiation and progression of CRC. Based on the association of these mutations with different steps in the multistep progression model of CRC formation, these mutations could provide unique insights into the process of tumorigenesis and could be used as molecular markers for the detection of colon adenomas or colon carcinomas.

The Transforming Growth Factor β (TGFβ) signaling pathway is thought to play a central role in colorectal cancer (CRC) particularly in tumor progression, invasion and metastasis[3]. The TGFβ superfamily consists of the TGFβ, Activin and Bone Morphogenetic Protein (BMP) subfamilies. The TGFβ superfamily plays an important role during embryogenesis and regulates many processes including cellular proliferation, differentiation and apoptosis[4].

BMPs and their receptors have previously been investigated in other solid tumours. The expression of BMP receptors (BMPR) correlates with tumour grade in human prostate cancer[5] and loss of BMPR2 may be a prognostic marker in prostate cancer patients[6]. The important evidence pointing to a likely role of BMPs in colonic neoplasia is the finding that BMPR1a and SMAD4 are frequently mutated in Juvenile Polyposis, an inherited syndrome with a high risk of developing colorectal cancer[7-9]. This is also supported by a transgenic mouse model of Juvenile Polyposis, the villin-noggin mouse in which the BMP signaling is completely abrogated and which develops neoplasia[10]. We have recently shown that BMP acts as a tumour suppressor promoting apoptosis in mature colonic epithelial cells and therefore perturbations in BMP signaling could lead to increased tumourigenesis[11].

BMPs initiate signaling by binding cooperatively to transmembrane serine-threonine kinase receptor types 1 and 2, triggering the phosphorylation and activation of the type 1 receptor by the type 2 receptor kinase. The activated type 1 receptor phosphorylates

SMADs 1,5 and 8 and this permits their association with SMAD4. This heterodimeric complex then translocates to the nucleus and activates gene transcription.

In this study we aimed to answer the questions whether and where during the adenoma-carcinoma sequence of CRC formation BMP signaling is disrupted. For this purpose we performed immunohistochemistry using a tissue microarray on material from 22 patients with simultaneous colorectal adenomas and carcinomas and determined the expression of BMP receptors and signal transduction elements specific for the BMP-pathway. We report that the expression of BMPRs is intact and the pathway is active at the adenoma stage of colon cancer progression. Conversely, BMPR2 and SMAD4 are frequently lost in cancer specimens and BMP signaling is lost in the majority of CRCs.

Materials and Methods

Selection of patient material

An overview of the clinicopathological data is given in Table1. Formalin-fixed and paraffin-embedded tissues from 22 patients with adenomas and CRCs between the years of 2002 and 2004 were used for the compilation of the tissue microarray (TMA). The inclusion criteria were presence of an adenoma and carcinoma in the colon resection specimen of the same patient and the approximately the same age of formalin-fixed, paraffin-embedded tissues (2002-2004 years of preparation) in order to prevent staining artifacts. Blocks were selected from the archives of the Pathology Department of the Academic Medical Center, Amsterdam. The study was approved by the investigators' institutional review board. Our study included 12 men (54.6%) and 10 women (45.4%), their ages ranging from 56 to 92 years, with a mean (±SD) of 74.5 (10.42) years and median 76.5 years. The primary carcinomas (localization was known for all tumours) originated in the colon (n = 19) and rectum (n = 3). The adenomas were classified histologically into four types; tubulovillous, villous, tubular and serrated.

A further series (n=13) of adenomas showing regions of high-grade dysplasia or containing carcinoma in situ were processed as standard whole tissue sections for immunostaining.

Tissue Microarray (TMA) Construction

For the TMA construction we used one H&E-stained slide from each block to define representative tumour regions. Tissue cylinders with a diameter of 0.6 mm were

Table 1. Characteristics of patients and tumors

			Adenoma			Cancer		
Patient	Sex	Age	Histological type	Size	Grade of dysplasia	Location	Grade	Dukes
1	f	56	Tubulovillous	4.5cm	LGD	colon	G	C
2	m	71	Serrated	<1cm	LGD	colon	P	A
3	m	77	Villous	3.5cm	LGD	rectum	G	A
4	f	56	Villous	2.8cm	LGD	colon	M	B
5	f	81	Serrated	<1cm	LGD	colon	P	C
6	f	70	Tubulovillous	1.5cm	LGD	rectum	M	C
7	m	81	Tubulovillous	<1cm	LGD	colon	M	B
8	m	66	Villous	3cm	LGD	colon	M	C
9	m	72	Villous	<1cm	LGD	colon	M	C
10	m	83	Villous	<1cm	LGD	colon	M	B
11	f	60	Tubular	<1cm	LGD	colon	M	B
12	m	79	Tubulovillous	<1cm	LGD	rectum	M	B
13	f	88	Tubular	<1cm	LGD	colon	P	B
14	m	87	Tubulovillous	3.5cm	LGD	colon	M	B
15	m	70	Tubulovillous	<1cm	LGD	colon	M	C
16	m	87	Tubular	1.2cm	LGD	colon	M	B
17	f	83	Tubulovillous	<1cm	LGD	colon	P	B
18	m	79	Tubular	<1cm	LGD	colon	M	C
19	m	62	Tubulovillous	<1cm	LGD	colon	P	B
20	f	79	Tubulovillous	<1cm	LGD	colon	P	C
21	f	72	Villous	<1cm	LGD	colon	M	A
22	f	92	Tubulovillous	3cm	LGD	colon	G	A

LGD- Low Grade Dysplasia,
G – well differentiated cancer, M- moderately differentiated cancer, P- poorly differentiated cancer

punched from the tumour areas of each block and brought into a recipient paraffin block using a Manual Tissue Arrayer MTA-1 (Beecher Instruments, Sun Prairie, WI, USA).

To overcome the problem of tissue microheterogeneity and to increase the number of evaluable cases, the TMA included three cores of tissue from each cancer specimen and two cores from each adenoma from the same patient. Thus, the analysis of protein expression represents the mean of the staining of three different cores from the same cancer and staining of two different cores from the same adenoma. For each case one tissue core from normal colon was included in TMA as a control.

Immunohistochemistry

TMA blocks were sectioned (4 μm), deparaffinised, blocked for endogenous peroxidase activity by immersion in 0.3% H_2O_2 in methanol for 20 min and heat treated at 100°C in Tris (pH 9) for 10 min (antigen retrieval). Nonspecific binding sites were blocked with 5% normal goat serum for 10 min followed by incubation for 1 hour with the primary antibody at room temperature. Primary rabbit polyclonal antibodies to BMPR2 were

kindly provided by Prof. ten Dijke (Dutch Cancer Institute, Amsterdam, The Netherlands) and were used at a concentration of 1:400. The specificity of the antibodies has been demonstrated previously [12]. Mouse monoclonal antibodies to SMAD4 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (1:1600). The Powervision+poly-HRP detection system (ImmunoVision Technologies, Daly City, CA, USA) was used to visualise the antibody binding sites with 3,3-diamino-benzidine+ as a chromogen. Sections were counterstained with haematoxylin. Negative control sections for all antibodies were processed in an identical manner after omitting the primary antibody and showed no staining.

Immunohistochemistry for phosphorylated SMAD1,5,8 (pSMAD1,5,8), BMPR1a and BMPR1b

Sections were first deparaffinised and immersed in 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed by boiling slides for 10 min in 0.01 mol/L sodium citrate, pH 6.0. Nonspecific binding sites were blocked with TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween 20, pH 8.0) for 30 min. Slides were incubated with primary rabbit polyclonal antibodies to Phospho-Smad1/5/8 which recognizes the doubly phosphorylated forms of Smad1 (Ser463/465), Smad5 (Ser463/465), and Smad8 (Ser426/428) (Cell signaling, Beverly, MA, USA) at a concentration of 1:50, or with goat polyclonal antibodies to BMPR1a (R&D) at a concentration of 1:100 or with mouse monoclonal antibodies to BMPR1b (R&D) at a concentration of 1:1000 overnight at 4°C in PBS with 0.1% Triton and 1% bovine serum albumin, then incubated with biotinylated secondary goat anti-rabbit, goat anti-mouse or rabbit anti-goat antibodies (Dako, Glostrup, Denmark) at a concentration of 1:200 at room temperature for 1 hour in PBS with 10% human serum. Slides were then incubated with streptavidin-biotin-horseradish peroxidase (Dako, Glostrup, Denmark) for 1 hour and peroxidase activity was detected with “Fast DAB” (Sigma, St. Louis, MO, USA).

TMA analysis

The cellular localization and pattern of immunoreactivity were examined by two investigators independently in a blinded fashion. BMPR1a, BMPR1b and BMPR2 expression was graded according to both the intensity of staining and the number of cells stained as shown in Table 2. Only staining of the cell membrane or

Table 2. Scoring system for BMPR1a, BMPR1b and BMPR2

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

intracytoplasmic staining was assessed. Scores of 0 and 1 were considered negative for BMPR expression, and scores of 2 and 3 were considered positive.

Table 3. Scoring system for SMAD4 and 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

For the evaluation of the SMAD4 and pSMAD1,5,8 expression only nuclear staining was assessed and was scored as shown in Table 3. Scores of 0 and 1 were considered negative for SMAD expression, and scores of 2 and 3 were considered positive.

Statistical Analysis

Statistical analysis was performed using the Statistical Package of Social Science (SPSS) version 11.5 for Windows. The χ^2 test and Fisher exact tests were used as appropriate. McNemar test was used to compare differences between adenomas and cancers. $P < 0.05$ was considered statistically significant.

Results

Expression of BMP receptors and signal transduction elements in the normal human colon and adenomas

The expression of BMPRs, SMAD4 and pSMAD1,5,8 was investigated in normal colon and adenomas using immunohistochemistry (Figure 1). In normal and adenomatous tissue, the expression of BMP pathway components is predominantly localized in the epithelial cells. SMAD4 is expressed in both epithelial and stromal cells. The cellular staining pattern for BMPRs is both membrane and cytoplasmic. BMPRs are expressed in all normal and all adenoma samples (Figure 1, 2). With SMAD4 and pSMAD1,5,8 we see cytoplasmic as well as clear nuclear staining. Positive nuclear staining for

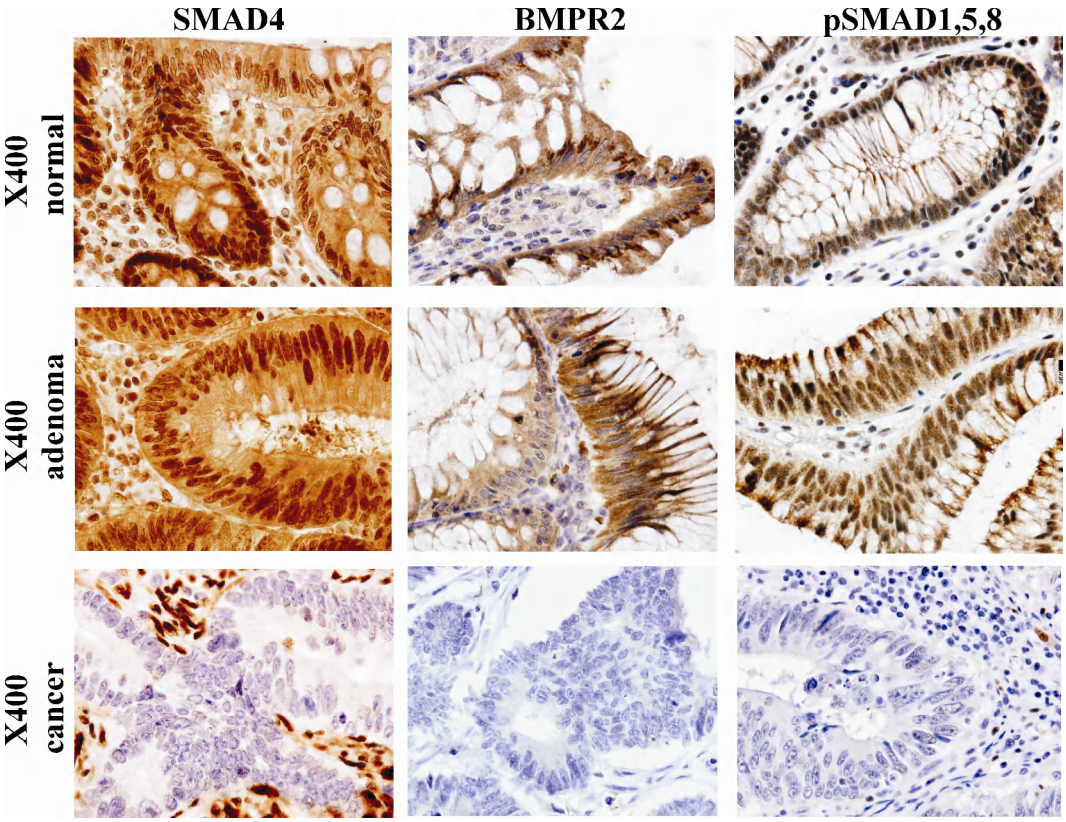


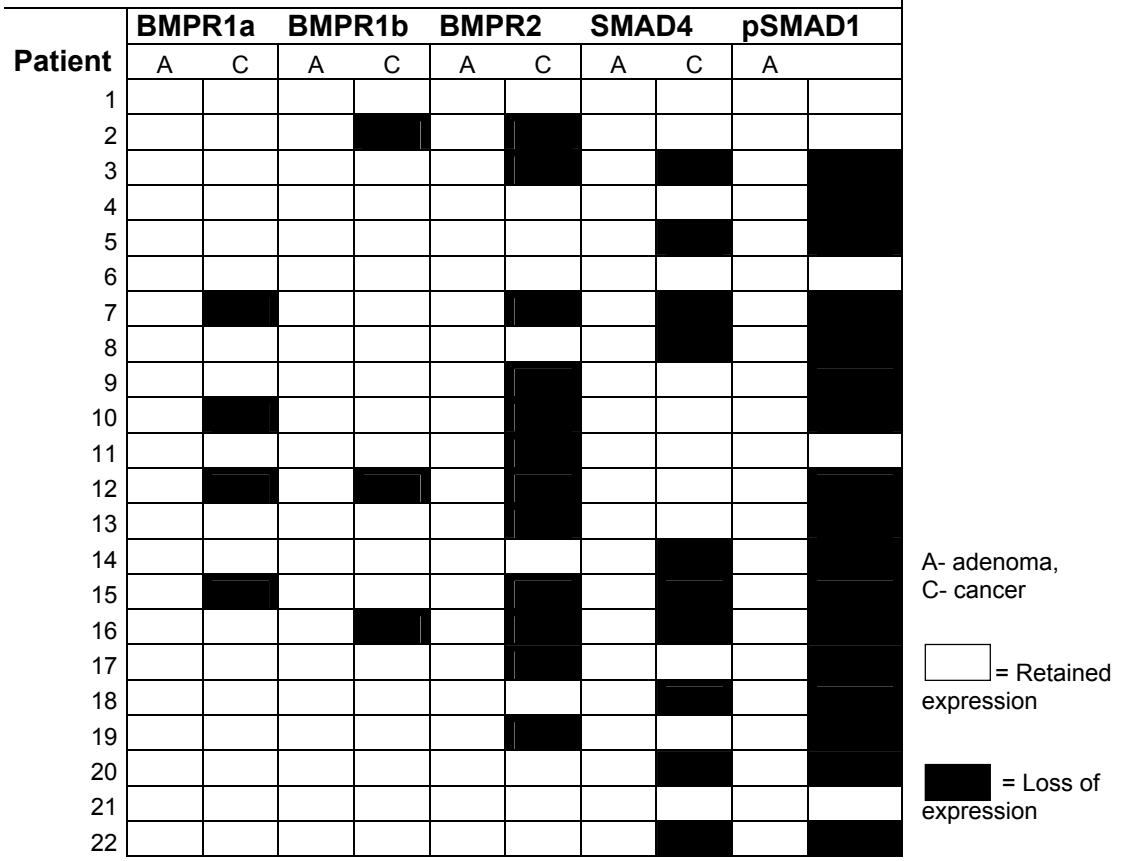
Figure 1. Expression of SMAD4, BMPR2 and pSMAD1,5,8 in normal human colon, in human colonic adenomas and in human colon cancer specimens.

SMAD4 is found in all normal and adenoma specimens. pSMAD1,5,8 is expressed in the nucleus of all normal specimens and in 20 out of 22 adenomas (adenomas vs normal, p=0,5, ns), suggesting that BMP pathway is active in normal colon tissue and in the majority of adenomas (Figure 2, Table 4).

Table 4. Expression of BMP pathway components in adenomas and CRC specimens

Characteristic	Adenoma		Cancer		
	positive	negative	positive	negative	*P*
BMPR1a	22	0	18	4	0.125
BMPR1b	22	0	19	3	0.25
BMPR2	22	0	10	12	<0.0001
SMAD4	22	0	12	10	0.002
pSMAD1,5,8	20	2	5	17	<0.0001

Figure 2. Schematic representation of the expression of BMP pathway components in 22 patients with adenomas and CRCs



Expression of BMP receptors and signal transduction elements in human colorectal cancer specimens

To determine whether or not a significant portion of human CRC cases have reduced levels of BMPRs compared with adenomas, the cancer tissue from the same patients were evaluated for BMPR expression using immunohistochemistry (Figure 1 and 2). The frequency of loss of expression of BMPR1a and BMPR1b does not differ significantly between cancers and adenomas. (Table 4). We found that cancers show frequent loss of BMPR2 and SMAD4 expression compared with adenomas ($p< 0,0001$ and $p=0.002$ respectively) (Table 4). Around 80% (17 out of 22) of CRC samples show negative nuclear pSMAD1,5,8 staining, implying reduced BMP-pathway activity in these cancers (cancer vs adenomas, $p<0.0001$). We found a strongly statistically significant association between defective expression of BMPRs or SMAD4 and negative nuclear staining for pSMAD1,5,8 ($p=0,024$) (Table 5) suggesting that the abnormal expression of the BMPRs and SMAD4 influences the activity of BMP pathway in CRC.

Table 5. Association between nuclear localization of pSMAD1,5,8 and expression of the components of BMP-pathway

n=22		pSMAD1,5,8		*P*
		negative	positive	
BMP pathway	**defective**[1]	16(72.7%)	2(9.1%)	0,024
	normal	1(4.5%)	3(13.6%	

Percentages in brackets refer to the percentage of the total number of cases
[1]Defective means negative staining in one or more of BMPR1a, BMPR1b, BMPR2 or SMAD4

We found a significant association between loss of BMPR2 and Dukes' stage. Negative expression of BMPR2 is more frequent in Dukes' B tumors (80%) when compared to Dukes's C tumors (25%) (see Table 6). No significant differences were observed for the remaining clinicopathological or biological variables (sex, age, location and grade of tumors).

Table 6. Expression of BMPR2 in CRCs in relation to Dukes' stage

Characteristic	Dukes A	Dukes B	Dukes C	*P*
BMPR2, n (%)				
Score 0	1 (25)	2 (20)	2 (25)	0.028
Score 1	1(25)	6 (60)	0 (0)	
Score 2	0 (0)	1 (10)	0 (0)	
Score 3	2 (50)	1 (10)	6 (75)	
Total cases	4 (100)	10 (100)	8 (100)	

Expression of BMP receptors and signal transduction elements in adenomas with high-grade dysplasia/carcinoma in situ

We then examined pSMAD1,5,8 staining in a series of adenomas containing areas of high-grade dysplasia/early carcinomas in order to determine at what morphological stage the loss of BMP pathway activity could be detected. In 10 out of 13 patients loss of nuclear pSMAD1,5,8 staining was observed. There was a clear difference in pSMAD1,5,8 nuclear expression between highly dysplastic/cancer loci and the surrounding adenomatous tissue (Figure 3). This fits well with our results obtained from TMA immunohistochemistry analysis and suggests that loss of BMP signaling at the advanced adenoma/early cancer stage.

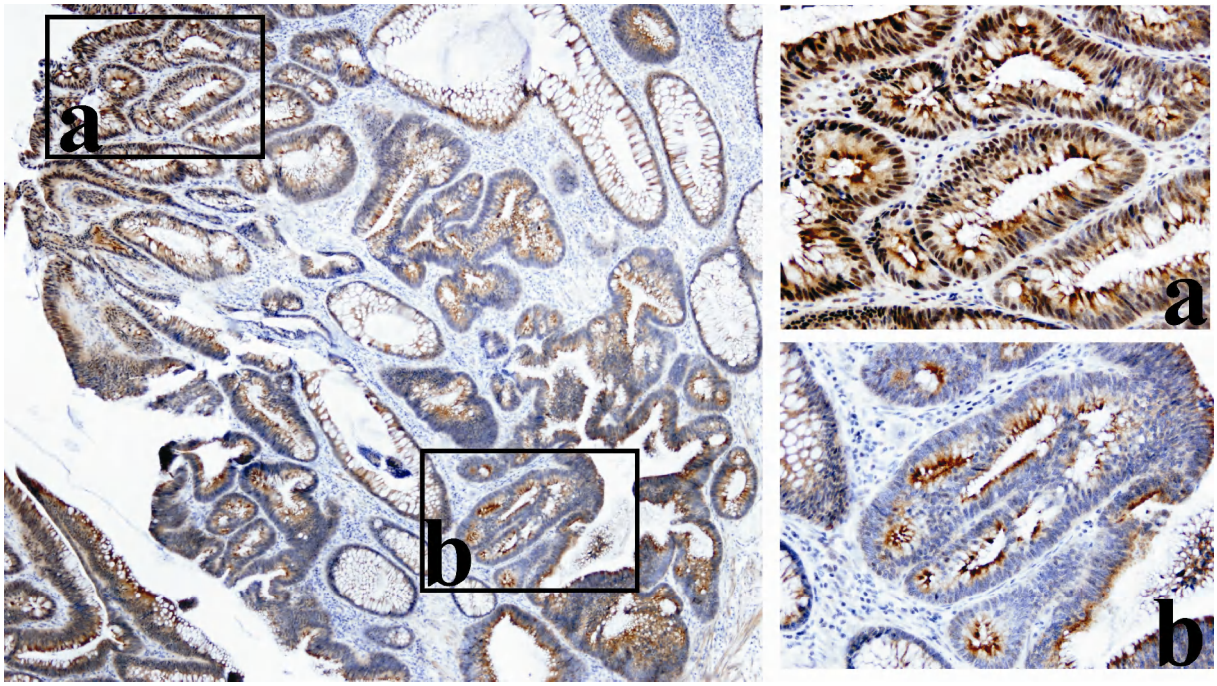


Figure 3. Selective loss of nuclear pSMAD1,5,8 expression in an area of high grade dysplasia within an adenoma. A, the area of adenoma with positive pSMAD1,5,8 nuclear expression. B, the area of high grade dysplasia with negative pSMAD1,5,8 nuclear staining.

Discussion

Disturbances in TGFβ superfamily signaling result in various human diseases including cancer. Several pathway elements act as tumour suppressors in a variety of human tumour types, including colon and pancreatic cancer[13, 14]. Mutational inactivation of TGFβR2 and SMAD4 are frequent events in colorectal cancers[15, 16]. The role of BMPs in colon cancer progression, members of the TGFβ superfamily, has so far received little attention despite the finding of BMPR1a germline mutations in families with Juvenile Polyposis, where affected individuals develop multiple intestinal polyps with a high chance of developing cancer[17].

We have previously shown that BMP signaling is required for apoptosis in colonic epithelial cells and acts as a tumour suppressor with similar characteristics to TGFβ[11]. Here we show that CRCs but not adenomas have frequently reduced levels of expression of BMPRs and SMAD4. We show that the BMP pathway is inactivated in 80% of CRCs, as judged by nuclear pSMAD1,5,8 expression, but active in adenomas. Selective loss of pSMAD1,5,8 nuclear staining in areas of high-grade

dysplasia/carcinoma in situ within adenomas suggests that loss of BMP signaling is an event associated with the progression of adenomas to carcinomas. Taken together, these observations provide an insight into the potential role of BMPs in the adenoma/carcinoma sequence.

In this study, no BMP pathway components abnormalities are detected in adenomas. This is in agreement with the fact that mutation of TGFβR2 is a late event in adenomas and correlates tightly with progression of these adenomas to cancer[18].

We observed that the tumours with attenuated BMPR2 expression were significantly more likely to be Dukes' B stage cancers than advanced cancers. These results correlate with the previously described association between Dukes B cancers and TGFβR2 mutations[19].

TGFβR2 inactivation is associated with an increased survival in the late stages of CRC patients[20, 21]. Despite the well known tumour suppressor functions of TGFβ, there is considerable evidence that this pathway promotes tumour progression, invasion and metastasis in the late stages of carcinogenesis[17]. Inactivation of TGFβ signaling at a point when cells are no longer sensitive to the growth inhibitory effect of TGFβ, and where the effects of TGFβ have switched from tumour supressive to tumour promotional, could explain the improved survival of patients with TGFβR2 inactivation. As BMP has similar characteristics to TGFβ it is possible that BMP could also act to promote tumour progression in the late stages of CRC progression. Inactivation of BMP signal transduction through inactivation of BMPR2 may initially release the adenoma cells from BMP-induced growth suppression, but later protect the host from metastasis by switching off the metastasis enhancing effects of BMPs. Further analysis is necessary to confirm this hypothesis.

We also assessed whether changes in the protein expression of BMPR2 or SMAD4 influence the activity of BMP-pathway as judged by pSMAD1,5,8 nuclear staining. Our results show that impaired BMPR2 or SMAD4 expression strongly correlates with reduced BMP-pathway activity. The influence of this inactivation of BMP signaling on cancer progression, invasiveness and eventually, patient outcome is a subject for further investigation.

We did not find a correlation between BMP signaling inactivation and cancer location or degree of differentiation as has been observed in case of TGFβ (rightsided, moderate

or poorly differentiated CRC). This could be due to the small sample size and more studies are needed to test these associations.

In summary our data demonstrate that BMP signaling is intact in normal colonic epithelium and adenomas of all types, but frequently inactivated in carcinomas similarly to TGFβ signaling (Figure 4). The change occurs at the advanced adenoma/early carcinoma stage.

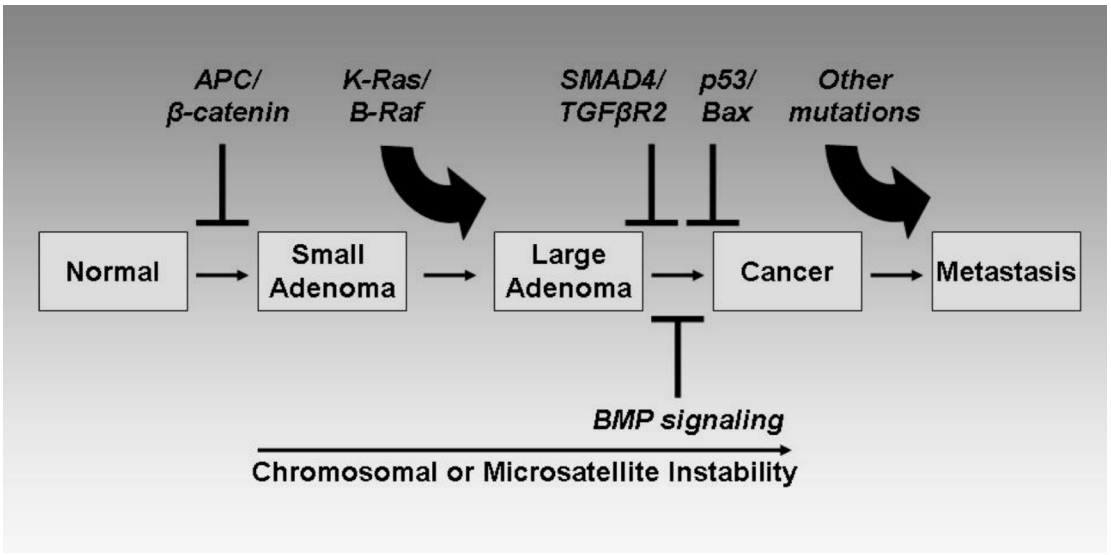


Figure 4. Modified representation of the molecular events associated with the polyp-carcinoma sequence according to Vogelstein with the addition of the BMP pathway acting as a tumor suppressor at the late advanced adenoma stage.

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Chapter 3

The Bone Morphogenetic Protein Pathway is inactivated in the majority of Sporadic Colorectal Cancers

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Submitted

Abstract

Background and Aims:

Transforming Growth Factor β (TGF β) plays an important role in colorectal cancer (CRC). The finding of Bone Morphogenetic Protein (BMP) Receptor 1a mutations in Juvenile Polyposis suggests that BMPs, a subgroup within the TGFβ superfamily, also play a role in CRC. We investigated the BMP pathway in sporadic CRC.

Methods:

We investigated BMP receptor (BMPR) expression using immunoblotting and sequenced microsatellites of BMPR2, TGFβReceptor 2 (TGFβR2) and Activin Receptor 2 in CRC cell lines. We assessed the expression of BMPRs, SMAD4 and pSMAD1,5,8 in 72 sporadic CRCs using a tissue microarray and immunohistochemistry. CRC specimens were analyzed for microsatellite mutations in BMPR2, TGFβR2 and ACVR2.

Results:

BMPR2 protein expression is abrogated in microsatellite unstable (MSI) cell lines while SMAD4 protein expression is abrogated in microsatellite stable (MSS) cell lines. BMPR2 is mutated in all MSI and in none of the MSS cell lines. BMPR2 expression is impaired more frequently in MSI CRCs than MSS (85% vs 29%:$p<0.0001$) and shows a mutually exclusive pattern of impaired expression compared to SMAD4 as seen in cell lines. Nine of eleven MSI-cancers with impaired expression of BMPR2 have microsatellite mutations. The BMP pathway is inactivated, as judged by nuclear pSMAD1,5,8 expression, in 70% of CRCs and this correlates with BMPR and SMAD4 loss.

Conclusions:

Our data suggest that the BMP pathway is inactivated in the majority of sporadic CRCs. In MSI CRC this is associated predominantly with impaired BMPR2 expression and in MSS CRC with impaired SMAD4 expression.

Introduction

The Transforming Growth Factor β (TGFβ) signaling pathway is thought to play a central role in colorectal cancer (CRC)[1]. The TGFβ superfamily consists of the TGFβ, Activin and Bone Morphogenetic Protein subfamilies. TGFβ Receptor 2 (TGFβR2) has long been considered the most frequently mutated gene in microsatellite unstable (MSI) cancers[2], but how this leads to cancer is unclear with conditional TGFβR2 knockout from the colonic epithelium in mice showing no CRC phenotype[3]. Disturbances of the downstream mediators of TGFβ function, the SMADs, do have a CRC phenotype in mice[4-6] and mutations of SMAD4 are frequently found in human CRC[7]. Signaling via SMAD4 is not exclusively activated by TGFβ but can also be activated via both the Activin and Bone Morphogenetic Protein (BMP) receptors. Recent work has shown that one of these two potential means of bypassing a TGFβR2 receptor defect is also dysfunctional in MSI cancers with very high rates of mutation of the Activin Receptor 2 (ACVR2)[8] but the possible involvement of the BMP pathway in sporadic colon cancer has received little attention.

Several recent findings suggest the involvement of BMPs in CRC. First, the mutations in SMAD4 frequently found in colon cancers[9] may implicate not only TGFβ but also BMPs in colon cancer progression, as SMAD4 is central to both BMP and TGFβ signal transduction. Second, up to 50% of individuals with Juvenile Polyposis, an inherited syndrome with a high risk of developing CRC, carry germline mutations in either BMPR1a or SMAD4 genes [10, 11, 12]. This is further supported by a transgenic mouse model of Juvenile Polyposis, the villin-noggin mouse in which BMP expression is completely abrogated, which also develops neoplasia[13]. Third, BMP acts as a tumor suppressor promoting apoptosis in mature colonic epithelial cells and therefore perturbations in BMP signaling could lead to increased tumorigenesis[14]. Finally, BMPs have been implicated in other epithelial cancers. Prostate cancers frequently show loss of expression of BMP receptors [15, 16] and bladder transitional cell carcinoma tissue has loss of BMPR2[17].

BMPs play an important role during development and regulate many processes including cellular proliferation, adhesion, differentiation, inflammation and apoptosis[18, 19]. BMPs initiate signaling by binding cooperatively to transmembrane serine-threonine kinase receptors types 1 and 2, triggering the phosphorylation and activation of the type

1 receptor by the type 2 receptor kinase. The activated type 1 receptor phosphorylates SMADs1,5 and 8 and this permits their association with SMAD4. This heteromeric complex then translocates to the nucleus and regulates the transcription of genes specific for the BMP pathway.

In this study we set out to investigate the expression of elements of the BMP pathway in CRC cell lines and patient specimens as a first step in determining whether the BMP pathway plays a role in sporadic CRC. Since TGFβR2 and ACVR2 losses are specific to MSI CRC [2,8] we were also interested to see whether the expression of components of the BMP pathway was related to MSI status.

Materials and Methods

Cell culture

CACO2, DLD1, SW480, LOVO, SW48, HT29 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 L-glutamine. This was supplemented with penicillin (50U/ml) and streptomycin (50µg/ml) and, when serum was used, with 10% fetal calf serum (Gibco, Paisley, Scotland). Cells were grown in monolayers in a humidified atmosphere containing 5% CO_2.

Immunoblotting

Cells at 60-80% confluence from 6 well plates (Greiner Bio-One B.V., Alphen a/d Rijn, Netherlands) were washed in ice-cold PBS and scraped into 200µl of 2×sample buffer (125mM Tris/HCl, pH 6,8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, 1mg bromophenol blue). Protein concentration was measured using the RC DC protein assay kit (Biorad, CA, USA). The lysates were sonicated and then heated at 95° for 5 min. 50µg of protein from each sample was loaded onto SDS-PAGE and blotted onto PVDF membrane (Millipore, Bedford, MA, USA). The blots were blocked in block buffer (2% low fat milk powder in Tris-buffered saline with 1% Triton (TBST)) for 1 hour at room temperature and washed 3×10 min in TBST before overnight incubation at 4°C with primary antibody in primary antibody buffer (TBST with 0.2% low fat milk powder). Primary antibodies to BMPR1a (goat polyclonal), BMPR1b (mouse monoclonal) and BMPR2 (goat polyclonal) were from R&D (Abingdon, UK). Mouse monoclonal antibodies to SMAD4 and rabbit polyclonal antibodies to β-actin were from Santa Cruz

Biotechnology (Santa Cruz, CA, USA). Blots were then washed 3×10 min in TBST and incubated for 1 hour at room temperature in 1:2000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit, rabbit anti-goat or goat anti-mouse secondary antibody (Dako, Glostrup, Denmark) in block buffer. After a final 3×10 min wash in TBST, blots were incubated for 5 min in Lumilite Plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Lumi-Imager (Boehringer-Mannheim, Mannheim, Germany).

Selection of patient material

An overview of the clinicopathological data is given in Table1.

Formalin-fixed, paraffin-embedded tissues from 72 CRC cases between the years of 2002 and 2004 were used for the compilation of the TMA. Blocks were selected from the archives of the Pathology Department at the Academic Medical Centre, Amsterdam. The study was approved by the investigator's institutional review board. Our study included 37 men (51.4%) and 35 women (48.6%), their ages ranging from 30 to 92 years, with a mean (±SD) of 69.8 (11.8) years and median 70 years. The primary tumors (localization was known for all tumors) originated in the colon (n = 45) and rectum (n = 27).

Table 1. Characteristics of patients and tumours

Characteristic	All cases	MMR-proficient CRC	MMR-deficient CRC	*P*
Patients, n. (%)	72 (100)	59 (81.9)	13 (18.1)	
Age at presentation, y				
Mean (±SD)	69.85 (11.8)	68.8 (12.2)	73.7 (9.7)	0.62
Median	70	68	73	
Range	30-92	30-92	51-88	
Sex, n. (%)				
Male	37 (51.4%)	27 (45.8)	10 (68.8)	0.065
Female	35 (48.6%)	32 (54.2)	3 (23,1)	
Site of tumour, n. (%)				
Colon	45 (62.5)	35 (59.3)	10 (76.9)	0.35
Rectum	27(37.5)	24 (40.7)	3 (23.1)	
Tumour grade, n (%)				
Well differentiated	9 (12.5)	8 (13.6)	1 (7.6)	0.24
Moderately differentiated	44 (61.1)	38 (64.4)	6 (46.2)	
Poorly differentiated	19 (26.4)	13 (22.0)	6 (46.2)	

Construction of the Tissue Microarray (TMA)

Tissue cylinders with a diameter of 0.6 mm were punched from the tumor areas of each block and brought into a recipient paraffin block using a Manual Tissue Arrayer MTA-1 (Beecher Instruments, Sun Prairie, WI, USA). Three cores of tissue from each cancer specimen were used and for each cancer case one core from the corresponding normal colon.

Immunohistochemistry

TMA blocks were sectioned (4 μm), deparaffinised, blocked for endogenous peroxidase activity by immersion in 0.3% H_2O_2 in methanol for 20 min and heat treated at 100°C (pH 9) for 10 min (antigen retrieval). Nonspecific binding sites were blocked with 5% normal goat serum for 10 min followed by incubation for 1 hour with the primary antibody at room temperature. Rabbit polyclonal antibodies to BMPR2 were used at a concentration of 1:400. The specificity of the antibodies has been demonstrated previously [20]. Mouse monoclonal antibodies to SMAD4 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (1:1600). The Powervision+poly-HRP detection system (ImmunoVision Technologies, Daly City, CA, USA) was used to visualise the antibody binding sites with 3,3-diamino-benzidine+ as a chromogen. Sections were counterstained with haematoxylin. Negative control sections for all antibodies were processed in an identical manner after omitting the primary antibody and showed no staining.

Immunohistochemistry for phosphorylated SMAD1,5 and 8 (pSMAD1,5,8)

Sections were first deparaffinised and immersed in 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed by boiling slides for 10 min in 0.01 mol/L sodium citrate, pH 6.0. Nonspecific binding sites were blocked with TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween 20, pH 8.0) for 30 min. Slides were incubated with primary rabbit polyclonal antibodies to Phospho-Smad1/5/8 which recognizes the doubly phosphorylated forms of Smad1 (Ser463/465), Smad5 (Ser463/465), and Smad8 (Ser426/428) (Cell Signaling, Beverly, MA, USA) at a concentration of 1:50 overnight at 4°C in PBS with 0.1% Triton and 1% bovine serum albumin, then incubated with biotinylated secondary goat anti-rabbit antibodies (Dako, Glostrup, Denmark) at a concentration of 1:200 at room temperature for 1 hour in PBS with 10% human serum. Slides were then incubated with HRP-conjugated ABComplex

(Dako, Glostrup, Denmark) for 1 hour and peroxidase activity was detected with "Fast DAB" (Sigma, St. Louis, MO, USA) used according to the manufacturer's instructions.

TMA analysis

The cellular localization and pattern of immunoreactivity were examined by two investigators independently in a blinded fashion. Expression was graded from 0 to 2 for BMPR1a, BMPR1b and BMPR2 (0 = no staining, 1 = weak membrane or intracytoplasmic staining in less than 10% of the cells, 2 = moderate to strong membrane or intracytoplasmic staining in more than 10% of the cells). Semi-quantitative evaluation of the SMAD4 was scored as follows: 0 = no staining, 1= weak nuclear staining or negative nuclear and weak intracytoplasmic staining in less then 10% of the cells, 2 = moderate to strong nuclear staining in more than 10% of the cells. A dichotomised scale was used to measure the intensity of the MLH1 and MSH2 expression. Samples with no nuclear staining in tumor cells were classified as negative, and as positive if more then 10% of cells had nuclear staining intensity greater than that of negative control slides. Expression of pSMAD1,5,8 was graded as negative if less then 30% of cells showed weak nuclear staining or less then 10% of cells showed strong nuclear staining.

Tissue Microdissection and DNA Extraction

Enriched tumor tissue (minimally 75% tumor cells) was microdissected from 4-µm formalin fixed, paraffin embedded tumor sections using a sterile needle and DNA was extracted using the DNeasy Tissue Kit (Qiagen, Venlo, The Netherlands).

Microsatellite analysis

MSI status was determined using 5 microsatellite markers (BAT25, BAT26, D2S123, D5S346, and D17S250, the Bethesda panel) as previously described[21]. Analysis was carried out using an automated ABI 377 or ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA) with a Genescan™ 350ROX size standard (Applied Biosystems) and the manufacturer's Genescan® 3.7 software. MSI tumors were defined as having ≥ 2 of 5 markers with novel alleles compared with matched normal DNA, whereas MSS tumors had 0 of 5 markers with novel alleles.

DNA sequencing

DNA samples were amplified with specific primers in a reaction containing PCR master-mix and 3µM of each primer. Polymerase chain reaction (PCR) was performed

over 40 cycles of 94ºC, 50ºC, and 72ºC of 1 min each, preceded by a 5-min denaturing step at 94ºC and followed by a 10-min extension step at 72ºC. The correct product size was verified on 2% agarose gel. The PCR product was purified using a PCR purification kit (Qiagen, Venlo, The Netherlands) and was sequenced on an ABI 377 or 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA) using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). For the GeneScan analysis PCR was performed with specific primers as shown in table 2. The forward primer was HEX-labelled. Then DNA standard size GENESCAN-500 ROX (Applied Biosystems, Foster City, CA, USA) was added to the PCR product, the mix was sequenced and the size of the DNA fragments containing the 7A microsatellite was measured using 3.1 Genescan analysis software.

BMPR2 was sequenced at the Leiden Genome Technology Center (www.lgtc.nl). Primers were designed with Primer3 software [22] to generate products of approximately 250bp to facilitate eventual sequencing from paraffin embedded tissue. 18mer M13 tails were added to the primers to improve the sequence results. PCR conditions: 3.2 µM of both primers; 200nM dNTP; 10mM Tris-Hcl, 50mM KCl, 2.0mM $MgCl_2$ and 50 units/ml of Amplitaq Gold Polymerase (PE Applied Biosystems Inc., Foster City, USA). 10 min at 96ºC and 38 cycles of 30 sec at 96ºC, 30 sec at 60ºC, 1 min at 72ºC, and a delay extension step of 5 min at 72ºC in a GeneAmp 9700 thermocycler (PE Applied Biosystems Inc., Foster City, USA). The sequences of all primers used are given in Table 2.

RNA isolation and RT-PCR

Total RNA was isolated from cell lines using Trizol Reagent (Life Technologies Inc, Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using oligo dT primers and Superscript II MMLV-reverse transcriptase according to the manufacturer's instructions (Life Technologies Inc, Invitrogen, Breda, the Netherlands). Subsequent PCR analysis for BMPR2 was carried out in 25 µL reactions containing 1 µL cDNA, 23 µL Reddy Mix PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer, as used in [23]. PCR was performed with 33 cycles of 94ºC, 58ºC, and 72ºC of 45 sec each, preceded by a 5-min denaturing step at 94ºC and followed by a 10-min

extension step at 72ºC. The products were electrophoresed on 1% agarose gel. β-actin was used as a control.

Table 2. Primers

Target	Forward Primer 5'- 3'	Reverse Primer 5'- 3'
BMPR2 11A	ATTAGGTCACTGAAAGAACT	GCATATTACTTAGCTTCTCT
BMPR2 7A	TCCATCATACTGACAGCATCG	TGTGGTGTTTGTGGTTGTTG
TGFβR2 10A	TTCTCTCTCTCCCTCTCCCC	TGCACTCATCAGAGCTACAGG
ACVR2 9A	GTTGCCATTTGAGGAGGAAA	CCTCTGAAAAGTGTTTTATTGGAA
BMPR2 RT-PCR	GATGGCAAATCAGGATCAGG	CCTCACAGTCCAGCAATTCAG
BMPR2 MSP U	TTAGGAGTTTAGAGTTGTGGGAGAAT	CTCCCATCAATAACTCCTATAAACAA
BMPR2 MSP M	GTTTAGAGTTGCGGGAGAAC	CGTCAATAACTCCTATAAACGAA
BMPR2 Bis.seq.	AATAATAGAGGGTAGTTTTGTTTTT	AAAAACACTTCCAATAACTCC
BMPR2 full seq.		
Exon 1	GCCGGTCTACTTCCCATATT	CGAAGGGCAAGCACAGG
Exon 2	TTTGTCATTCCTTTATTTCCTTT	AACACAGTCATTTCAGGTAAGG
Exon 3	TCTTTATCATATTGTCTCCTTTTT	GGAAATACAAAGAAAAGTTGGTT
Exon 4	TGACATTTCAAAATTTGTTTTC	CGGAATTTAAAAGGAGCAAA
Exon 5	TTCTGCAGCTCTTCTTTTTAAG	TCACAGTAGAAACAACAGTCCAT
Exon 6.1	GAGAGCTGTAGCATTCTGTTT	TTGTCATGTTCCATCAAAGG
Exon 6.2	CCTTTGATGGAACATGACAA	AAGTGATCCACCTGCCTTAG
Exon 7	TGCAAATTCTTTATAAGGATGC	CCCACATGAGTGTCAATTTC
Exon 8	TTTCATGTTCAATAGTCCCTTTT	CATCAGTGTGATACCTTTTGT
Exon 9	TCAGAATATGCTACGTTCTCTCTC	TAACTGCTTCACTTCAAAAA
Exon 10	GAAATTTTATTCTGTCATTCTTTTC	TTCAGTCATAAGTCCTCTCTTT
Exon 11	TTTAAAGACACATGGTTTGACAT	ATAGATGCCACACCCCTTAG
Exon 12.1	TCATAAATGTACGTTCTCAATGTG	TTGTGCTTGCTGTCGTTC
Exon 12.2	GCACACCTTTGACTATAGGG	TGTAAGCAGACAGGGGTTG
Exon 12.3	ACCACAAATGTTGCACAGTC	CTGCTGTCCAGTTGCTTCTA
Exon 12.4	AGTTCTAGCTTGCTTTACCC	CAAGTTTGATTTGTGCTTGC
Exon 12.5	TAGTTTGCCTTTGAACACCA	TGGTTGTTTGGCCAGATAGT
Exon 12.6	ACAACCCAATATGCCAATG	TAGTTCGGCCACCTTCTAGT
Exon 12.7	GAAGGTGTTCTGGATCGTCT	CTGTATACTGCTGCCATCCA
Exon 12.8	ACAAGATGTTCTTGCACAGG	TTATTTAAATGGCCCCAAAA
Exon 13.1	CATCCCTTACCCGTTATTTC	GGAATGAACTGCCCTGTTAC
Exon 13.2	CTGGGTCATCTCCACTGAAT	GCATGTTTAAATGATGCAAAA

Luciferase reporter assay

Cells were transiently transfected with either a BMPR2 plasmid or pmaxGFP control vector (from Amaxa GmbH, Cologne, Germany) in combination with the BRE-Luc vector[24] and a cytomegalovirus (CMV) promoter-driven Renilla luciferase vector (Promega, Madison, Wisconsin, USA) using Lipofectamine Plus (Invitrogen, Breda,

The Netherlands). After 24 hours of treatment with 100ng/ml of BMP2 luciferase activity was assayed according to the Dual-Glo Luciferase Assay System (Promega) protocol on a Lumat Berthold LB 9501 Luminometer (Berthold Technologies, Bad Wildbad, Germany). Each firefly luciferase value was corrected for its cotransfected CMV-driven Renilla Luciferase standard to correct for transfection efficiency or dilution effects.

Methylation analysis

Bisulfite treatment was performed using the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA). Bisulfite sequencing and MSP were performed as described previously[25, 26]. The region within the BMPR2 promoter CpG island examined by bisulfite sequencing contains 52 CpG dinucleotides and lies between -1051 and -497bp upstream of the predicted transcriptional start site[27]. MSP examined a region between -389 and -272bp. Primers were designed with MethPrimer software [28] and were designed to analyze the most proximal BMPR2 promoter region as predicted by Proscan[29] and previous functional analysis of the BMPR2 promoter[27]. This region also falls within a CpG island (Criteria used: Island size > 400, GC Percent > 50.0, Obs/Exp > 0.6). This is a region where methylation correlates with transcriptional repression in other genes as reviewed in [30]. Human genomic DNA from peripheral blood lymphocytes was used as the unmethylated control. Human genomic DNA treated *in vitro* with Sss I methyltransferase (New England Biolabs, Beverly, Massachusetts, USA) was used as positive control for the methylated reaction.

Statistical Analysis

Statistical analysis was performed using the Statistical Package of Social Science (SPSS) version 11.5 for Windows. The χ^2 test and Fisher exact tests were used as appropriate. $P < 0.05$ was considered statistically significant.

To look for the presence of subsets of carcinomas with comparable patterns of BMP-pathway protein expression and possible associations with mismatch repair status, two-way hierarchical cluster analysis of the TMA data was performed (JMP 6.0.0, SAS Institute, Cary, NC, USA). For the hierarchical cluster analysis positive expression of protein was coded as 1, weak as 0 and negative as -1. To avoid overinterpretation of the data, only first- and second-generation clusters (the first- and second-level branches of the tree) were used for classification purposes.

Results

Expression of the human BMP receptors and SMAD4 in colorectal cancer cell lines

Seven CRC cell lines were investigated for the expression of BMP receptors and SMAD4 at protein level by immunoblotting (Figure 1). BMPR1a is expressed at the protein level in 6 CRC cell lines and absent in DLD1. BMPR1b is expressed in all cell lines tested. BMPR2 protein expression, however, is reduced or absent in microsatellite unstable HCT116, DLD1, SW48 and LOVO cells while its expression is normal in microsatellite stable SW480, HT29 and CACO2 cells. Interestingly, the expression of SMAD4 shows a completely reverse pattern being negative in all MSS and positive in all MSI cell lines, as shown previously.[31]

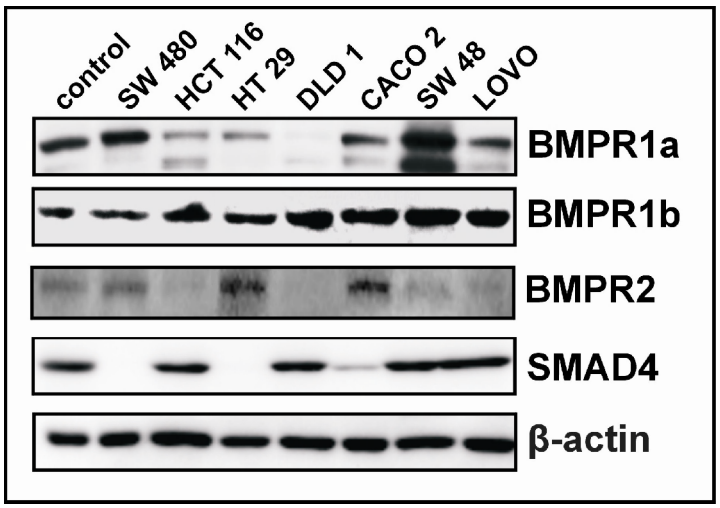

Figure 1. Immunoblot for BMPR1a, BMPR1b, BMPR2 and SMAD4 in seven colon cancer cell lines. 50µg of protein from cell lysates was loaded per lane and analyzed by blotting with the corresponding specific antibody. SAOS-2 osteosarcoma cells were used as a positive control. Equal loading was confirmed by showing equal β-actin levels.

As there is a clear association between altered BMPR2 protein expression and microsatellite instability, we checked the mRNA sequence of BMPR2 for the presence of microsatellites. We found a long 11 adenine tract in 3'-UTR of the BMPR2 and sequenced it as well as the A_{10} of TGFβR2 and A_8 of ACVR2 (Table 3), both coding microsatellites described by previous authors [2,8]. The results show that BMPR2 is mutated at A_{11} in all MSI cell lines tested and in none of the MSS cell lines (Figure 2 and Table 3). All our MSI cell lines also show frameshift mutations within A_{10} of TGFβR2 and A_8 of ACVR2.

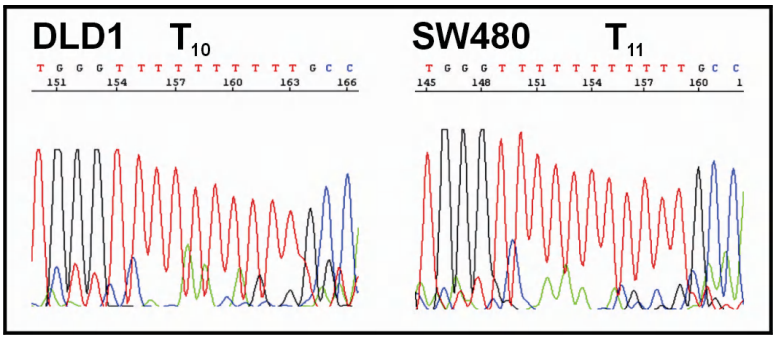

Figure 2. DNA sequence analysis of the A_{11} BMPR2 microsatellite tract in CRC cell lines. MSI DLD1 cells show a 1-base pair contraction to T_{10} in the reverse strand, while MSS SW480 cell line shows wild-type T_{11} in the reverse strand.

Table 3. BMPR2, ACVR2, TGFβR2 mutations in colon cancer cell lines

Cell line	MSI status	BMPR2 A_{11}	ACVR2 A_8	TGFβR2 A_{10}
SW480	MSI-	A_{11}	A_8	A_{10}
HT29	MSI-	A_{11}	A_8	A_{10}
CACO 2	MSI-	A_{11}	A_8	A_{10}
HCT116	MSI+	A_{10}	A_7	A_9
DLD1	MSI+	A_{10}	A_7	A_9
SW48	MSI+	A_{10}	A_7	A_9
LOVO	MSI+	A_8	A_7	A_9

To investigate mRNA levels of BMPR2, we performed RT-PCR in seven colorectal cancer cell lines. mRNA levels of BMPR2 as judged by semi-quantitative RT-PCR correlate well with the protein levels found by western blot with only one exception. SW48 cells show attenuated levels of BMPR2 protein but normal levels of mRNA (Figure 3A).

In order to exclude other possible mutations explaining BMPR2 loss, sequencing of the whole coding sequence of BMPR2 was performed in all 7 cell lines. We found a monoallelic frameshift mutation within a 7 adenine tract in exon 12 of BMPR2 in 2 MSI cell lines – HCT116 and LOVO (1742delA) leading to a stop codon. The sequencing results were also confirmed by the results of Genescan analysis of a fragment of BMPR2 containing this 7A microsatellite (Figure 3B). We then went on to check for this mutation in MSI high cancer specimens with attenuated BMPR2 expression (n=11) by direct sequencing. None of the 11 human cancer specimens contained this mutation (data not shown). In DLD-1 we found a monoallelic point mutation in exon 6 (631C>T) leading to a stop codon (data not shown).

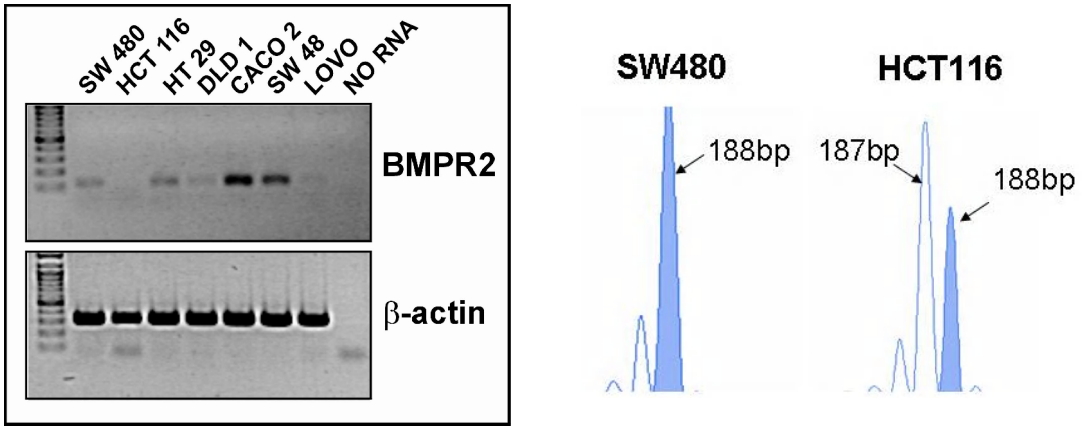

Figure 3. **A**, RT-PCR for BMPR2 in seven colon cancer cell lines. A single product of the expected size (115 bp) was seen as shown above. β-actin was used as a control. **B**, GeneScan analysis of the 188bp DNA fragment containing the 7A microsatellite. SW480 cells have only the wild type 7A tract and all fragments have length of 188bp, while HCT116 cells have a mutation in the 7A tract therefore fragments have 187bp and 188bp in length, suggesting a heterozygous pattern of this mutation.

An alternative explanation for BMPR2 protein and mRNA loss could be promoter hypermethylation leading to gene silencing in MSI cancers with a CpG island methylator phenotype and consequent loss of MLH1 expression. This mechanism would, however, not explain the loss of BMPR2 expression we see in tumors and cell lines with loss of MSH2 expression. We performed both methylation specific PCR and bisulfite sequencing for the BMPR2 promoter CpG island region. All seven CRC cell lines contain only unmethylated alleles of BMPR2 (Figure 4). Bisulfite sequencing in the 7 cell lines showed no methylation of the promoter CpG island within the examined region.

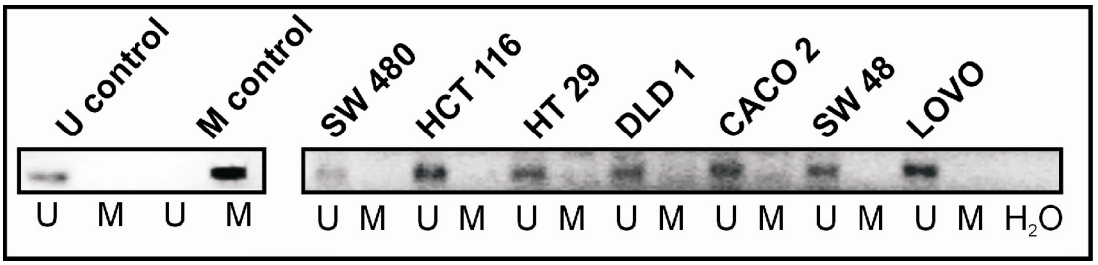

Figure 4. Methylation specific PCR analysis of the CpG island in the BMPR2 5'region in colon cancer cell lines. PCR products specific for unmethylated (U) and methylated (M) CpG sites were analysed in 2.5% agarose gels. U control – human genomic DNA from peripheral blood lymphocytes. M control - human genomic DNA treated in vitro with Sss I methyltransferase.

Expression of the BMP receptors and signal transduction elements in human sporadic colorectal cancers

To further investigate the role of BMP pathway in CRC we constructed a TMA. The clinical characteristics of the study population are listed in Table 1. Based on the absence of hMLH1 and hMSH2, as judged by immunohistochemistry, 13 patients (18.1%) were classified as MMR-deficient and 59 (81.9%) as MMR-proficient.

We performed immunohistochemical staining for BMPR1a, BMPR1b, BMPR2 and SMAD4 (Figure 5). MMR-deficient CRCs show frequent loss of expression of BMPR2 ($p<0.0001$) compared with MMR-proficient cancers (Table 4), in agreement with our immunoblot results. The frequency of the loss of BMPR1a and BMPR1b expression does not differ significantly between these two groups of cancers.

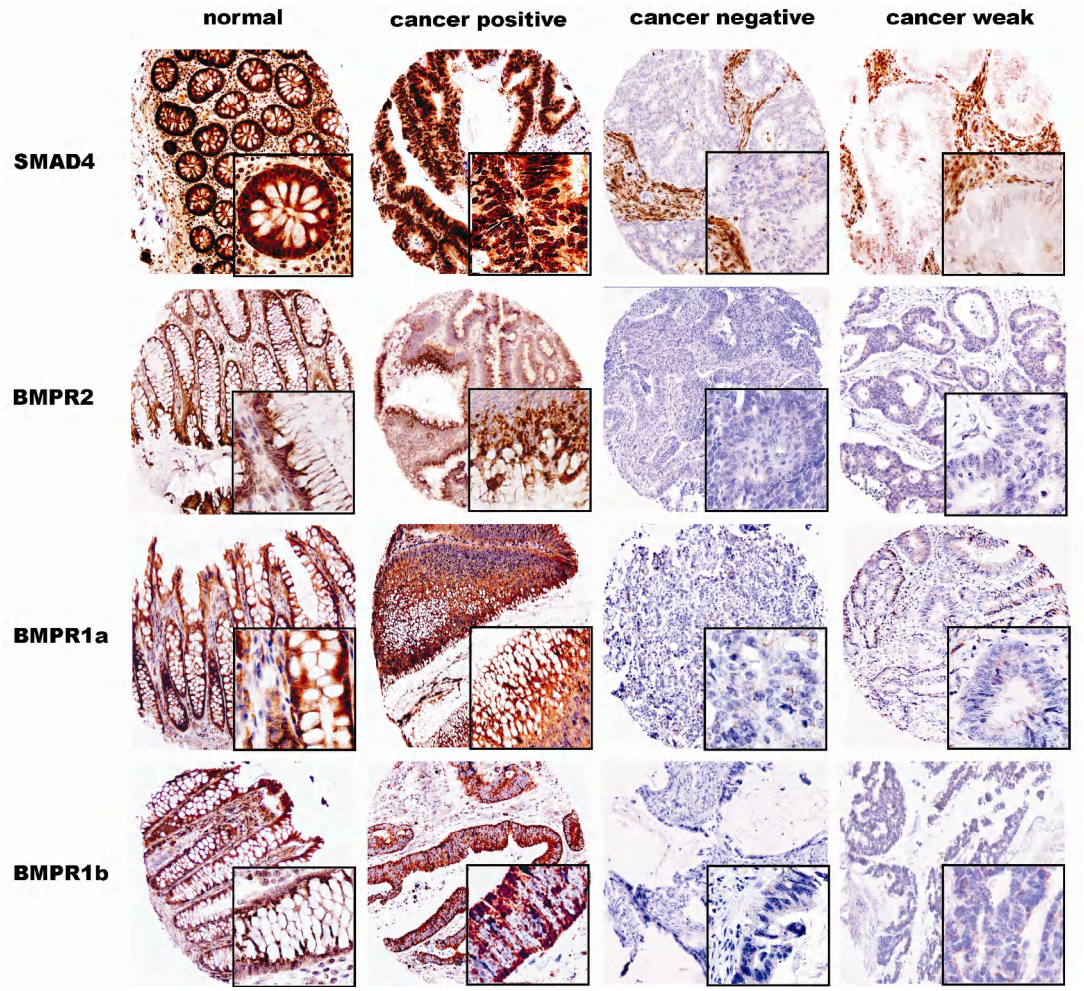

Figure 5. Immunohistochemistry for SMAD4, BMPR2, BMPR1a and BMPR1b in normal human colon and colon tumours. Representative cores from normal and cancer specimens to show positive, negative or weak expression of SMAD4, BMPR2, BMPR1a and BMPR1b.

Table 4. Expression of BMPRs and SMAD4 in CRC specimens

Characteristic	All cases (n=72)	MMR-proficient (n=59)	MMR-deficient (n=13)	*P*
BMPR1a, n (%)				
0 (negative)	2 (2.8)	1 (1.7)	1 (7.7)	0.082
1 (weak)	15 (20.8)	10 (16.1)	5 (38.5)	
2 (positive)	55 (76.4)	48 (83.9)	7 (53.8)	
BMPR1b, n (%)				
0 (negative)	4 (5.6)	3 (5.2)	1 (7.7)	0.148
1 (weak)	7 (9.9)	4 (6.9)	3 (23.1)	
2 (positive)	60 (84.5)	51 (87.9)	9 (69.2)	
BMPR2, n (%)				
0 (negative)	9 (12.5)	4 (6.8)	5 (38.5)	<0.0001
1 (weak)	19 (26.4)	13 (22.0)	6 (46.2)	
2 (positive)	44(61.1)	42 (71.2)	2 (15.4)	
SMAD4, n (%)				
0 (negative)	7 (9.7)	7 (11.9)	0 (0.0)	0.41
1 (weak)	19 (26.4)	16 (27.1)	3 (23.1)	
2 (positive)	46 (63.9)	36 (61.0)	10 (76.9)	

Percentages in brackets refer to the percentage of cases in each individual column. For example, BMPR1a staining was positive in 76.4% of all cancers, 83.9% of MMR-proficient cancers and 53.8% of MMR-deficient cancers. Statistical analysis was performed using the Fisher exact test for a significant difference between MMR-proficient and MMR-deficient cancers over the table of six values obtained for each characteristic.

Interestingly, we observed a mutually exclusive pattern of loss of expression of BMPR2 and SMAD4 in sporadic CRC (Table 5 and Figure 6). None of the cancers show negative expression of both BMPR2 and SMAD4. This is the same expression we observed in cell lines. Only 4% (3) of cancers have weak staining for both and 33% (24) cancers are positive for both. All other cancers exhibit either BMPR2 or SMAD4 loss.

Table 5. Mutually exclusive nature of BMPR2 and SMAD4 loss

n=72		BMPR2 negative	BMPR2 weak	BMPR2 positive
SMAD4	negative	0(0.0%)	0(0.0%)	7(9.7%)
	weak	3(4.2%)	3(4.2%)	13(18.1%)
	positive	6(8.3%)	16(22.2%)	24(33.3%)

Percentages in brackets refer to the percentage of the total number of cases

Negative/weak for both | Mutually exclusive | Positive for both

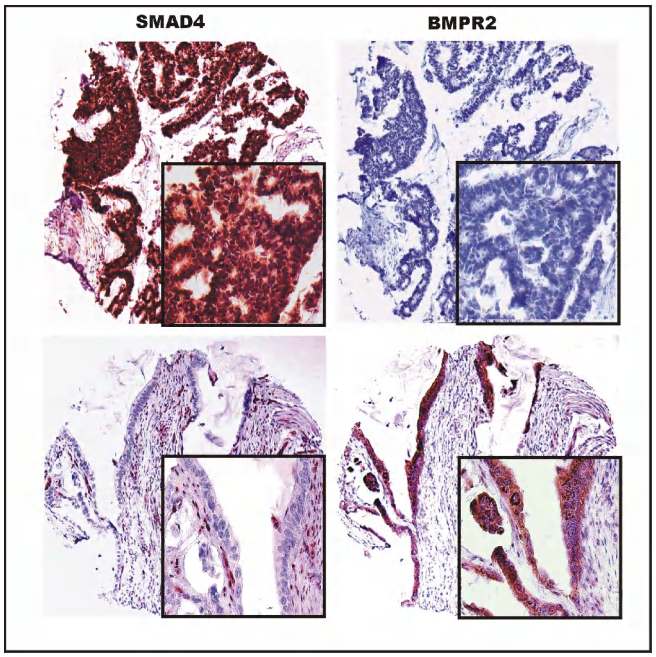

Figure 6. Immunohistochemistry for SMAD4 and BMPR2 demonstrating the mutually exclusive nature of SMAD4 and BMPR2 expression in colon cancer specimens. The same tumour stained for SMAD4 or BMPR2 is shown in each case. Note that the tumour with positive expression of SMAD4 has negative BMPR2 protein expression and the tumour with negative SMAD4 staining strongly expresses BMPR2 protein.

To clearly demonstrate the selective loss of BMPR2 expression in tumor tissue we performed IHC on sections containing normal and cancer tissue on one slide (Figure 7). Here it can be seen that loss of BMPR2 staining occurs specifically in the tumor.

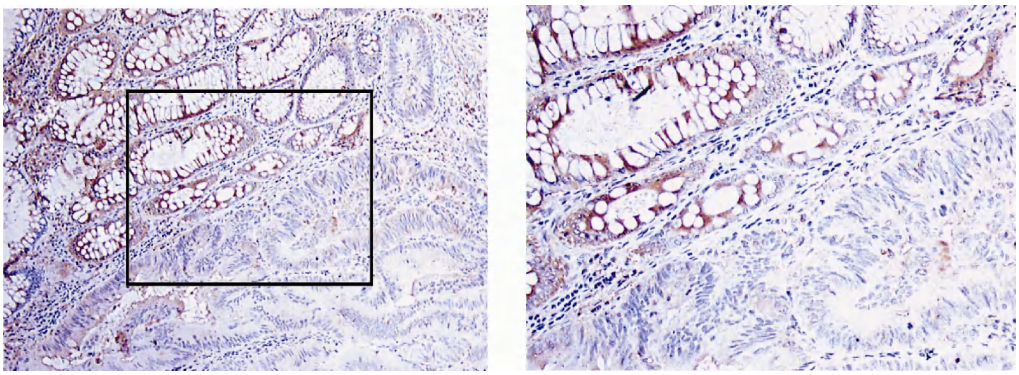

Figure 7. Selective loss of expression of BMPR2 in the MSI tumour. Note the expression of BMPR2 protein in normal colonic epithelium and loss of expression in the tumour. Stromal tissue is negative in both normal and carcinoma tissue.

To investigate the influence of the abnormal expression of BMPRs and SMAD4 on the activity of BMP pathway in sporadic colon cancers, we performed immunohistochemical staining for pSMAD1,5,8 (Figure 8) and found a very strong statistically significant association between defective expression of the BMP pathway components and negative nuclear staining for pSMAD1,5,8 ($p<0.0001$) (Table 6).

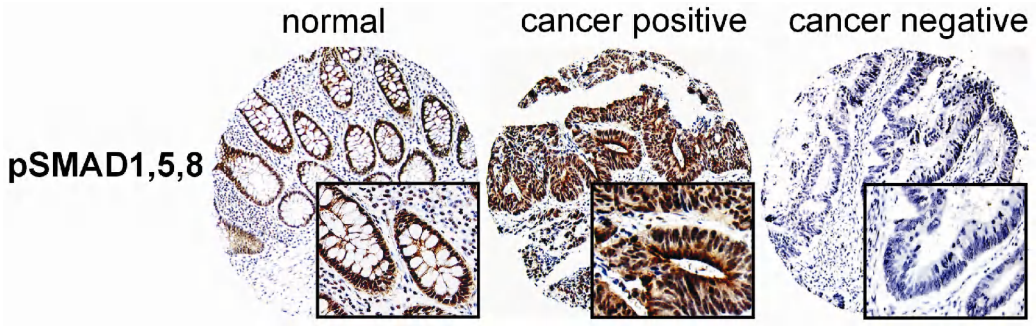

Figure 8. Immunohistochemistry for pSMAD1,5,8 in normal human colon and colon tumours. Representative cores from normal and cancer specimens to show positive and negative expression of pSMAD1,5,8.

Table 6. Association between nuclear localization of pSMAD1,5,8 and expression of the components of BMP-pathway

n=72	pSMAD1,5,8		*P*
	negative	**positive**	
BMP pathway defective[1]	44(61.1%)	6(8.3%)	<0.0001
Normal	6(8.3%)	16(22.2%)	

Percentages in brackets refer to the percentage of the total number of cases
[1]Defective means negative or weak score in any one staining (BMPR1a, BMPR1b, BMPR2 or SMAD4)

To look for further correlations we performed hierarchical cluster analysis of the TMA data. This results in 4 clusters of carcinomas (based on the first- and second-level branches of the tree) (Figure 9). Carcinomas in cluster C1 are the only carcinomas with normal BMP pathway activity as assessed by pSMAD1,5,8.

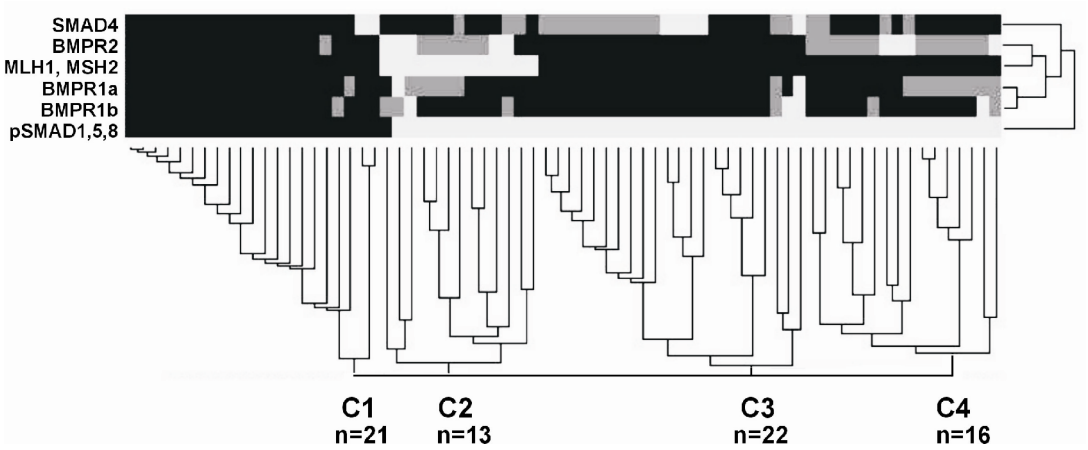

Figure 9. Heat map and dendrograms to show the results of hierarchical cluster analysis of the expression of MMR-proteins, BMPRs, SMAD4 and pSMAD1,5,8 in 72 CRC specimens. Rows represent protein expression and columns represent individual tumours. Black cells represent positive expression, grey cells weak expression and white cells negative expression.

They express all components of the BMP pathway and the mismatch repair proteins normally. Carcinomas in cluster C2 are MMR-deficient cancers with aberrant BMPR2 and positive SMAD4 expression as main characteristics. In contrast, cluster C3 consists of MMR-proficient cancers with aberrant SMAD4 and positive BMPR expression. Cancers in cluster C4 are also MMR-proficient but with negative BMPR2 and high numbers of BMPR1a negative expression. Clusters 2, 3 and 4 all show attenuated BMP-pathway activity with negative nuclear pSMAD1,5,8 expression.

Association between BMPR2 altered protein expression and A_{11} tract mutations of BMPR2 in microsatellite unstable colon cancers

We selected all (n=11) tumors with attenuated expression of BMPR2 and loss of MLH-1 or MSH-2 expression and firstly determined MSI status in these samples. All 11 tumors were MSI high (data not shown). We then sequenced A_{11} of BMPR2 (Figure 10), A_{10} of TGFβR2 and A_8 of ACVR2.

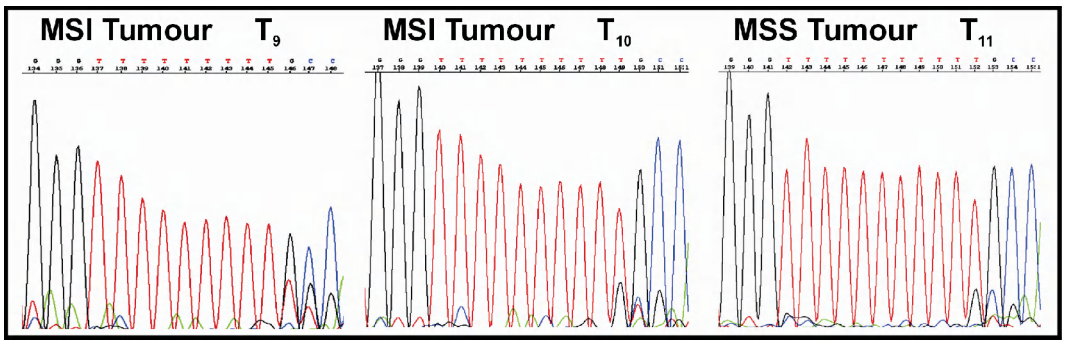

Figure 10. Representative examples of DNA sequence analysis of the A11 BMPR2 microsatellite tract in MSI and MSS tumours. The MSI tumours show a 1- or 2- base pair contraction to T10 or T9 in the reverse strand, while the MSS tumour shows wild-type T11 in the reverse strand.

Nine out of 11 colon carcinomas analyzed show deletions of one or two adenines from the A_{11} of BMPR2 (Table 7). In contrast, none of the MMR-proficient tumors with positive expression of BMPR2 used as control (n=8) show this deletion. We also analyzed the same samples for mutations within A_{10} of TGFβR2 and A_8 of ACVR2. Five out of 11 colon carcinomas had a one adenine deletion in the polyadenine tract of TGFβR2 and 6 out of 10 carcinomas had mutations in A_8 of ACVR2. None of the control tumors were mutated. Our results show a very tight correlation between attenuation of BMPR2 expression in MSI cancers and mutations in A_{11} of BMPR2.

Table 7. Summary of BMPR2, ACVR2 and TGFβR2 mutations in MMR-deficient colon cancer specimens with altered BMPR2 protein expression

BMPR2 A_{11}	ACVR2 A_8	TGFβR2 A_{10}
A_9	A_8	A_{10}
A_9	A_8	A_9
A_9	A_7	A_9
A_{10}	A_7	A_9
A_{10}	A_7	A_9
A_{10}	A_7	A_9
A_{10}	#	A_{10}
A_{10}	A_8	A_{10}
A_{10}	A_7	A_{10}
A_{11}	A_7	A_{10}
A_{11}	A_8	A_{10}

Insufficient DNA

Reconstitution of BMPR2 negative cells with BMPR2 leads to activation of the BMP pathway

To investigate the functional consequences of BMPR2 loss, we used a reporter of BMP pathway activity and a wt-BMPR2 plasmid. BMPR2 negative HCT116 cells were co-transfected with either the BMP pathway activity reporter construct (BRE-Luc) and GFP, or with BRE-Luc and wt-BMPR2. Reconstitution of HCT116 cells by transient transfection with wt-BMPR2 induces 20-fold activation of BMP pathway activity (Figure 11) even in the absence of exogenous BMP. The addition of exogenous BMP2 leads to only a modest further increase in BMP pathway activity.

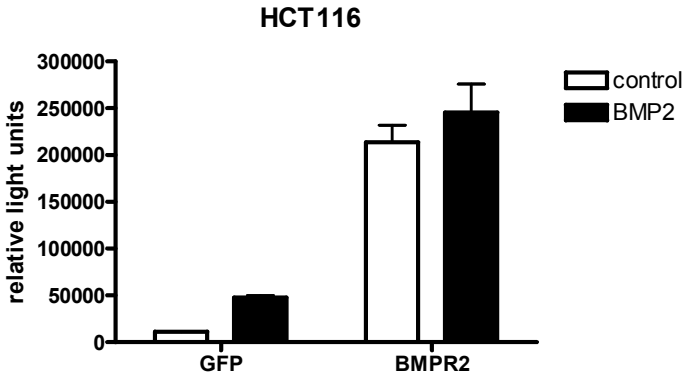

Figure 11 HCT116 cells were transfected with either BMPR2 or a GFP control vector and the activity of the BMP pathway was determined using the BRE-Luc reporter construct. After 24 hours of treatment with 100 ng/ml of BMP2, luciferase activity was assayed. GFP transfected cells show slight upregulation of BRE-luc activity after BMP2 treatment. Transfection with BMPR2 increases BRE-luc activity 20-fold. This high activity increases only modestly with BMP2 treatment.

Discussion

Several lines of evidence point towards an important role for the BMP pathway in CRC. Germline BMPR1a mutations lead to Juvenile Polyposis[10, 12, 32] and to a high risk of developing CRC[33], but whether the BMP pathway is involved in sporadic CRC has received little attention. In the only other study to address this question Beck et al[34] investigated the expression of BMP pathway components in 2 cell lines and 13 primary cancer specimens and concluded that the BMP pathway was intact. However, they did not investigate BMPR2 and did not specifically determine nuclear localization of pSMAD1,5,8 in their tumor specimens. This might explain the discrepancy between our findings and theirs. We find that MSI cell lines with loss of TGFβR2 and ACVR2 also have aberrant BMPR2 expression. These cells therefore have multiple defects in their ability to respond to the growth inhibitory stimuli of TGFβ superfamily ligands. In contrast, all MSS cell lines express type 2 receptors, but do not express SMAD4, a key molecule for TGFβ, activin and BMP pathways.

Evidence is accumulating that there is some redundancy at the receptor 2 level in TGFβ family signaling. Despite homozygous mutations of the TGFβR2, some colon cancer cell lines (LOVO and SW48) respond to TGFβ1, showing growth inhibition[35]. Interestingly, these are cell lines which show low but some level of BMPR2 in our immunoblots. Others have shown TGFβ specific gene activation on overexpression of ACVR2 in TGFβR2 and ACVR2 mutated colon cancer cell lines[36]. Similarly, BMPs can signal via ACVR2, have overlapping binding specificities with activins, and share some of the functional effects of activins [37,38] despite the fact that earlier binding studies indicated ligand-receptor 2 interaction specificity [39]. This would suggest that loss of one of the three type 2 receptors is insufficient for complete pathway disruption and that simultaneous loss of several of these receptors is required.

Heterozygous mutations in BMPR2 are associated with human familial and idiopathic pulmonary arterial hypertension and reduced levels of expression of BMPR2 protein have been found in the lungs of all pulmonary hypertension patients examined[40-42]. The human BMPR2 gene contains 7 kb of 3'-untranslated region (UTR) sequence which is thought to contribute to posttranscriptional regulation of mRNA turnover. It has been suggested that the reduced pulmonary endothelial expression of BMPR2 found in patients without the classical mutations in the coding regions might arise from

posttranscriptional downregulation of BMPR2 RNA, and it has been demonstrated that reduced BMPR2 expression could be enhanced by medicines increasing the posttranscriptional stability of BMPR2 mRNA[27]. Our finding that the long polyadenine tract in the 3'UTR of BMPR2 mRNA is mutated with high frequency in MMR-deficient cancers and coupled with altered BMPR2 expression might indicate a causative link. This is supported by two lines of evidence. Firstly, there is evidence for the selection of specific mutations within microsatellites in the 3'UTR of genes, suggesting that such mutations are not merely bystander events consequent to the mismatch repair defect. Secondly, an 8 base-pair 3'UTR microsatellite has been shown to control mRNA stability in a detailed functional study [43]. Investigation into how 3'UTR mutations in BMPR2 lead to loss of protein expression is beyond the scope of this paper. Our RT-PCR data show reduced mRNA levels of BMPR2 in essentially the same pattern seen at protein level, except for the SW48 cell line. This suggests that protein levels are being influenced at the mRNA level possibly by influencing mRNA stability. Our findings in SW48 cells could be explained by mechanisms operating at the level of translation. We excluded alternative explanations for BMPR2 loss such as coding sequence mutations and promoter methylation as far as possible. This is in agreement with the results of screens in CRC for mutations in gene coding sequences[44] and for genes silenced by methylation[45] which have not identified BMPR2. We do find heterozygous coding mutations in several MSI cell lines, but this is an unlikely explanation of loss of BMPR2 protein expression especially since we do not find this in patient specimens. However, in primary pulmonary hypertension heterozygous mutations are believed to be the cause of the abrogated BMPR2 expression seen[42].

To investigate promoter methylation we used two complementary techniques. Methylation specific PCR is highly sensitive but only assesses the methylation status of a very small number of CpG dinucleotides [25]. We have therefore also used direct bisulfite sequencing of the BMPR2 promoter region. This has the advantage of directly testing the methylation status of a large number of CpG dinucleotides although it is less sensitive to lower levels of methylation.

LOH due to chromosomal instability is not seen in MSI tumours making large deletions an unlikely explanation for BMPR2 loss in this tumour type[46]. Furthermore, we find a heterozygous pattern in the intronic BMPR2 sequence in most of the cell lines

suggesting the presence of two alleles, and studies of chromosomal instability in colorectal cancer have not revealed LOH in chromosome 2 [47].

We explored whether changes in the protein expression of BMPR2 or SMAD4 influence the activity of BMP-pathway as judged by pSMAD1,5,8 nuclear staining. This is the only available method to indirectly assess BMP-pathway activity in archived patient material. According to our data, 70% of sporadic colon carcinomas exhibit negative pSMAD1,5,8 nuclear staining. With the corroborating evidence provided by the SMAD4 and BMPR staining it seems likely that this indicates an inactive BMP-pathway in these tumors. Reduced BMP signaling may lead to reduced apoptosis and could be associated with increased tumorigenesis.

To confirm the functional importance of BMPR2 loss we reconstituted HCT116 cells by reintroducing wild-type BMPR2. This induces a 20-fold increase in BRE-Luc reporter construct activity indicating strong activation of the BMP pathway even without the addition of exogenous BMP. Similar results have previously been obtained when the SW480 (SMAD4-null) cell line was reconstituted with wt-SMAD4. Strong activation of BRE-Luc in the absence of exogenous BMP ligands was observed and only a modest further increase in BRE-Luc activation when treated with BMPs[34].

A standard method for data analysis looking for associations within small tables of data is Hierarchical cluster analysis. While most associations within our relatively small table of data from the TMA were immediately obvious, cluster analysis has the advantage of being unsupervised and comprehensive. The results visually demonstrate the association between aberrant BMPR2 expression and MMR-deficiency (cluster2) and the mutually exclusive nature of BMPR2 and SMAD4 loss (clusters 2, 3 and 4). Interestingly, carcinomas with abnormal SMAD4 expression express all of the BMP receptors normally (cluster3) while clusters 2 and 3 show multiple abnormalities at the receptor level, but almost none at the level of SMAD4. This coupled with the sequencing analysis of BMPR2, TGFβR2 and ACVR2 in cell lines, would support the hypothesis that while a mutation in one of the SMADs is sufficient to cause neoplasia, mutations at the TGFβ superfamily receptor 2 level must occur in combination. The mouse knockout data, where SMAD4 and SMAD3 knockouts show a cancer phenotype[6, 48] and mice with conditional TGFβR2 knockout from the colonic epithelium do not[3], further support this hypothesis

In summary our data demonstrate extensive loss of BMP signaling in sporadic colon cancer. The loss of BMPR2 expression in MSI cancer ties up neatly with the known defects in TGFβR2 and ACVR2 expression in MSI cancer and confirms the importance of the TGFβ superfamily in colorectal cancer.

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Chapter 4

Wnt and Bone Morphogenetic Protein Pathway activity is inversely correlated in polyps of patients with Familial Adenomatous Polyposis

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Abstract

Background and Aims: Studies of the transgenic mouse models have implicated interaction between the Wnt and the Bone Morphogenetic Protein (BMP) pathways in colorectal carcinogenesis, but the relationship between these signaling pathways in humans has not been studied. We investigated the Wnt-BMP interaction in different groups of inherited polyposis syndromes and in sporadic polyps.

Methods: We employed adenomas from patients with Hereditary Nonpolyposis Colorectal Cancer (HNPCC), Familial Adenomatous Polyposis (FAP), Juvenile Polyposis (JP) and sporadic polyps to assess the activity of the BMP and the Wnt signaling by immunohistochemistry. We investigated the activity of the Wnt pathway by evaluating nuclear β-catenin expression and the activity of the BMP signaling by nuclear pSMAD1,5,8 expression.

Results: The activity of the Wnt pathway is significantly upregulated in adenomas from FAP patients ($p<0.01$ vs JP and vs HNPCC). Six of 9 FAP adenomas show negative pSMAD1,5,8 expression and therefore inactivated BMP signaling and these patients have the highest scores for β-catenin. The remaining 3 FAP adenomas show local upregulation of Wnt signaling and co-localized losses of pSMAD1,5,8. Only around 25% of adenomas from HNPCC and JP patients have impaired BMP pathway activity and all sporadic polyps show intact BMP signaling.

Conclusions: Our data suggest that the Wnt pathway is overactive in the majority of adenomas from FAP patients. This is associated with inactivation of the BMP pathway, suggesting interaction of these pathways in this group of patients and indicating a possible role for this interaction in the process of colon cancer initiation and progression.

Introduction

Morphogens are crucial in embryonic gastrointestinal tract development and in maintaining the gut homeostasis by controlling stem cell division and cell fate choices[1]. The Wnt, TGF-β, BMP, and Notch morphogenetic pathways are the major players in homeostatic control of the adult epithelium. Several hereditary cancer syndromes deregulate these signaling cascades through mutational (in)activation. Moreover, these mutations often also occur in sporadic tumors.

Wnt signaling is required for the establishment of the progenitor compartment in the normal intestinal epithelium [2-4]. Wnt proteins also promote the terminal differentiation of Paneth cells, residing at the bottoms of the crypts [5]. Mutation in Adenomatous Polyposis Coli (APC) gene resulting in premature stop codons and in truncated APC protein[6] causes Familial Adenomatous Polyposis (FAP) syndrome [7]. The central tumor promoting effect of these mutations is to lead to overactivation of Wnt/β-catenin pathway, which controls the transcription of genes involved in cell adhesion, migration and proliferation. In the absence of Wnt signaling, β-catenin is phosphorylated by Glycogen Synthase Kinase – 3β (GSK-3β) in the so-called β-catenin degradation complex consisting of GSK-3β, APC, axin and β-catenin. The phosphorylation of β-catenin targets it for ubiquitin-mediated proteosomal degradation.

Truncating APC mutations prevent this process from happening. β-catenin therefore accumulates to high level in the cytoplasm, translocates to the nucleus, interacts with other transcription factors like T-cell factor/lymphoid enhancing factor (TCF/Lef) and activates transcription of target genes that favor cell growth and proliferation. The mutation and dysfunction of APC with consequent constitutive activation of the Wnt signal transduction pathway has been shown to be critical in the development of sporadic polyps and CRCs as well. Around 70% of sporadic CRCs have a somatic mutation of APC gene[8].

The Bone Morphogenetic Protein (BMP) pathway also plays a key role during gastrointestinal development[9] and in the maintainance of tissue homeostasis in the adult[10, 11]. BMPs initiate signaling by binding cooperatively to transmembrane serine-threonine kinase receptors types 1 and 2, triggering the phosphorylation and activation of the type 1 receptor by the type 2 receptor kinase. The activated type 1 receptor phosphorylates SMADs1,5,8 (receptor-regulated Smads(R-Smads)) and this permits

their association with SMAD4 (common-partner Smads (Co-Smads)). This heterodimeric complex then translocates to the nucleus and modulates the transcription of genes specific for the BMP pathway[12, 13].

Juvenile Polyposis (JP) is an autosomal dominant disease in which individuals are predisposed to hamartomatous polyps and gastrointestinal cancer [14, 15]. Genetic studies of affected families have revealed germline mutations in SMAD4 and BMPR1a in approximately 20% of patients for each gene [16-18]. While SMAD4 is a central element in the signal transduction pathway of all TGFβ superfamily members, the finding of frequent BMPR1a mutations in JP is important evidence pointing specifically to a likely role of BMPs in colonic neoplasia.

Previous studies have shown interactions between Wnt and BMP signaling pathways. In Xenopus Smad4-Lef1/Tcf and Smad4/ β-catenin molecular complexes have been demonstrated, and it has been shown that Smad4 and Lef1 cooperate to control the expression of the homeobox gene *Xtwn* in Spemann's organizer[19]. BMP counteracts proliferation promoted by Wnt in spinal cord neuroepithelial cells and Wnt antagonizes BMP-dependent neuronal differentiation [20]. In mice, deficiency of one Smad4 allele synergizes with deficiency of APC to increase the number and invasiveness of intestinal tumors [21]. Haramis et al. showed that villin-noggin transgenic mice in which BMP expression is completely abrogated develop polyps with high levels of nuclear β-catenin [22]. Further, BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt- β-catenin signaling and BMPR1a mutant mice develop intestinal polyposis with nuclear accumulation of β-catenin [23].

Inactivation of the MMR system due to germline mutations in Mismatch Repair (MMR) genes cause Hereditary Nonpolyposis Colorectal Cancer (HNPCC) [24, 25]. Uncorrected mutations during replication due to an inactive MMR system lead to the genome wide accumulation of mutations within short repetitive gene sequences (microsatellites) and to microsatellite instability (MSI) [26]. HNPCC is an autosomal dominant condition, giving rise to early onset CRC with a predisposition for proximal colonic involvement. HNPCC is responsible for 3-5% of CRCs. Somatic inactivation of the MMR system is found also in approximately 15% of sporadic colon cancers. The classical example of a gene that is affected by MSI is the Transforming Growth Factor Receptor 2 (TGFβR2)[27].

Despite all the evidence for the importance of the Wnt and BMP pathways in colon cancer, and data from mice studies suggesting interaction between these two signaling pathways, the BMP-Wnt interaction has not been studied in the human colon. In this pilot study we evaluated the activity of the BMP pathway and Wnt signaling in the small groups of patients with rare gastrointestinal polyposis syndromes: FAP, HNPCC and JP as well as in sporadic polyps. We report the mutually exclusive activity of BMP and Wnt signaling in FAP patients and JP patients and no visible associations in the other groups of polyps.

Materials and methods

Selection of patient material

Formalin-fixed, paraffin-embedded tissues from 35 patients – 7 HNPCC, 9 FAP, 10 JP and 9 SP cases between the years of 1996 and 2004 were used for the study. Blocks were selected from the archives of the Pathology Department at the Academic Medical Centre, Amsterdam. The study was approved by the investigator's institutional review board. Our study included 15 men (42.9%) and 20 women (57.1%), their ages ranging from 10 to 88 years, with a mean (±SD) of 53.1 (23.3) years and median 57 years.

Immunohistochemistry for phosphorylated SMAD1,5,8 (pSMAD1,5,8)

Sections were first deparaffinised and immersed in 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed by boiling slides for 10 min in 0.01 mol/L sodium citrate, pH 6.0. Nonspecific binding sites were blocked with TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween 20, pH 8.0) for 30 min. Slides were incubated with primary rabbit polyclonal antibodies to Phospho-Smad1/5/8 which recognizes the doubly phosphorylated forms of Smad1 (Ser463/465), Smad5 (Ser463/465), and Smad8 (Ser426/428) (Cell signaling, Beverly, MA, USA) at a concentration of 1:50 overnight at 4°C in PBS with 0.1% Triton and 1% bovine serum albumin, then incubated with biotinylated secondary goat anti-rabbit (Dako, Glostrup, Denmark) at a concentration of 1:200 at room temperature for 1 hour in PBS with 10% human serum. Slides were then incubated with HRP-conjugated ABComplex (Dako, Glostrup, Denmark) for 1 hour and peroxidase activity was detected with “Fast DAB” (Sigma, St. Louis, MO, USA) used according to the manufacturer’s instructions.

Immunohistochemistry for β-catenin

TMA blocks were sectioned (4 μm), deparaffinised, blocked for endogenous peroxidase activity by immersion in 0.3% H_2O_2 in methanol for 20 min and heat treated at 100°C in Tris (pH 9) for 10 min (antigen retrieval). Nonspecific binding sites were blocked TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween 20, pH 8.0) for 30 min. Slides were incubated with primary antibody overnight at 4°C in PBS with 0.1% Triton and 1% bovine serum albumin. The Powervision+poly-HRP detection system (ImmunoVision Technologies, Daly City, CA, USA) was used to visualize the antibody binding sites with 3,3-diamino-benzidine+ as a chromogen. Sections were counterstained with haematoxylin. Negative control sections for all antibodies were processed in an identical manner after omitting the primary antibody and showed no staining.

Analysis of immunohistochemistry

The cellular localization and pattern of immunoreactivity were examined by two investigators independently in a blinded fashion. For the evaluation of the pSMAD1,5,8 expression only nuclear staining was assessed and was scored as shown in Table 1. Scores of 0 and 1 were considered negative for pSMAD1,5,8 expression, and scores of 2 and 3 were considered positive.

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

For β-catenin expression positive nuclei were scored in 4 power fields 100X of each polyp specimen. Then the amount from these scores was divided by 4 and the results represent the mean of each specimen.

Statistical analysis

Statistical analysis was performed using the Statistical Package of Social Science (SPSS) version 11.5 for Windows. Data were analyzed by Kruskal-Wallis test with Mann-Whitney U as post test. $P < 0.05$ was considered statistically significant.

Results

The activity of the Wnt and BMP pathways in normal human colon

In the majority of normal colonic epithelial cells, β-catenin exists in two cellular pools, a structural pool important for the cytoskeleton and a free cytoplasmic pool. It is this latter pool that plays a part in the Wnt pathway. We performed immunohistochemistry for β-catenin on normal human colon tissue. We see a strong intracytoplasmic and membrane staining for β-catenin in normal colon. As reported previously, only few cells at the colonic crypt base, presumably stem cells, show positive nuclear β-catenin staining and active Wnt signaling (Figure 1).

The positive nuclear staining for pSMAD1,5,8 indicates active BMP signaling. We show here that pSMAD1,5,8 is expressed in human colon at the epithelial surface with a gradient decreasing downwards towards the crypt base. We see cytoplasmic staining as well as clear nuclear staining for pSMAD1,5,8 in normal human colon indicating active BMP signaling (Figure 1).

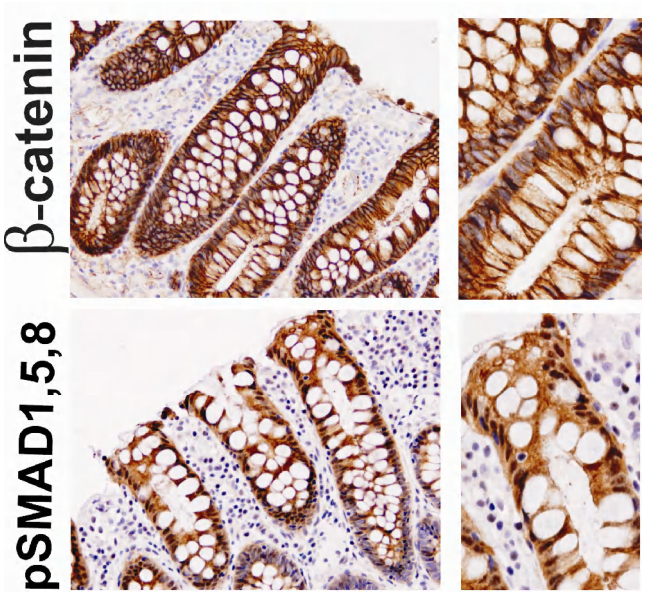

Figure 1. The expression of β-catenin and pSMAD1,5,8 in normal human colon. Magnifications 200X and 400X are shown.

The activity of the Wnt pathway in different groups of adenomas

In order to evaluate the activity of the Wnt pathway in different groups of adenomas, we performed immunohistochemistry for β-catenin and counted positive nuclei in adenomas. As perhaps expected, the highest Wnt pathway activity is observed in FAP patients (Figure 2A,B). As shown in Figure 2B, the mean score for this group is 110 positive nuclear per 100X power field. The amount of positive nuclei in adenomas from FAP patients is significantly higher then in JP adenomas (mean score 14) and in HNPCC adenomas (mean score 15) ($p<0.01$, Mann-Whitney U test), but not significantly different form sporadic polyps (mean score 49).

The activity of BMP pathway in different groups of adenomas

To investigate the activity of the BMP pathway in human adenomas we performed analysis of the nuclear localization of pSMAD1,5,8. The results reveal that only 2 adenomas from the HNPCC group (28,5%), 3 adenomas from JP group (30%), and no adenomas from sporadic polyp group are negative for nuclear pSMAD1,5,8. In contrast, the majority of FAP adenomas (6 out of 9 (70%) do not express nuclear pSMAD1,5,8 and therefore have inactivated BMP signaling (Figure 2A, C).

The activity of the BMP pathway in different groups of adenomas

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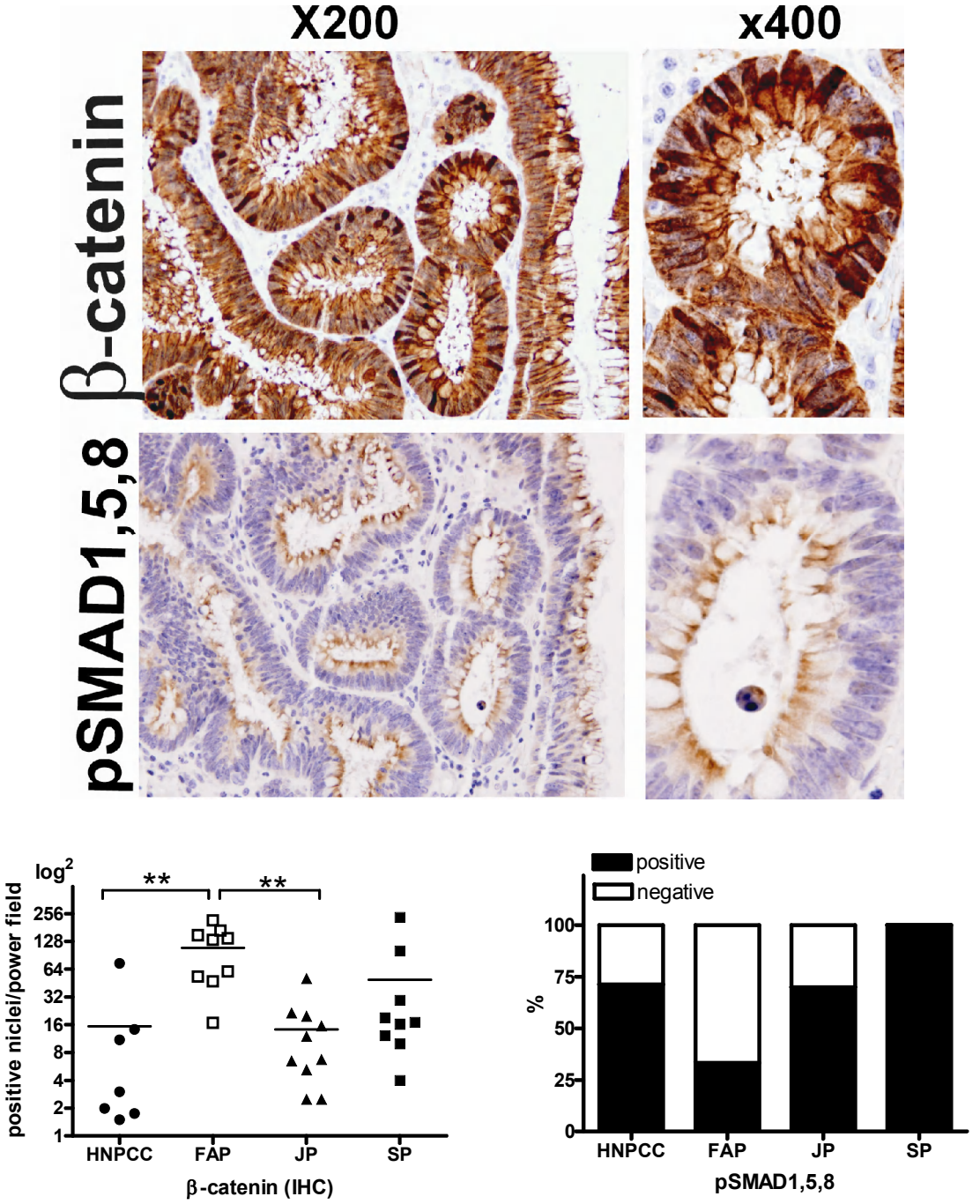

Figure 2. A, Immunohistochemistry for β-catenin and pSMAD1,5,8 in a FAP associated adenoma . The adenoma exhibits large numbers of positive nuclei for β-catenin and simultaneous negative pSMAD1,5,8 nuclear staining. **B**, Immunohistochemical analysis of β-catenin expression in four different groups of polyps. ** p<0.01. **C**, , Immunohistochemical analysis of pSMAD1,5,8 expression in four different groups of polyps.

Mutually exclusive pattern of Wnt and BMP pathway activity.

It has been recently shown in transgenic mice models that inactivation of the BMP pathway leads to polyp formation and upregulation of Wnt signaling in these polyps[22, 23]. To investigate weather aberrant accumulation of β-catenin is associated with the activity of BMP pathway in human adenomas, we decided to elucidate whether there was any association between activities of these two pathways in all groups of polyps. Interestingly, our results show that 6 adenomas from FAP patients with inactivated BMP signaling have the highest scores for nuclear β-catenin (Figure 2A, Table 2). Moreover, the remaining 3 FAP adenomas show local losses of pSMAD1,5,8 and co-localized overactivation of Wnt signaling (accumulation of β-catenin) (Figure 3). Similar results are seen in the JP polyposis group, however not to such an extent. All three JP adenomas showing inactivated BMP signaling have relatively high scores for nuclear β-catenin for this group (20; 22 and 51, mean = 14). In the HNPCC group we have not found any relationships between the activity of the BMP and Wnt pathways. In the sporadic polyp group all adenomas express nuclear pSMAD1,5,8 and therefore we see no association between BMP and Wnt signaling in this group of adenomas.

Table 2. The activity of BMP (nuclear pSMAD1,5,8) and Wnt (β-catenin) pathways in adenomas from 9 FAP patients

N	pSMAD1,5,8	β-catenin (positive nuclei/100xpower field)
1	n	61
2	p+local loss	53
3	n	138
4	n	169
5	n	149
6	n	216
7	p+local loss	47
8	p+local loss	17
9	n	132

Discussion

The importance of Wnt signaling in the initiation of colon cancer has been established by numerous studies (reviewed in [28]). There is accumulating evidence for the role of BMP signaling in the process of colon cancer initiation and progression and a tumor suppressor pathway[11, 16, 22]. In Chapter 2 and 3 of this book we have shown that loss of BMP signaling correlates tightly with the progression of adenomas to cancer and that the BMP pathway is inactivated in the majority of colorectal carcinomas. Interactions

between the Wnt and BMP signaling pathways have been reported during development[19] and in the transgenic mice models[22, 23].

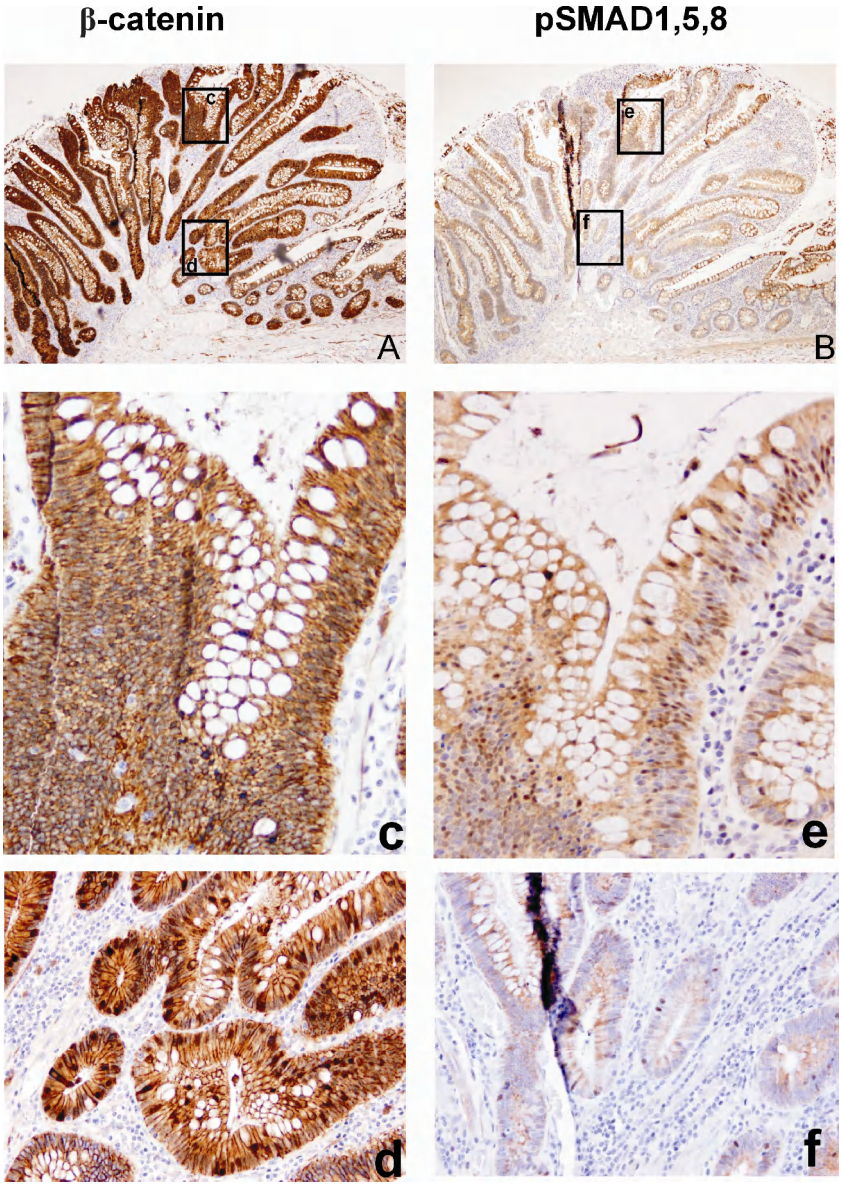

Figure 3. Immunohictochemistry for β-catenin (A, c, d) and pSMAD1,5,8 (B, e, f) in an adenoma from a FAP patient. This adenoma shows strong cytoplasmic and membrane staining for β-catenin (c) co-localized with positive nuclear pSMAD1,5,8 expression (e), but only local accumulation of nuclear β-catenin (d) which is co-localized with loss of nuclear pSMAD1,5,8 expression (f). Original magnification: A, B 5X; c-f: 20X.

In this pilot study we investigated associations between the activities of the BMP and Wnt signaling in four different groups of polyps. The use of tissue from patients with inherited colon cancer syndromes allows us to examine the consequences of a known genetic defect in human material and gives us the possibility to elucidate a "cause-

effect" relationship between the activities of these two pathways. We know that patients with FAP have an overactive Wnt pathway due to germline mutation in APC gene[6, 7]. Around 50% of JP patients have inactivated BMP signaling due to germline mutations in the BMPR1a and SMAD4 genes[16-18]. HNPCC patients have a defective MMR system and inactivated TGFb signaling due to mutation in microsatellite in the BMPR2 gene[24, 27].

We report that Wnt is more highly generally activated in FAP adenomas than adenomas with other genetic backgrounds. The majority of adenomas in this group show inactivated BMP signaling. Moreover, the adenomas showing negative pSMAD1,5,8 nuclear staining possess the highest activity of the Wnt pathway. The remaining adenomas in this group have local losses of nuclear pSMAD1,5,8 and co-localized accumulation of nuclear β-catenin. These observations indicate that there is a clear inverse association between BMP and Wnt signaling in adenomas from FAP patients. Presumably, in FAP adenomas β-catenin dysregulation and nuclear translocation due to inactivating mutation in APC gene occur first with subsequent downregulation of proapoptotic and antiproliferative BMP signaling.

FAP adenomas represent the familial counterparts of sporadic polyps, the only difference is that neoplasia in FAP is "one step ahead" because of the first mutation existing in the germline. In our study we also see the similarity between FAP adenomas and sporadic polyps regarding the activity of the Wnt pathway. While nuclear β-catenin expression is significantly higher in FAP adenomas then in JP and HNPCC adenomas, it is not significantly different from that seen in sporadic polyps. Controversially, the majority of FAP adenomas exhibit an inactivated BMP pathway, while all adenomas in the sporadic polyp group show intact BMP signaling. This is in agreement with data we present in chapter 2 showing that the inactivation of BMP signaling occurs in the transition point from sporadic adenomas to sporadic cancer. FAP adenomas possibly represent more advanced lesions and are closer to cancer then early sporadic adenomas.

In the group of JP adenomas we also see the association between inactivation of the BMP pathway and upregulation of the Wnt pathway. However, the scores for nuclear β-catenin in this group of adenomas are significantly lower then in FAP adenomas. These data suggest that inactivation of BMP signaling as a first step only leads to modest upregulation of the Wnt pathway. These data are supported by mice transgenic models,

where inactivation of BMP signaling leads to upregulation of Wnt pathway and to adenoma formation[22, 23].

We have not found any associations between BMP and Wnt pathways activities in adenomas from HNPCC patients, but analysis of a larger cohort of patients is needed to make a final conclusion. Additional work on *in vitro* cell line models with experiments elucidating how the activity/inactivation of one of the pathways influences the activity of another would help us to expand our knowledge about the BMP-Wnt interaction and its role in carcinogenesis further.

Based on the results of our study, we hypothesize that there is a reciprocal interaction between BMP and Wnt signaling. The proapoptotic BMP signaling counterbalances proproliferative Wnt and if BMP signaling is disrupted, as in case of JP adenomas, increased accumulation of nuclear β-catenin takes place even without mutations in APC or β-catenin. The Wnt pathway is overactive in FAP adenomas and this leads to downregulation of the BMP pathway and to a further imbalance between proliferation and apoptosis, giving survival advantage to these cells and playing an important role in colon cancer initiation and progression.

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Chapter 5

Altered bone morphogenetic protein signaling in the Helicobacter pylori-infected stomach

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Abstract

Background and Aims

Morphogens regulate epithelial cell fate decisions in the adult gastrointestinal tract. We hypothesized that influx of inflammatory cells into the lamina propria may disturb the normal expression gradients of morphogens (morphogenetic landscape) in gastrointestinal epithelia.

Methods

Changes in the activity of the bone morphogenetic protein (BMP) pathway in normal and *Helicobacter pylori* infected gastric mucosa were examined by immunohistochemistry and analyzed statistically.

Results

The BMP receptors, the activated (phosphorylated) form of the intracellular BMP signal transduction protein SMAD1, and BMP target ID2 all localize to gastric epithelial cells that are at the end of the axis of epithelial renewal in normal mucosa. Colonization of human gastric mucosa with *H. pylori* was associated with an increase in BMP2 expression due to influx of inflammatory cells that produce BMP2. No BMP4 was detected in the normal antrum, focal infiltrates of BMP4-expressing cells were found in the *H. pylori*-infected stomach. This influx of BMP-expressing cells was associated with an increase in epithelial BMP signaling. A shift in activity of the BMP pathway was observed towards the precursor cell compartment (isthmus) of the gastric units.

Conclusions

H. pylori infection results in an influx of inflammatory cells that disturb the normal activity gradient of a morphogenetic pathway with an established role in epithelial cell fate regulation. The data suggest that morphological changes in epithelial histology may result from alterations in the morphogenetic landscape secondary to changes in the cellular composition of the lamina propria.

Introduction

Maturation of epithelial cells is regulated by extrinsic signals that couple cellular position along the axis of renewal to the appropriate cellular differentiation state. During embryogenesis, such extrinsic signals are provided by soluble signaling molecules termed morphogens. These morphogens fall into a small number of families with common signaling receptors such as the Hedgehog, Wnt, fibroblast growth factor, and transforming growth factor (TGF) families[1]. Several morphogens and their antagonists are expressed in a developing tissue and form a matrix of activity gradients, or a morphogenetic landscape, that enables cells in the tissue to adopt the appropriate cell fate for a given cellular position. Recent studies suggest that morphogens play a similar role in epithelial differentiation in the adult gastrointestinal (GI) tract and that the deregulation of the activity of these morphogens plays an important role in the development of GI cancers[2, 3].

During inflammation of the GI mucosa, the dynamics of epithelial turnover are changed, with increased cell turnover and disturbance of the epithelial differentiation programme such as in metaplastic change[4]. Inflammatory cells are known to produce the same morphogens that regulate epithelial cell fate[5, 6]. We therefore hypothesized that influx of inflammatory cells may disturb the normal expression gradient of morphogens in GI epithelia. *H. pylori* infection of the antral gastric mucosa can serve as a model to study such changes. The gastric epithelium of the antrum consists of tubular units with a stem cell somewhere one-third up this unit in a precursor cell compartment that is called the isthmus. The precursor cells in the isthmus populate two functionally different compartments of daughter cells that migrate in opposite directions. Cells that migrate down (to the bottom of the antral unit) form the gland compartment comprising endocrine cells and mucinous cells that secrete mucin 6[7, 8]. Cells that migrate up (towards the lumen) form the pit compartment with pit cells (or foveolar cells) that secrete mucin 5AC[8, 9]. Infection with *H. pylori* leads to chronic inflammation of the antral mucosa with an inflammatory infiltrate that accumulates mainly under the superficial mucosa and around the isthmus of the gastric units. This inflammatory process is associated with changes in epithelial turnover and the development of intestinal metaplasia, gastric gland atrophy, and finally, in some patients, the development of epithelial dysplasia and gastric cancer[4].

We have previously demonstrated that members of the BMP family are expressed in the adult stomach[10]. BMPs are part of the large TGF morphogen family. BMPs signal through the membrane-bound heteromeric receptor complex of type I and II serine/threonine kinase receptors. Activated receptors phosphorylate BMP-specific SMADs, which form complexes with SMAD4. The SMAD complexes translocate into the nucleus, where they act as transcription factors that induce changes in the expression of target genes such as inhibitor of differentiation-2 (ID2)[11, 12]. We have recently shown that BMP signaling acts on terminally differentiated colonic enterocytes, inducing apoptosis in human adult colon epithelium[13]. Here we use antibodies against the BMP signaling receptors, the phosphorylated (active) form of downstream receptor associated SMAD1, and transcriptional target ID2 to identify the target cells of BMP signaling in the human gastric mucosa. We examine possible changes in the activity of the BMP pathway and its target cells in the *H. pylori*-infected gastric epithelium.

Materials and Methods

Antibodies

The antibodies are listed below. A rabbit polyclonal anti-Id2 (sc489, 1 : 1000) and a goat polyclonal anti-BMPR1a (1 : 250, E-16)[14] were from Santa Cruz (Santa Cruz, CA, USA). A mouse monoclonal anti-BMP2 (MAB355, 10 µg/ml, 1 : 1000), a mouse monoclonal anti-BMP4 (MAB757, 10 µg/ml, 1 : 50), a goat polyclonal anti-BMPRIa (AF346, 10 µg/ml), and a mouse monoclonal anti-BMPRIb (MAB505, 10 µg/ml, 1 : 500), were all from R&D Systems; a rabbit polyclonal anti-pSMAD1 (9511, 1 : 40) and anti-cleaved caspase-3 Asp175 (9661, 1 : 150) were from Cell Signaling (Beverly, MA, USA).

Immunohistochemistry

Carefully orientated formalin-fixed, paraffin-embedded human biopsy specimens of the antral mucosa of 70 consecutive patients were obtained from the archives of the Department of Experimental Internal Medicine, Academic Medical Center, Amsterdam, The Netherlands. Biopsies were collected in the context of a study of patients with dyspeptic complaints that was approved by the Ethical Board of the Academic Medical Center. The presence of *H. pylori* in these biopsies was assessed by scoring routine haematoxylin and eosin (H&E) sections, and confirmed by culturing the bacterium. An

independent pathologist scored the severity of inflammation according to the Sydney classification system[15]. Immunohistochemistry was performed as described in detail previously[16]. Briefly, sections (4 µm) were deparaffinized and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched; antigen retrieval was performed by heating for 10 minutes at 100 °C in 0.01 M sodium citrate; and non-specific staining was reduced by a blocking step with TENG-T [10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (v/v) Tween 20, pH 8.0] for 30 minutes. For BrdU visualization, sections were incubated in 2 N HCl at 37 °C for 1 h and thereafter washed in boric acid (pH 8.5) before the blocking step. The primary antibody was applied in PBS containing 1% bovine serum albumin and 0.1% Triton X-100, and incubated overnight at 4 °C. The following day, a three-step detection method was used using a biotinylated goat anti-rabbit (E0432, 1 : 200), rabbit anti-goat (E0466, 1 : 200), or goat anti-mouse (E0433, 1 : 200), all from DAKO (Glostrup, Denmark). Sections were incubated with HRP-conjugated ABComplex (DAKO) for 60 minutes and peroxidase activity was detected with 3,3-diaminobenzidine (Fast DAB; Sigma, St Louis, MO, USA) used according to the manufacturer's instructions in 0.05 M Tris, pH 7.4. Cells were briefly counterstained with haematoxylin solution according to Mayer (Fluka, Steinheim, Germany), dehydrated in graded alcohols, and mounted in Entellan (Merck, Darmstadt, Germany) under coverslips. Controls consisted of omitting the primary and secondary antibodies and use of appropriate Ig control.

Apoptotic cell death was determined by a modified TUNEL technique according to the manufacturer's instruction (*In Situ* Cell Detection Kit POD, Roche, Penzberg, Germany). Briefly, sections (4 µm) were deparaffinized and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched in 0.3% H_2O_2 in 50% ethanol/PBS for 30 minutes. After washing once with PBS, sections were permeabilized for 10 minutes with 50 µg/ml proteinase K in 10 mM Tris, pH 7.5 at room temperature. Sections were washed in PBS and blocked in TENG-T supplemented with 1 : 100 goat serum to reduce background staining. The sections were equilibrated in label buffer diluted 1 : 10 in 100 mM Tris, pH 7.5 in a humidified chamber for 1 h at 37 °C. After washing in PBS, sections were incubated in POD solution diluted 1 : 15 in 100 mM Tris (pH 7.5) and 250 mM NaCl for 30 minutes in a humidified chamber for 1 h at 37 °C. Peroxidase activity detection, counterstaining, dehydration, and mounting steps were as

described previously[16].

Scoring and statistical analysis

Counting of BMP2-positive cells was performed in a blinded manner. Three digital pictures were taken of each section at 40x (BMP2) magnification containing at least five antral units. Sections were scored with the use of an image analysis program (Image-ProPlus, MediaCyberNetics, Silver Spring, MD, USA). Data are presented as mean ± SEM. Comparisons between groups of data were made using a one-way analysis of variance followed by a Tukey *post-hoc* test. *p* values less than 0.05 were considered as statistically significant.

Results

Expression of BMP pathway components in gastric epithelium

We first examined the localization of BMP pathway components in histologically normal human gastric mucosa in at least ten patients per protein by immunohistochemistry. The expression pattern observed was consistent between different patients. The specificity of antibodies used was confirmed previously[13, 14, 17]. We investigated the expression patterns of the BMP signaling receptors BMPR1a, BMPR1b, signal transduction proteins pSMAD1, and transcriptional target ID2 as a read-out for the activity of the BMP pathway in the stomach. BMPR1a was expressed mainly by epithelial cells in the glands of the gastric antrum (Figures 1A and 1B), whereas BMPR1b was expressed by epithelial cells in both pit and gland (Figures 1C-1E). The expression of the BMP signaling receptors showed a bi-directional gradient along the bi-directional axis of renewal, with low expression in the precursor cell compartment at the isthmus of the gastric unit and increasing expression towards the top of the gastric pit and the base of the gastric gland, with the highest expression in the cells that were at the end of the axis of epithelial renewal. The staining pattern for pSMAD1 in epithelial cells was in accordance with the expression of the BMP signaling receptors. The pSMAD1 antibody stained mainly the nuclei of epithelial cells at the base of the gastric gland and the top of the gastric pit (Figures 1F-1H). Finally, the expression of the BMP target ID2 mirrored the expression pattern of pSMAD1, with

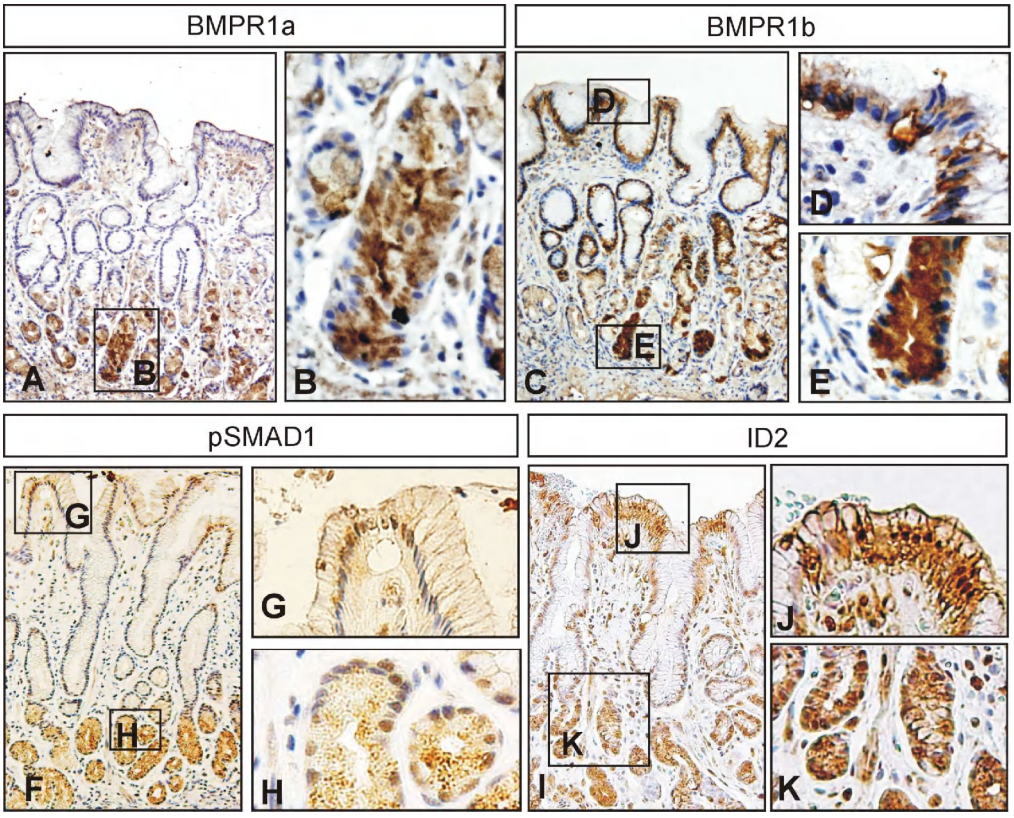

Figure 1 Immunohistochemical analysis of BMP signaling in human gastric antral epithelium. BMP receptor 1a (**A**) was expressed in gastric gland cells: the area in the box is enlarged in panel B. BMPR1b (**C**) was expressed by both pit (**D**) and gland (**E**) epithelial cells. Phosphorylated SMAD1 (**F**) was detected in the terminally differentiated pit (**G**) and gland cells (**H**). Expression of BMP target ID2 (**I**) was found in the same cells that showed phosphorylation of SMAD1 (**J**, **K**). Original magnifications: (A, C, F, I) 100 ; (B, D, E, G, H, J, K) 400

high nuclear expression in the cells at the end of both gastric axes of renewal (Figures 1I-1K).

The BMP pathway in H. pylori-infected gastric epithelium

We examined the expression of BMP2 and BMP4 in histologically normal and *H. pylori*-infected patients (*n*= at least ten well-orientated sections). In the histologically normal gastric antrum, BMP2 was expressed by gastric gland cells and a few cells in the mesenchyme. BMP2 expression was strongly increased in the *H. pylori*-infected antrum. This increase was due to a diffuse influx of BMP2-producing inflammatory cells (Figures 2A-2D). No BMP4 expression was detected in the histologically normal antrum of the human stomach. In *H. pylori*-infected patients, focal infiltrates of BMP4-expressing cells were observed (Figures 2E-2H). Since BMP2-expressing inflammatory cells were diffusely spread throughout the mucosa, these cells could be counted. We therefore examined the expression of BMP2 in a larger set of patients with

histologically normal gastric epithelium (*n*=32), *H. pylori* infection (*n*=28), and in patients in whom *H. pylori* had been recently (weeks) eradicated (*n*=10).

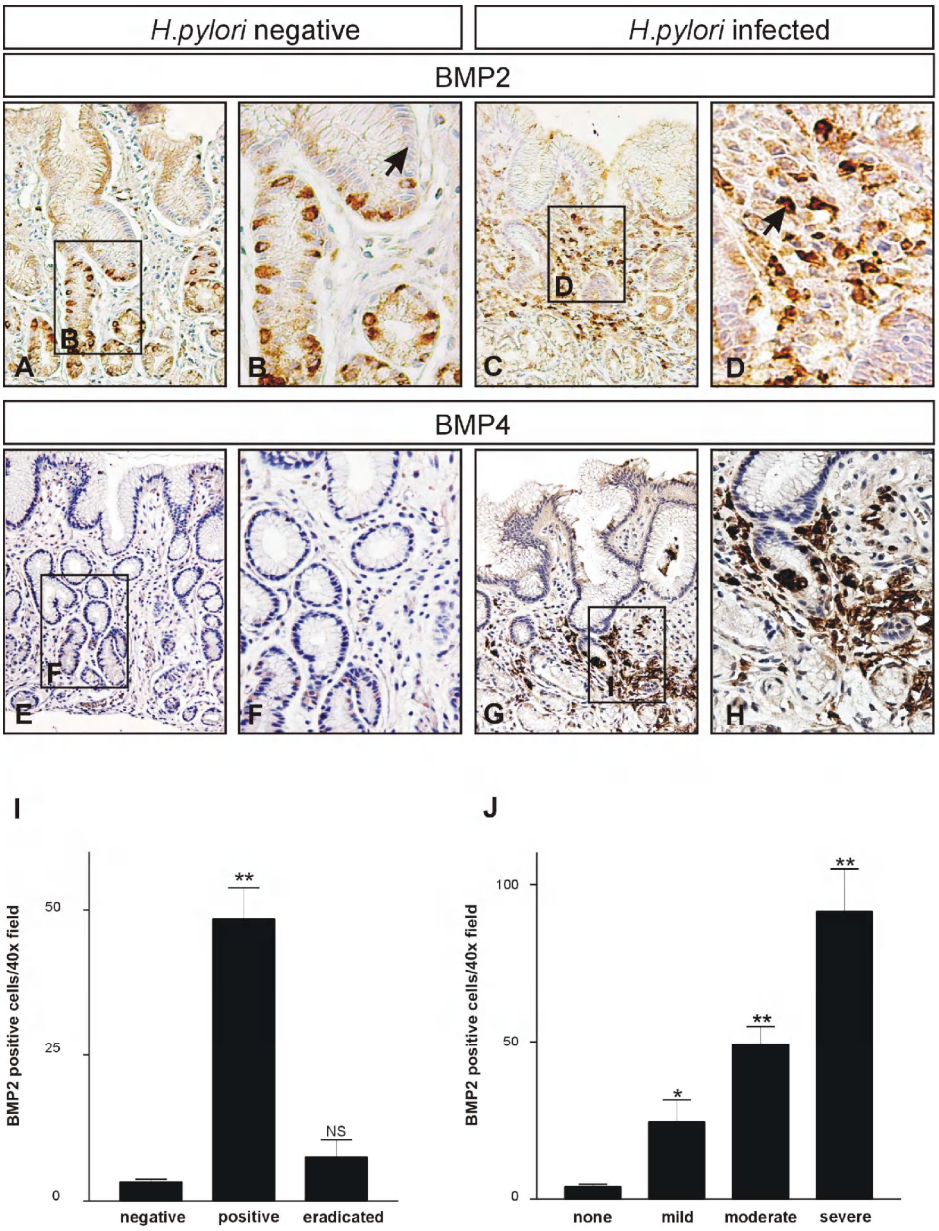

Figure 2 Immunohistochemical analysis of BMP2 and BMP4 expression showing influx of BMP-producing inflammatory cells in *H. pylori* infection. (**A**, **B**) Representative example of normal mucosa showing BMP2 expression by epithelial cells in gastric glands (enlarged in panel B; arrow indicates BMP2-positive cell). In *H. pylori*-infected gastric epithelium (**C**, **D**), BMP2 expression was increased as a result of the influx of BMP2-expressing inflammatory cells (arrow in D). (**E**, **F**) No BMP4 expression was found in the normal antrum. (**G**, **H**) Some focal infiltrates of BMP4-expressing cells were found in *H. pylori*-infected patients. (**I**) The number of BMP2-expressing cells per 40x microscope field was significantly increased in biopsies from *H. pylori*-infected patients (n = 28) compared with uninfected controls (n = 32, $p < 0.001$). After eradication (n = 10), BMP2 expression returned to normal levels (I, third bar). (**J**) The number of BMP2-positive cells per 40x field increased with the activity of gastritis, which was scored according to the Sydney classification. Original magnifications: (A, C, E, G) 100x; (B, D, F, H) 200x

The number of BMP2-producing inflammatory cells increased from 3 cells per 40x field in controls to 48 cells per 40x field ($p<0.0001$) in *H. pylori*-infected patients (Figure 2I). The number of BMP2-producing inflammatory cells decreased to near-normal values (7 cells per 40x field) weeks after eradication (Figure 2I).

The number of BMP2-positive inflammatory cells increased with the activity of gastritis as scored according to the Sydney classification system (Figure 2J). The presence of a large number of BMP-producing inflammatory cells suggested that the concentration gradient of BMP in the gastric mucosa is altered and that BMP signaling may be disturbed as a result. We therefore examined the expression of both BMP signaling receptors in the *H. pylori*-infected stomach (n=10 well-orientated sections).

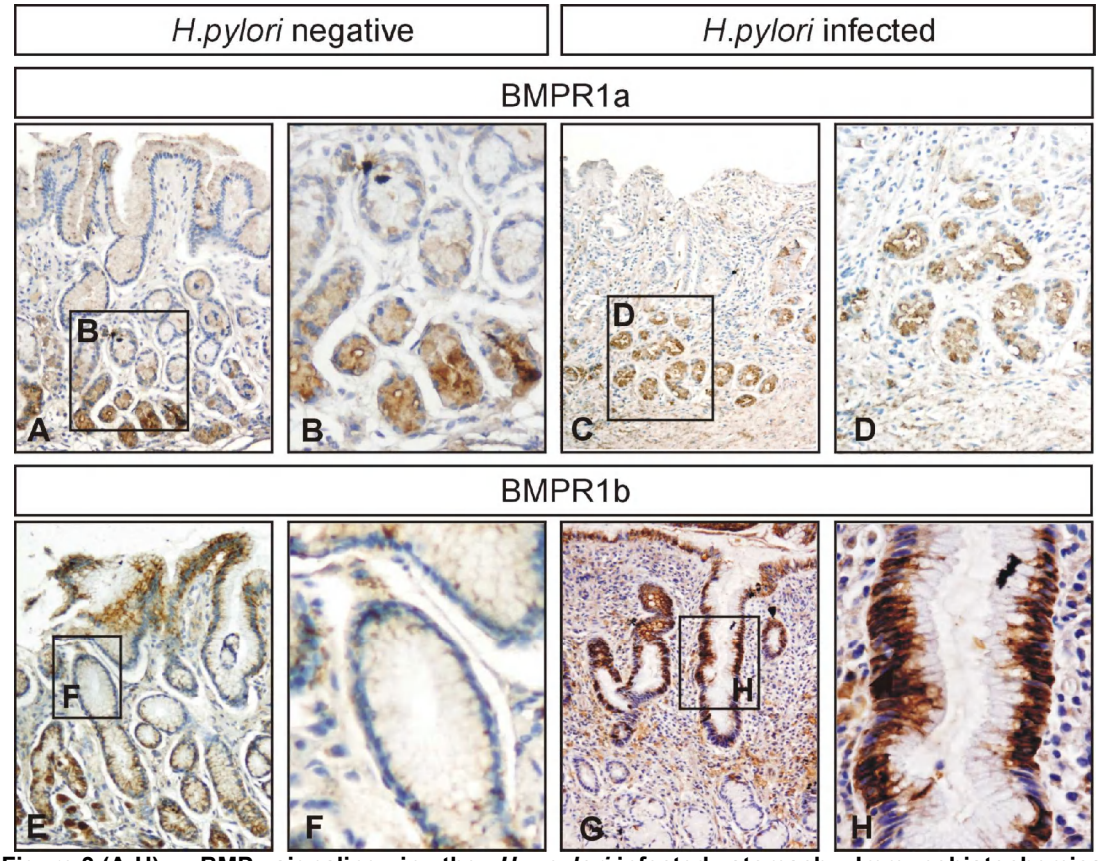

Figure 3 (A-H) BMP signaling in the *H. pylori*-infected stomach. Immunohistochemical analysis of BMPR1a and BMPR1b in histologically normal and *H. pylori*-infected human stomach. (**A** – **D**) BMPR1a was expressed by gastric gland cells in both *H. pylori*-negative (**A**, **B**) and *H. pylori*-positive (**C**, **D**) patients. (**E** – **H**) BMPR1b was expressed by differentiated pit and gland cells in the healthy mucosa (**E**), whereas cells in the isthmus were normally BMPR1b-negative (**F**). In *H. pylori*-positive patients, epithelial BMPR1b expression was increased (**G**), with strong induction of BMPR1b in the isthmus of the gastric units (**H**).

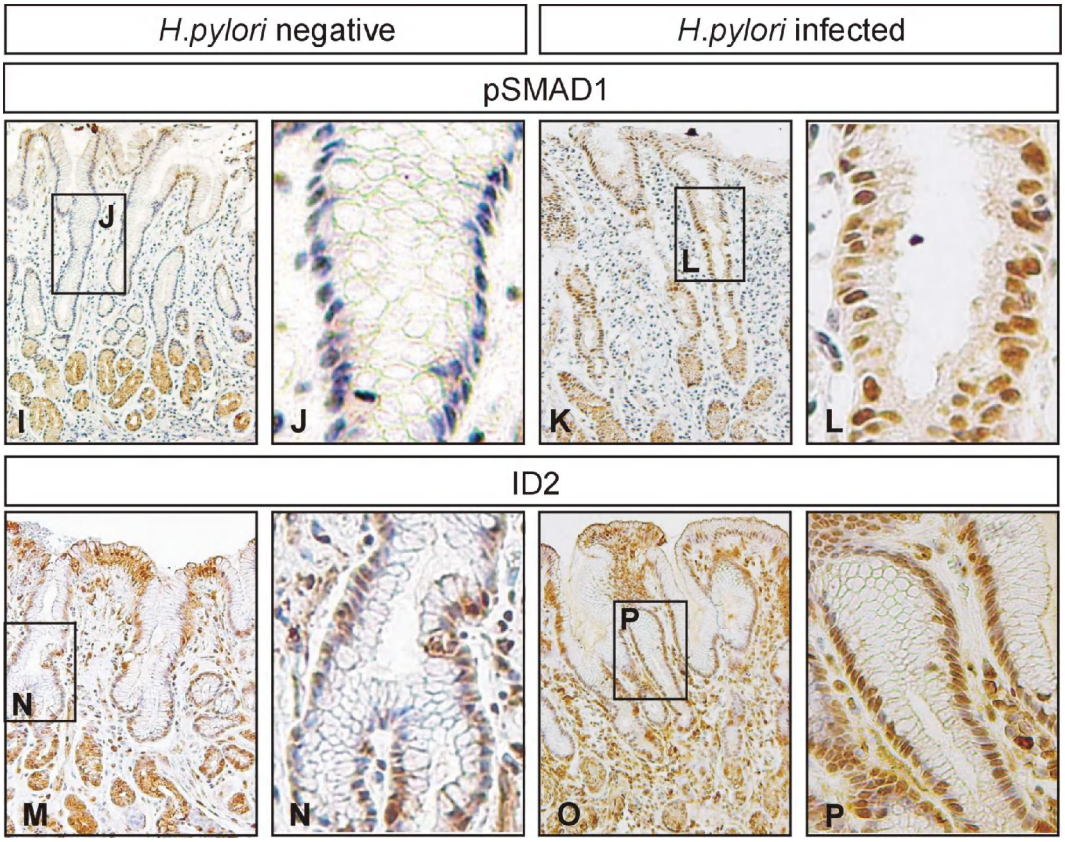

Figure 3 (I-L) BMP signaling in the *H. pylori*-infected stomach. Immunohistochemical analysis of pSMAD1, and ID2 in histologically normal and *H. pylori*-infected human stomach. (**I** – **L**) pSMAD1 was expressed by differentiated pit and gland cells in healthy mucosa (I), whereas epithelial cells in the isthmus were pSMAD1-negative (**J**). In *H. pylori*-infected patients, epithelial pSMAD1 expression was strongly increased (**K**), with highest expression in the isthmus of the gastric unit (**L**). (**M** – **P**) ID2 expression was similar to that of both BMPR1b and pSMAD1, with expression in differentiated pit and gland cells in normal mucosa (M, N) and up-regulation of ID2 expression in *H. pylori*-infected patients (**O**, **P**). Original magnifications: (A, C, E, G, I, K, M, O) 100x; (B, D, F, H, J, N, L, P) 400x

No gross differences were observed in BMPR1a expression between infected and uninfected patients (Figures 3A-3D). Expression of BMPR1b was up-regulated in the gastric epithelium of *H. pylori*-infected patients (n=10 well-orientated sections). The most notable observation was that the expression gradient of BMPR1b was reversed. In the normal mucosa, BMPR1b is not expressed in the isthmus of antral units (Figures 3E and 3F). In contrast, we found high expression of BMPR1b in the isthmus of the antral units in the inflamed mucosa (Figures 3G and 3H).

We observed a similar shift in the localization of both pSMAD1 and ID2. Whereas both are present in the superficial pit cells only in the histologically normal stomach (n=10 well-orientated sections), their localization shifts towards the isthmus of the gastric units in *H. pylori*-infected patients (n=10 well-oriented sections, Figures 3I-3P).

Interestingly, we found a corresponding shift in the localization of apoptotic cell death towards the isthmus of the gastric units (Figure 4). Staining of adjacent sections for active caspase-3 and pSMAD1 revealed that the apoptotic cells in the gastric isthmus are also positive for pSMAD1 (Figure 4).

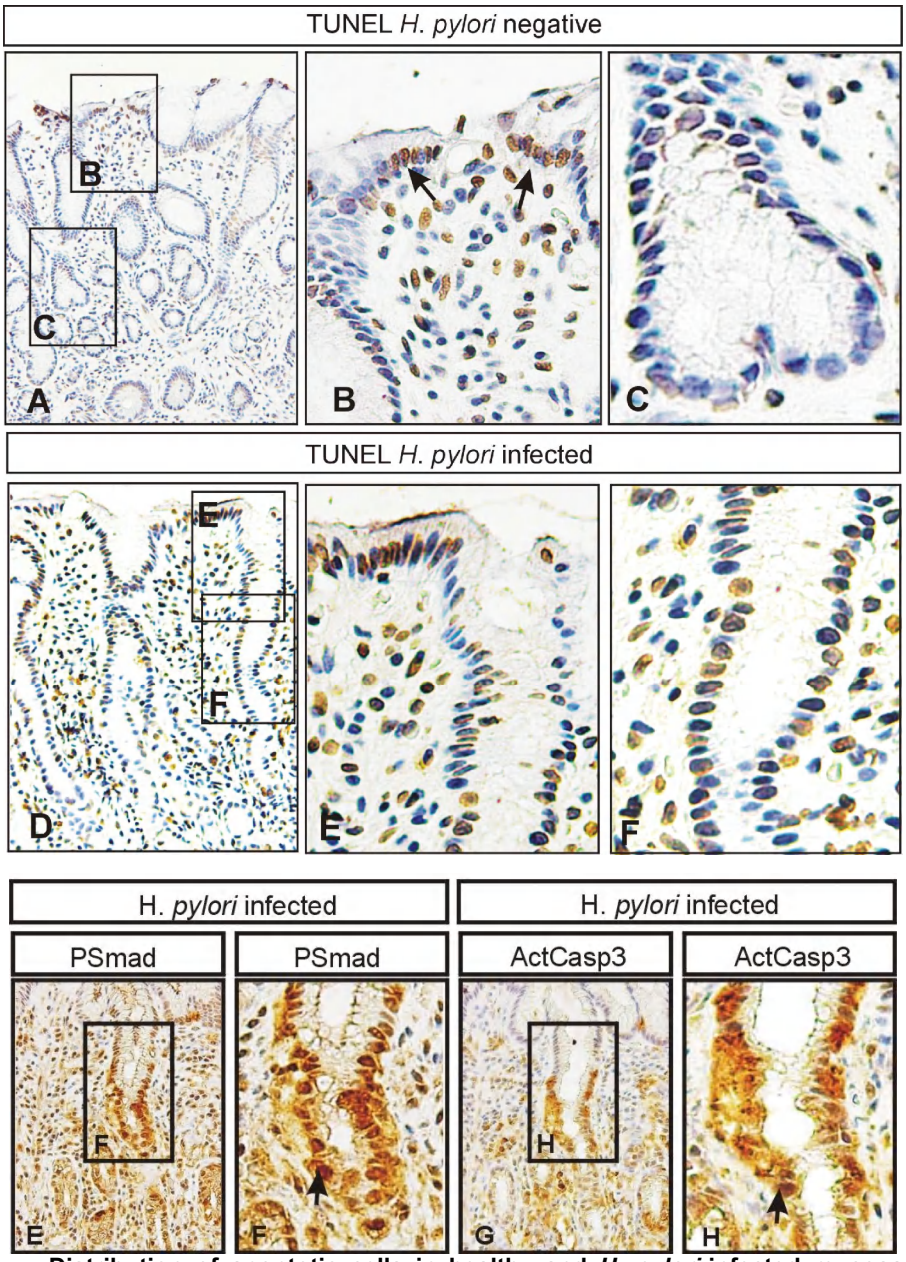

Figure 4 Distribution of apoptotic cells in healthy and *H. pylori*-infected mucosa. (**A** – **F**) TUNEL staining of apoptotic cells. In *H. pylori*-negative patients (**A**), TUNEL-positive cells were found mainly in the superficial terminally differentiated pit cells (arrows in B), whereas the epithelial cells in the precursor cell compartment of the isthmus were TUNEL-negative. In *H. pylori*-infected patients (**D**), TUNEL-positive cells were found in the differentiated pit cells (**E**) as well as in the precursor cells in the isthmus (**F**). (**G** – **J**) Adjacent sections stained for pSMAD1 and the cleaved (active) form of caspase 3. The shift of pSMAD1 to the isthmus of the gastric units in *H. pylori*-infected patients (**G**, **H**) was associated with high expression of cleaved caspase 3 in the same cells (**I**, **J**)

Discussion

Epithelia such as those of the skin and the intestine are in a state of constant renewal during adult life to maintain their integrity. These epithelia have a strong capacity to retain a stable micro-architecture throughout life. Renewal of adult epithelium has several interesting similarities with patterning events during embryonic development or morphogenesis and is therefore sometimes called morphostasis[18]. The molecular controls of morphogenesis are relatively well characterized. Cell fate determination is regulated by proteins in the cell such as transcription factors (intrinsic regulators) that are controlled by signals from outside the cell (extrinsic regulators). Morphogens are soluble proteins that form concentration gradients through tissues and form the most important extrinsic information during morphogenesis[1].

Recent work from several laboratories is beginning to reveal how controls of morphogenesis translate to morphostasis of the gastrointestinal (GI) tract. Morphogens have been shown to control proliferation, differentiation, and apoptosis in the homeostatic system of GI epithelial renewal[2, 3, 19]. The activity of these morphogens can be deregulated by genetic mutations in the genes that encode their receptors such as in the Hedgehog and BMP pathway[20, 21] or intermediate signaling molecules such as in the WNT and TGF family pathways[3, 22]. Differentiation of inflammatory cells is also controlled by morphogens[5] and recently, peripheral CD4+ T-lymphocytes have been shown to produce, for example, Sonic Hedgehog[6]. We therefore hypothesized that influx of morphogen-producing inflammatory cells would be an alternative way to disturb morphogen signaling in the GI epithelium. Such deregulation may be a novel way in which inflammation is linked to GI carcinogenesis.

Here we have used BMP signaling in the *H. pylori*-infected stomach as a model to study changes in epithelial homeostasis during inflammation. Chronic inflammation of the mucosa in *H. pylori* infection causes alterations of epithelial homeostasis, accompanied by both increased proliferation and apoptosis[23, 24]. We have shown that the BMP signaling receptors, pSMAD1, and a BMP target ID2 are expressed by cells at the end of the axis of epithelial renewal in the pit and the gland. BMPs regulate cellular proliferation, differentiation, and apoptosis, depending on the target cell type. We found previously that BMP signaling regulates apoptosis in adult colon epithelium[13] and BMP signaling has been shown to induce cell cycle arrest in gastric epithelial cells *in vitro*[25].

Since the activity of the BMP pathway localizes to epithelial cells at the end of the axis of epithelial renewal in the stomach, this is suggestive for a role in apoptosis in these cells.

We have previously described BMP4 expression by fibroblast-like cells in the fundic region of the stomach and have shown that BMP4 expression was regulated in a paracrine manner by Sonic Hedgehog that is produced by parietal cells[10]. In the present study, we did not find any BMP4 expression in the antrum of the healthy stomach and this may correlate well with the fact that Shh is not expressed in the normal antrum[26]. We found strong focal staining for BMP4 in infiltrates that surrounded some of the glands in *H. pylori*-infected patients. We observed expression of BMP2 by epithelial gland cells and a few inflammatory cells in the histologically normal human stomach. The number of cells increased strongly in *H. pylori*-associated gastritis and returned to normal after eradication of the bacterium. In contrast to BMP4-positive cells, BMP2-positive cells were localized more diffusely throughout the mucosa. The influx of BMP-producing cells correlated with a change in the localization of BMPR1b, pSMAD1, and the expression of BMP target ID2. The expression of all three proteins was strongly increased and displayed a shift from the superficial pit cells in the histologically normal mucosa to the isthmus of the gastric units in the *H. pylori*-infected patients. Since BMP signaling is known to regulate apoptosis in intestinal epithelial cells, we next examined if there would be a similar change in the localization of apoptotic cell death in the inflamed mucosa. Indeed, we observed increased apoptotic cell death in the isthmus of the gastric units in *H. pylori*-infected patients in exactly the same region as the maximal pSmad1/ID2 staining. This shift of apoptotic cells towards the isthmus in *H. pylori*-infected patients has been found by others previously[24]. The association between the shift of the activity of BMP pathway towards the precursor cell compartment and the very similar shift in the localization of apoptotic cell death suggests that there may be a causal relationship. Such a causal relationship awaits further experimental evidence. Furthermore, our study has focused on changes in BMP signaling target cells in the normal and inflamed human antrum. Studies on the role of BMP signaling in gastric fundic glands and in pathological conditions such as intestinal metaplasia, gastric atrophy, and carcinogenesis require further experimentation.

In summary, our data demonstrate that BMP signaling is restricted to the epithelial cells at both ends of the axes of renewal of the gastric units in the normal mucosa. *H. pylori* infection leads to an increase in BMP expression, mainly caused by an influx of BMP2-producing cells. The influx correlates with an increase in the activity of the BMP pathway. The activity of the pathway shifts from the differentiated epithelial cells in the gastric pit cell region to the precursor cells in the isthmus. This shift is associated with a corresponding shift in apoptotic cell death. Our data show that the morphogenetic landscape is altered in the inflamed mucosa of the GI tract by the production of morphogens by inflammatory cells.

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Chapter 6

The effect of statins in colorectal cancer is mediated through the Bone Morphogenetic Protein pathway

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Abstract

Background and Aims:

Epidemiological evidence suggests that statins prevent colorectal cancer (CRC) but the biological mechanism remains obscure. Statins induce Bone Morphogenetic Protein (BMP) expression in bone cells. We have previously shown that BMPs act as tumor suppressors in CRC. We hypothesized that the action of statins in CRC involves the induction of BMPs.

Methods:

We investigated the effects of statins on colorectal cancer cell lines using immunoblotting, measurements of apoptosis and cell proliferation, and luciferase reporter assays. The effect of statins was confirmed in a xenograft mouse model.

Results:

CRC cell lines show widely differing sensitivities to statin treatment. Sensitive cell lines show induction of BMP2 protein levels and a BMP2 reporter construct, activation of the BMP pathway, and induction of the BMP target gene ID-2 while resistant cell lines do not. The addition of the specific inhibitor of BMPs, Noggin, completely prevents Lovastatin-induced apoptosis in sensitive cells. Sensitive cell lines express the central BMP pathway element SMAD4 while the resistant cell lines do not. Targeted knock-out of SMAD4 leads to the loss of statin sensitivity and reconstitution with SMAD4, to the restoration of statin sensitivity. In a xenograft mouse model, tumors from sensitive and insensitive cell lines were treated with oral Simvastatin. Significant inhibition of tumor growth using sensitive cells but increased tumor growth when using insensitive cells was observed.

Conclusions:

Statins induce apoptosis in CRC cells through induction of BMP2. Statin therapy may only be effective in SMAD4-expressing CRCs and may have adverse effects in SMAD4-negative tumors.

Introduction

A recent trial specifically designed to investigate the incidence of colorectal cancer in statin users showed a highly significant 47% reduction [1]. Several authors are now suggesting that the time is ripe for large scale prospective clinical or observational trials of statin therapy as a chemopreventative or adjuvant treatment in colorectal cancer [2, 3], but the epidemiological evidence for a beneficial effect is conflicting [4] and the biological mechanism of action of statins in colorectal cancer remains obscure.

HMGCoA reductase inhibitors are extensively used to reduce serum cholesterol and to decrease the incidence of cardiovascular and cerebrovascular events [5, 6]. Statins prevent formation of mevalonate from HMGCoA by inhibiting the enzyme HMGCoA reductase and thereby inhibit cholesterol synthesis [7]. As well as reducing cholesterol levels, statins inhibit the generation of other products of the mevalonate pathway, including mevalonate and the downstream isoprenoids (farnesylpyrophosphate and geranylgeranylpyrophosphate). Posttranslational isoprenylation is important in determining the membrane localization and function of many cellular proteins including small GTPases like Ras and Rho [8]. *In vitro* studies show that statins inhibit cellular proliferation and induce apoptosis in colon cancer cells[9, 10]. Since Ras mutations are frequent in tumors [11] and Rho proteins participate in growth factor signaling [12], the study of the action of statins in tumor cells has largely focused on their ability to inhibit these small GTPases [13]. However, there is evidence that this may not be the only mechanism by which statins inhibit proliferation and induce apoptosis[2, 14].

A screen of 30,000 compounds for their ability to activate a BMP2 promoter construct in mouse osteoblast cell lines found that two statins (Simvastatin and Lovastatin) had the highest activity and went on to show the anabolic properties of statins on bone *in vivo* in mice[15].

BMPs are part of the Transforming Growth Factor-β superfamily. They signal by cooperatively binding type I and type II BMP receptors which in turn phosphorylate the BMP-specific SMADs1,5 and 8 which then complex with SMAD-4. The complex translocates to the nucleus and modulates gene transcription of BMP target genes such as ID-2. The BMP pathway has recently been implicated in colorectal cancer with the identification of germline mutations in BMPR1a and SMAD4 in families with familial Juvenile Polyposis Syndrome [16]. Affected individuals have a greatly increased risk of

developing cancer [17, 18]. We have shown that BMP 2 acts to promote apoptosis in mature epithelial cells in the colon[19].

Here we have investigated the effects of statins on colorectal cancer cell lines and xenografts in order to test the hypothesis that statins act through their actions on the BMP pathway. We show that statins induce BMP2 in colorectal cancer cells and that Noggin specifically blocks statin-induced apoptosis in colon cancer cell lines. Our results suggest that loss of SMAD4 confers resistance to statins and leads to growth promotional effects. This implies that statin therapy may only be effective in a subgroup of SMAD4-expressing colorectal cancers.

Materials and Methods

Cell Culture

DLD1, SW480, HT29 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 L-glutamine. This was supplemented with penicillin (50U/ml) and streptomycin (50µg/ml) and, where serum was used, with 10% fetal calf serum (Gibco, Paisley, Scotland). HCT116 SMAD4-/- cells and HCT116 SMAD4+/+ parental cell line were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA)). HCT116 SMAD4-/- cells were cultured in McCoy's 5A medium supplemented with 0,4 mg/mL G418 and 0,1 mg/mL hygromycin B. Cells were grown in monolayers in a humidified atmosphere containing 5% CO_2.

Cell count

Cells were trypsinized and 20µl of cell suspension were taken for counting using Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter B.V., Mijdrecht, The Netherlands) according to the manufacturer's instructions.

MTT assay

Cells were trypsinized and taken up in DMEM with 0.5 % fetal calf serum. 5×10^3 cells were seeded in flat-bottomed tissue culture treated 96-well plates (Greiner Bio-One B.V., Alphen a/d Rijn, Netherlands) and allowed to adhere for 12 hours. Cells were then stimulated with different concentrations of statins 24 and 48 hours. After treatment, MTT solution was added (final concentration 0.5 mg/ml, stock solution 5 mg/ml MTT in PBS), for 3 hours. Medium was discarded and cells were lysed in acidified 2-

propanol. Absorbance was measured at 550-560nm. 10 wells were used for each treatment condition (n=10).

Immunoblotting

Cells at 60-80% confluence from 6 well plates (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands) were washed in ice-cold PBS and scraped into 200µl of 2×sample buffer (125mM Tris/HCl, pH 6,8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, 1mg bromophenol blue). Protein concentration was measured using the RC DC protein assay kit (Biorad, CA, USA) according to the manufacturer's instructions. The lysates were sonicated and then heated at 95° for 5 min. 50µg of protein from each sample was loaded onto SDS-PAGE and blotted onto PVDF membrane (Millipore, Bedford, MA, USA). The blots were blocked in block buffer (2% low fat milk powder in Tris-buffered saline with 1% Triton (TBST)) for 1 hour at room temperature and washed 3×10 min in TBST before overnight incubation at 4°C with primary antibody in primary antibody buffer (TBST with 0.2% low fat milk powder). Primary antibodies to BMP2 (mouse monoclonal) were from R&D (Abingdon, UK). Goat polyclonal antibodies to pSMAD1,5 and 8 were from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal antibodies to SMAD4, rabbit polyclonal antibodies to Id1 and rabbit polyclonal antibodies to β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Blots were then washed 3×10 min in TBST and incubated for 1 hour at room temperature in 1:2000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit, rabbit anti-goat or goat anti-mouse secondary antibody (Dako, Glostrup, Denmark) in block buffer. After a final 3×10 min wash in TBST, blots were incubated for 5 min in Lumilite Plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Lumi-Imager (Boehringer-Mannheim, Mannheim, Germany).

Annexin V and 7-amino-actinomycin D assays

Control and treated cells were collected and resuspended in 1×binding buffer (0.01M Hepes/NaOH, pH 7.4, 0.14mM NaCl and 2.5mM $CaCl_2$) at a concentration of $2x10^7$ cells/ml. Subsequently 100µl of cell suspension was transferred to a 5ml tube and Annexin V-APC (5µl) and 7-amino-actinomycin D (7-AAD) - (5µl) was added. The cells were incubated at room temperature for 15 min, after which 400µl of 1×binding buffer was added, and apoptosis analyzed by flow cytometry (Becton Dickinson

FACSCalibur, Rockville, MD, USA) and data were analyzed using the software Cell Quest Pro (BD Biosciences Pharmingen).

Luciferase reporter assay

Cells were transiently transfected either with BMP2- Luc vector (kindly provided by Lilly Research Laboratories, Indianapolis, Indiana, USA), or BRE-Luc vector (a generous gift of Prof. ten Dijke (Leiden University Medical Center, Leiden, The Netherlands)) reporter in combination with a cytomegalovirus (CMV) promoter-driven Renilla luciferase vector (Promega, Madison, Wisconsin, USA) using Lipofectamine Plus (Invitrogen, Breda, The Netherlands) according to manufacturer's instructions. After 24 hours of treatment with Lovastatin cells were lysed with passive lysis buffer as provided by Promega and luciferase activity was assayed according to the Dual-Glo Luciferase Assay System (Promega) protocol on a Lumat Berthold LB 9501 Luminometer (Berthold Technologies, Bad Wildbad, Germany). Each firefly luciferase value was corrected for its cotransfected CMV-driven Renilla luciferase standard to correct for transfection efficiency or dilution effects.

Plasmids and transfection procedure

HCT116 SMAD4-/- cells were transiently transfected either with HAHA-SMAD4 plasmid (kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA)), or pmaxGFP control vector (from amaxa GmbH, Cologne, Germany)) using Lipofectamine Plus (Invitrogen, Breda, The Netherlands) according to manufacturer's instructions. Efficiency of transfection was determined by the measurement of GFP-positive cells and was at least 70%.

Tumor growth in nude mice

Female NMRI nu/nu mice were obtained from Charles River (Maastricht, The Netherlands). The study was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, The Netherlands. Mice were sacrificed when their tumors reached 1000mm^3 in size to avoid undue animal suffering. Groups of 8 female NMRI nu/nu mice were injected subcutaneously in the flank with 1×10^6 HCT116 cells or with 5×10^6 HT29 cells in Matrigel (BD, Breda, The Netherlands). Mice were fed *ad Libitum* with food containing Simvastatin (Arie Blok BV, Woerden, The Netherlands), thereby receiving 50 mg/kg/day for the duration of the experiment, initiated when the tumor volume reached

100-200 mm^3. We chose Simvastatin as being the second most potent statin in our *in vitro* experiments and because Simvastatin is licensed for use in humans in the Netherlands while Lovastatin is not [20]. The treatment was well tolerated by all animals with no signs of toxicity (loss of body weight, behavior changes). Controls received identical food without Simvastatin. Tumor volumes were determined by external measurement performed by two different independent animal technicians. Tumor volume was determined according to the equation $V=[L\times W^2]\times 0.5$, where V is volume, L is length, and W is width. After the mice were sacrificed the tumors were harvested, frozen in liquid nitrogen and later homogenized in cell lysis buffer (Cell Signaling) with 1mM Pefabloc (Sigma).

Statistical Analysis

Statistical analysis was performed using two-tailed Student's *t*-test and $P < 0.05$ was considered statistically significant. Data are shown as mean ± SEM.

Results

Colorectal cancer cell lines differ in their sensitivity to statin treatment

Using a panel of colorectal cancer cell lines a wide variation in the ability of Lovastatin to induce cell death was found using the same doses as previous *in vitro* studies [9] (Figure 1A). HCT116 and DLD-1 cell lines were significantly more sensitive than SW480 and HT29. At lower doses there was even a slight growth stimulatory effect of statins in HT29 and SW480 cells. The cell lines that were resistant to statin treatment are known to express no SMAD4 protein while the sensitive cell lines express SMAD4 normally [21] as can been seen in figure 1B. We further investigated the effect of 3 different statins, Lovastatin, Simvastatin and Pravastatin, in HCT116 and HT29 cells. Simvastatin and Lovastatin show the same effect with both leading to slight but significant increases in the growth of HT29 cells at a dose of 2 μM and reduced cell viability at higher doses, while leading to significant reductions in HCT116 cell viability at all doses (Figure 1C). Pravastatin also leads to a similar increase in the growth of HT29 cells but no effect on HCT116 cells. This is most likely due to the low lipophylicity of Pravastatin leading to low uptake into non-hepatic cells [22].

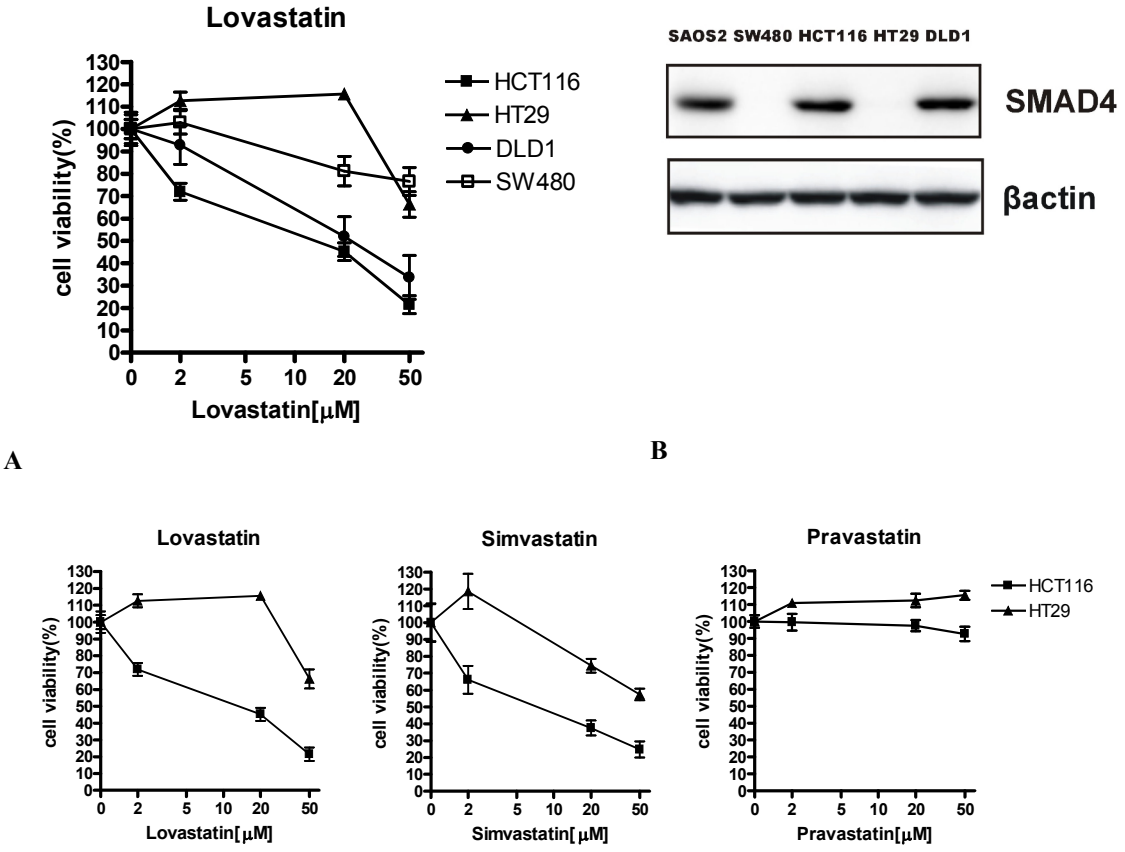

Figure 1. The effects of statins on the growth of colorectal cancer cell lines. (A) The MTT cell viability assay was performed in four colon cancer cell lines treated for 24h with various concentrations of Lovastatin. Values are expressed as a percentage of controls. Data are from three experiments ± s.e.m (n=10). **(B)** Immunoblots of SW480, HCT116, HT29 and DLD1 colon cancer cell lines for SMAD4 with β-actin as a loading control. **(C)** HCT116 and HT29 cells were treated for 24h with various concentrations of Lovastatin, Simvastatin or Pravastatin and the MTT assay was performed as in (A).

Lovastatin induces activation of the canonical BMP pathway in HCT116 cells but not in SW480 cells

In statin-sensitive HCT116 cells treated with Lovastatin, levels of BMP2 protein increased with a corresponding increase in levels of the activated form of the BMP specific SMADs (pSMAD1,5 and 8) and ID2, while in SMAD4 negative SW480 cells Lovastatin did not lead to increases in BMP2, pSMAD 1,5,8 or ID2 (Figure 2A). Similarly, HCT116 SMAD4 -/- cells generated by targeted deletion of the SMAD4 gene [23] show no upregulation of BMP2, pSmad1,5,8 or ID2 (Figure 2A). Confirming the importance of BMP pathway activity in statin-induced apoptosis, the addition of a specific BMP-inhibitor, Noggin [24] in the form of an FC-chimera was able to prevent Lovastatin-induced apoptosis in HCT116 cells as effectively as adding back mevalonate

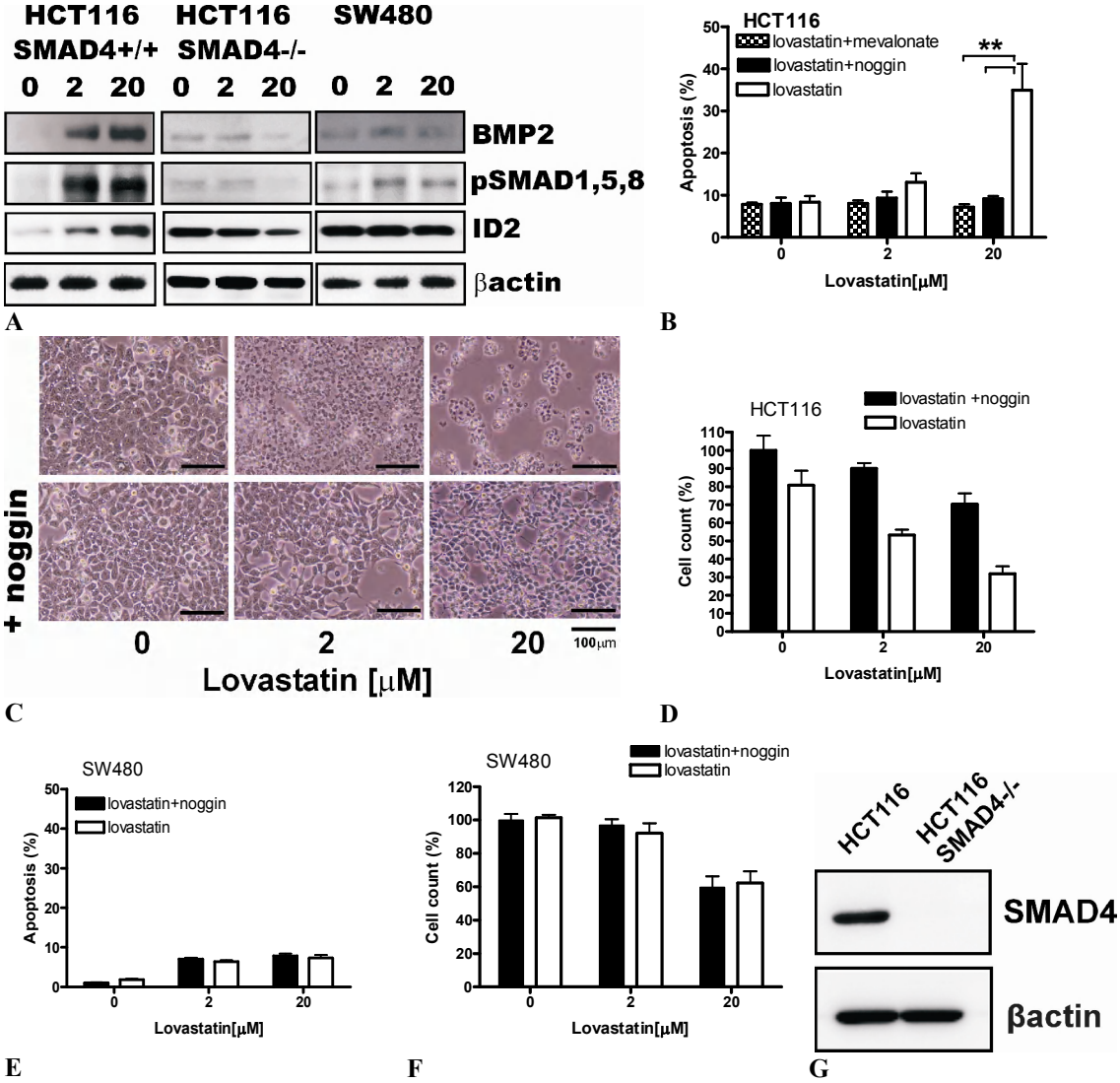

Figure 2. Lovastatin activates the BMP pathway in HCT116 cells, but not in SW480 cells. (A) Immunoblots of colon cancer cells treated with various concentrations of Lovastatin (μM) for 24h. The expression of proteins was analyzed using the corresponding specific antibody. β-actin served as a loading control. **(B)** HCT116 cells were treated with Lovastatin, Lovastatin in combination with Noggin (0,5μg/mL), or Lovastatin in combination with Mevalonate (100 μM) for 24h and apoptosis was quantified by flow cytometry. Data are from three experiments ±s.e.m.(n=3). **(C)** HCT116 cells were treated with Lovastatin or Lovastatin in combination with Noggin (0,5μg/mL) for 24h and then photographed under a microscope. **(D)** HCT116 cells were treated with Lovastatin or Lovastatin in combination with Noggin (0,5μg/mL) for 24h and subsequently counted. Values are expressed as a percentage of controls. Data are from three experiments ±s.e.m.(n=4) **(E)** SW480 cells were treated with Lovastatin or Lovastatin in combination with Noggin (0, 5μg/mL) for 24h and apoptosis was quantified by flow cytometry. Data are from three experiments ±s.e.m.(n=3). **(F)** as in (E) except that cells were counted. Values are expressed as a percentage of controls. Data are from three experiments ±s.e.m. **(G)** Immunoblot of HCT116 SMAD4-/- and +/+ cell lines for SMAD4 with β-actin as a loading control.

(Figure 2B, Supplementary Figure 1), with near complete inhibition of statin-induced apoptosis (Figure 2B,C). Similar data were obtained when cells treated with Lovastatin or with Lovastatin and Noggin were counted (Figure D). Noggin treatment was not able to reverse the low levels of apoptosis induced in SMAD4 negative SW480 cells (Figure

2E) and cell counting did not show any difference between Lovastatin and Lovastatin plus Noggin treated SW480 cells. (Figure 2F).

When Noggin was used in combination with other cytotoxic and proapoptotic agents such as 5-fluorouracil, Aspirin (Figure 3A, B) and FAS ligand, it had no effect (Figure 3D) suggesting that Noggin specifically blocks the cytotoxic effects of statins. The FC-chimera, Rituximab was used to control for non-specific effects of the FC-chimera itself (Figure 3C).

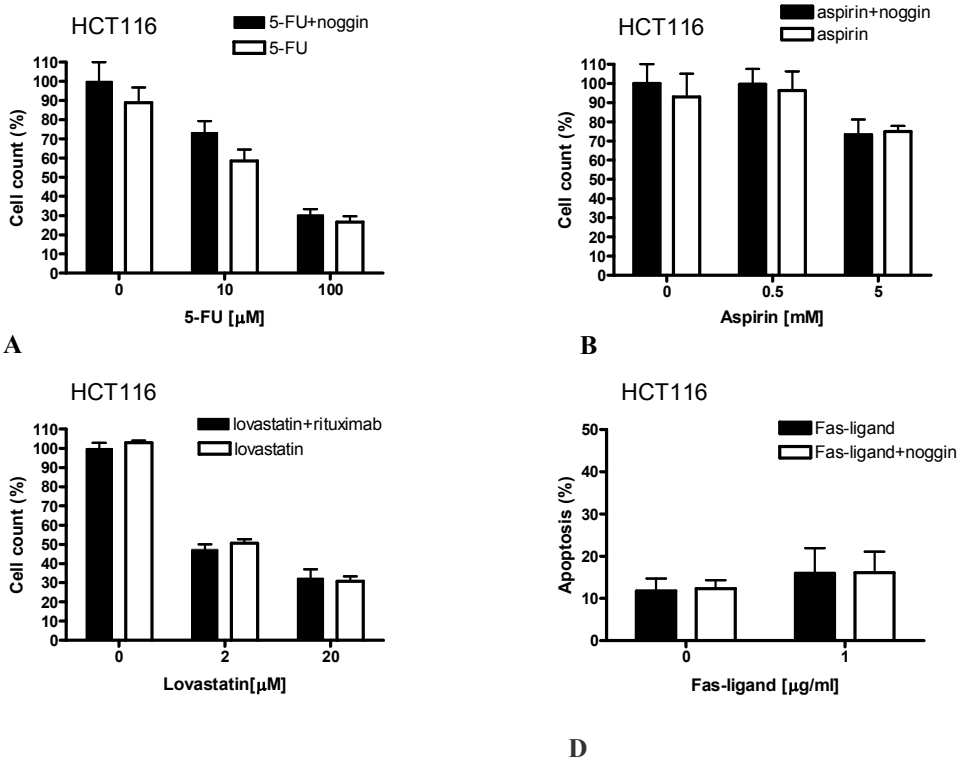

Figure 3. (A) HCT116 cells were treated with 5-FU or 5-FU in combination with Noggin (0,5μg/mL) for 24h and subsequently counted. Values obtained in the absence of compound have been set at 100. Results represent the mean ± s.e.m. (n=4) of three experiments. **(B)** the same as in A, only cells were treated with aspirin or aspirin combination with Noggin (0,5μg/mL). (**C**) the same as in A, only cells were treated with Lovastatin or Lovastatin in combination with Rituximab (0,5μg/mL).(**D**) HCT116 cells were treated with FAS ligand or FAS ligand in combination with Noggin(0,5μg/mL) for 24h and apoptosis was quantified by flow cytometry as the percentage of Annexin V-APC positive cells. Results represent the mean ± standard deviation of three experiments.

The sensitivity of colon cancer cell lines to statins is influenced by SMAD4 expression

To further investigate the importance of SMAD4 for the sensitivity of cell lines to statins we studied HCT116 cells with a targeted deletion of SMAD4 (SMAD4 -/- cells) and compared them with their SMAD4 expressing counterparts. HCT116 SMAD4 -/- cells express no SMAD4 protein as shown in figure 2G. Theses SMAD4 -/- cells are far

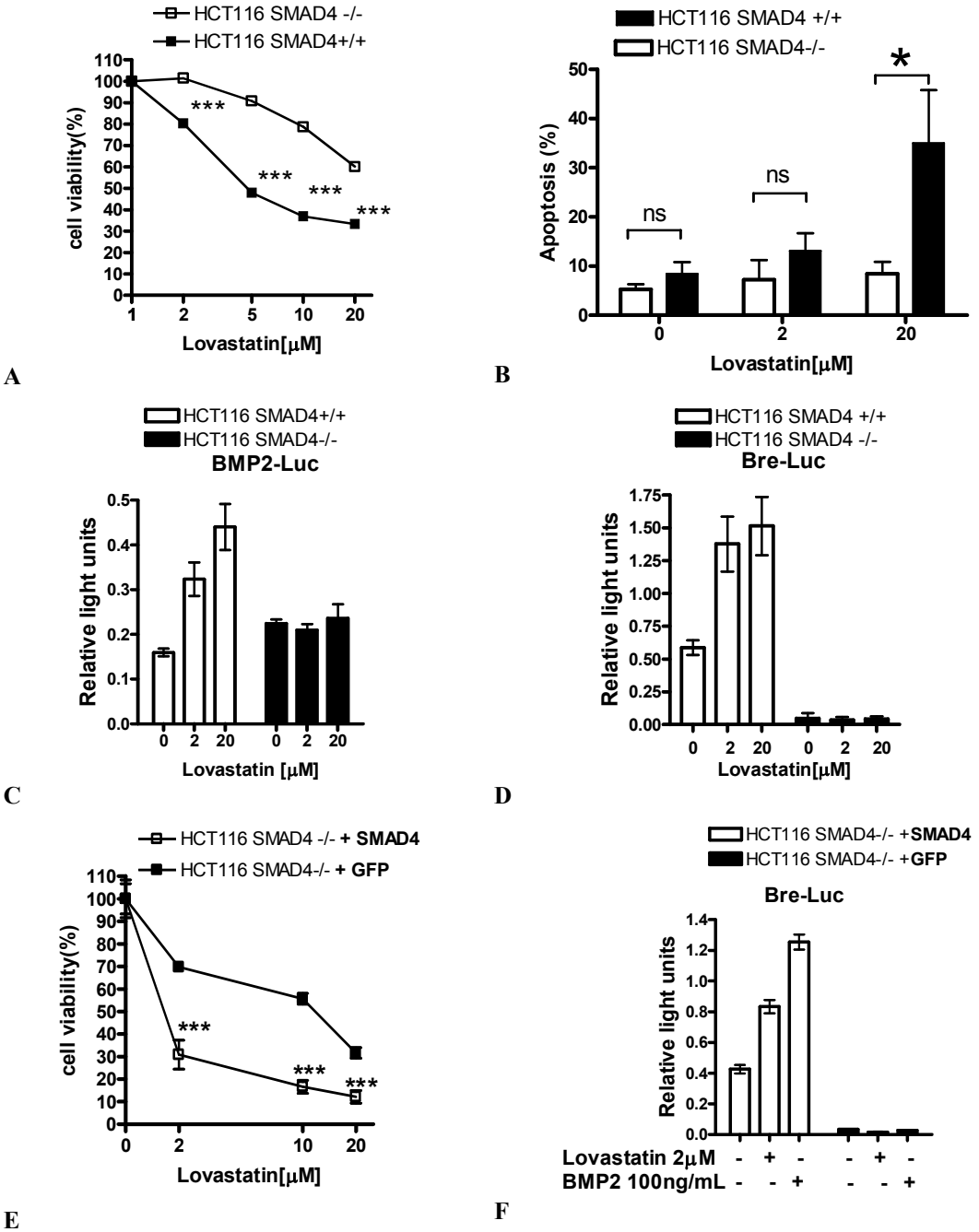

Figure 4. The response of HCT116 SMAD4+/+ and HCT116 SMAD4 -/- cell lines to Lovastatin treatment. (A) The MTT assay was performed in HCT116 SMAD4 +/+ or HCT116 SMAD4 -/- cells treated for 24h with various concentrations of Lovastatin. Values are expressed as a percentage of controls. Data are from three experiments ±s.e.m. *** $p<0.001$ (n=10). **(B)** HCT116 SMAD4 +/+ or HCT116 SMAD4 -/- cells were treated with Lovastatin for 24h and apoptosis was quantified by flow cytometry. Data are from three experiments ±s.e.m. * $p<0.05$ (n=3) **(C)** HCT116 SMAD4 +/+ or HCT116 SMAD4 -/- cells were transiently transfected with BMP2- Luc vector and treated with various concentrations of Lovastatin for 24h. Data were normalised to Renilla luciferase activity. Data are from three experiments ±s.e.m (n=4). **(D)** as in (C), except cells were transfected with the BRE-Luc vector. **(E)** HCT116 SMAD4 -/- cells were transiently transfected either with SMAD4 or with GFP construct, treated for 24h with various concentrations of Lovastatin and the MTT assay was performed as in A. **(F)** HCT116 SMAD4 -/- cells were transiently cotransfected either with SMAD4 and BRE-Luc or with GFP and BRE-Luc constructs, treated for 24h with 2 μM of Lovastatin or 100ng/mL recombinant human BMP2 and transcriptional activity was measured and normalised to Renilla-luciferase activity. Data are from three experiments ±s.e.m (n=4).

less sensitive to Lovastatin treatment than their SMAD4 expressing counterparts (Figures 4A and B). This is in contrast to their relative sensitivities to 5-fluorouracil where SMAD4 -/- cells are more sensitive (data not shown).

A reporter construct containing repeats of a BMP-responsive element (BRE) from the ID-1 promoter and a full length BMP2 promoter reporter construct were both activated when transiently transfected HCT116 cells were treated with Lovastatin. In contrast, HCT116 SMAD4 -/- cells show no increased BRE-Luc or BMP2-Luc reporter activity upon Lovastatin treatment (Figure 4C and D). SMAD4 +/+ cells also show higher basal levels of BRE-Luc activation even in the absence of exogenous ligand consistent with previous reports [25]. Reconstitution of HCT116 SMAD4-/- cells by transient transfection with SMAD4 restored Lovastatin sensitivity and Lovastatin-induced activation of BRE-Luc (Figure 4E and F).

The sensitive cell lines studied (HCT116 and DLD1) are also both microsatellite unstable cell lines while the resistant cell lines (SW480 and HT29) are microsatellite stable. To investigate the possibility that microsatellite instability could be responsible for statin sensitivity, HCT116 cells where the mismatch repair defect has been corrected by the addition of wild type chromosome 3 were compared to standard HCT116 cells. The correction of microsatellite instability did not alter the statin sensitivity of HCT116 cells (Supplementary Figure 2).

Statin treatment inhibits the growth of HCT116 xenografts in mice and promotes the growth of HT29 xenografts

Next we evaluated the effect of orally administered Simvastatin on growth of the SMAD4-wt HCT116 cell line and SMAD4 negative HT 29 cell line when these cells were implanted as xenografts in nude mice. Treatment of the nude mice with Simvastatin inhibited tumor growth in HCT116 xenografts but enhanced the growth of HT 29 xenografts compared to controls (Figure 5). These results are in agreement with our *in vitro* data where lower concentrations of Lovastatin have growth promotional effects in HT29 cells, while HCT116 cells are highly sensitive to statin induced cell death. To investigate whether the effects we see on the xenografts *in vivo* are associated with the activation of the BMP pathway, we assessed levels of pSMAD1,5 and 8 in tumor lysates. As shown in Figure 5C and D, levels of pSMAD1,5,8 are significantly

increased in the HCT116 xenografts in mice receiving Simvastatin compared to controls. Levels of pSMAD1,5,8 are equal and almost undetectable in both groups with HT29 xenografts (Figure 5C).

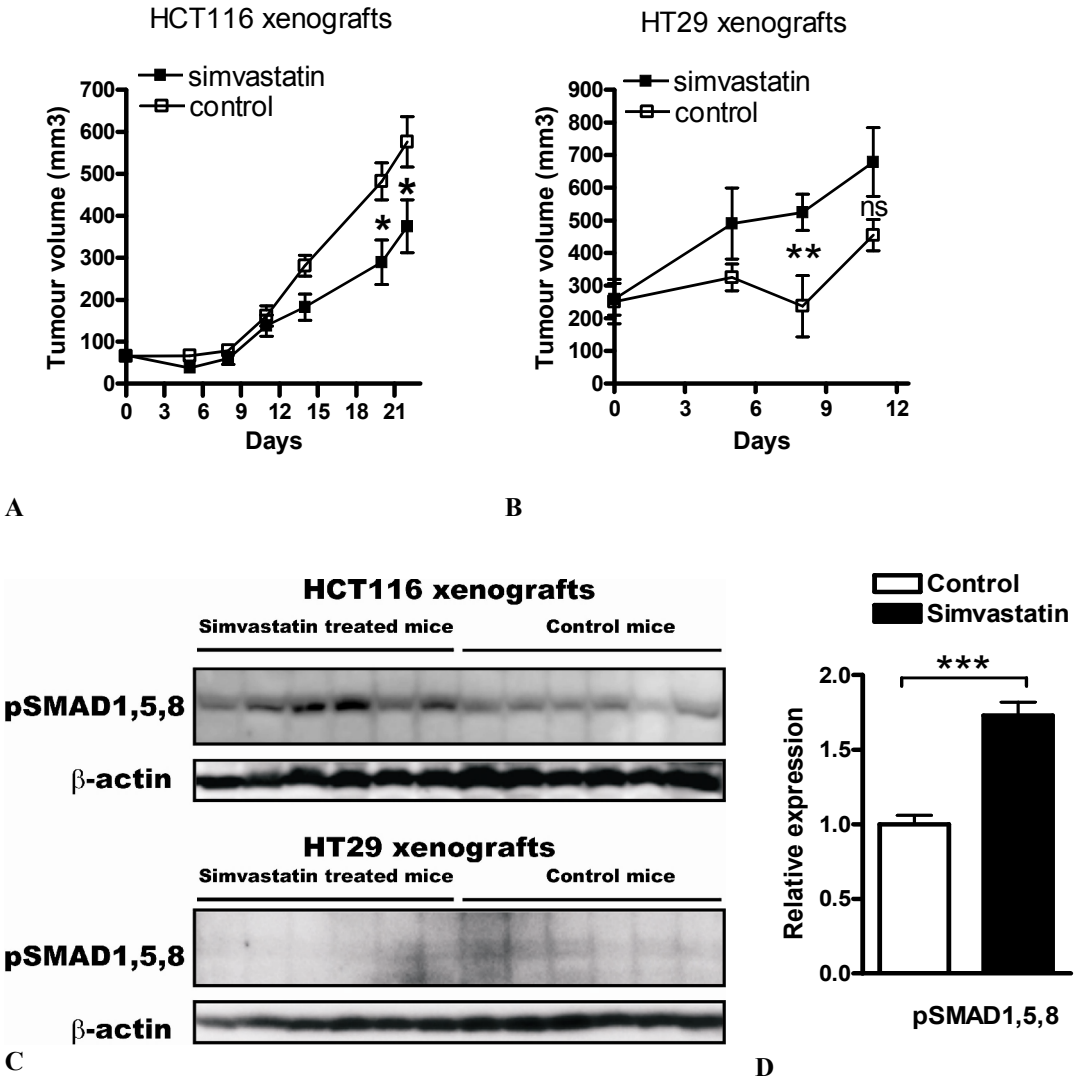

Figure 5. Growth curves of HCT116 and HT29 implants in nude mice. (A) Groups of 8 female NMRI nu/nu mice were injected subcutaneously in the flank with 1×10^6 HCT116 cells in Matrigel. Mice were fed *ad Libitum* with food containing Simvastatin, thereby receiving 50 mg/kg/day for 3 weeks, initiated when the tumor volume reached 100-200 mm^3. Controls received identical food without Simvastatin. Tumor volumes were determined by external measurement. Results represent the mean ±s.e.m. * P<0.05, **P<0.01. **(B),** as in (A), except 5×10^6 HT29 cells were used and Simvastatin treatment was for 12 days before mice had to be sacrificed due to tumor size. **(C)** Immunoblot analysis of lysates of HCT116 and HT29 xenografts from mice treated with Simvastatin and from controls. 50µg of total protein was loaded per lane. Equal loading was confirmed by assessing β-actin. Simvastatin treatment increases phosphorylated SMAD1,5,8 expression suggesting activation of BMP signaling. **(D)** Graph to show the relative mean expression of pSMAD1,5,8 in lysates of HCT116 xenografts from mice treated with Simvastatin and from controls analyzed by immunoblotting. Expression in control mice was set at 1. Error bars represent the s.e.m. *** P<0.001.

Low dose Statin treatment leads to cell death and increased BMP2 levels in vitro

Much of the evidence for the efficacy of statins in CRC comes from cohorts of patients taking standard cholesterol-lowering doses of statins where serum statin concentrations do not reach those commonly used in *in vitro* studies. To investigate the effect of much lower concentrations of statins, we assessed both cell viability and levels of BMP2 in HCT116 cells treated with low dose Lovastatin for longer periods. In Figure 6 we show that Lovastatin leads to significant reductions in cell viability and to increased levels of BMP2 even at concentrations approximating those achieved with standard cholesterol lowering doses of statins (~ 0.1μM) [26].

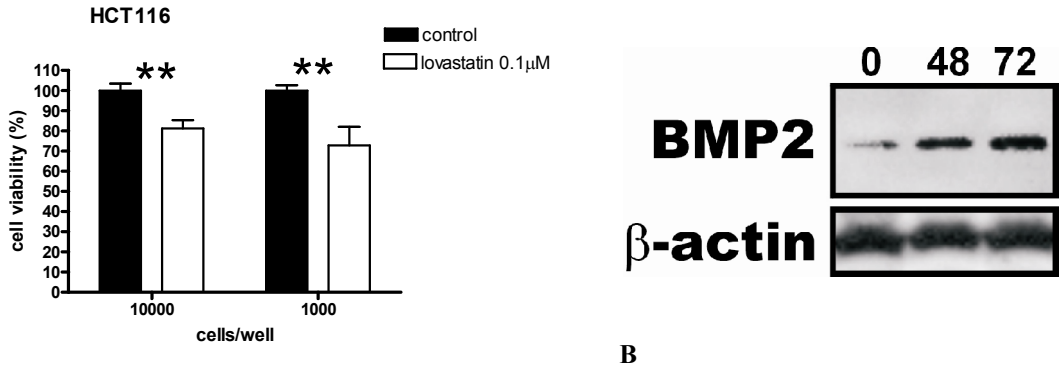

Figure 6. Low concentrations of Lovastatin induce BMP2 and reduce cell viability. (**A**) HCT116 cells were plated in concentration 1000 and 10000 per well in the 6 well plates and treated for 3 weeks with Lovastatin (0.1 μM). Cell viability was assessed using the MTT assay. Values are expressed as a percentage of controls. Data are from three experiments ±s.e.m (n=10). ** P<0.01. (**B**) Immunoblots of HCT116 cells treated with Lovastatin (0,15 μM) for 48h and 72h. The expression of BMP2 was analyzed using the corresponding specific antibody. β-actin served as a loading control.

Discussion

The effects of statins on CRC have been the subject of extensive previous investigation as reviewed by Demierre et al [2]. In humans the evidence for the antitumor efficacy of statins in CRC comes largely from epidemiological studies in statin users. One such study showed that using statins for at least 5 years was associated with significant (47%) reduction in the risk for developing CRC providing evidence for a CRC chemopreventative effect of statins in humans [1]. However, results from further observational studies in humans remain conflicting[4, 27], while an observed reduction in stage IV cancers in users may support a role for statins in the post-initiation phase of CRC in humans[27].

Statins have been shown to be effective anti-tumor agents in various different rodent models of CRC, both alone [28-30] and in combination with NSAIDs[9, 28] and COX-2

inhibitors [28, 31]. Efficacy has been demonstrated in models of tumor initiation[31, 32], tumor growth[33] and metastasis[33] compatible with both chemotherapeutic effects at doses achievable in the short term in humans[34], and chemopreventative effects using doses at or below those currently used for hypercholesterolemia in humans[29].

Previous *in vitro* studies have used doses of between 5 and 50μM [9, 14]. The mild growth stimulatory effects we observe in selected CRC cell lines are only seen at doses of 2μM. This may explain why this effect has not been previously reported. That different cell lines have different sensitivities to statins has previously been reported with HCT116 noted to be particularly sensitive and HT29 resistant, consistent with our findings[10].

Our studies were designed to investigate the mechanism behind the effects of statins in CRC. The model systems available to investigate this better mimic the chemotherapeutic use of statins than their chemopreventative use. However, as outlined above, previous studies using a wide range of doses and in models that better mimic the chemopreventative setting have already been performed. Our experiments *in vivo* investigate whether the differences we see in statin sensitivity between CRC cells of different genetic backgrounds *in vitro* translate into the same differences *in vivo*. This is particularly relevant given the previous finding that statins block tumor angiogenesis [35]. We have therefore chosen a xenograft model of CRC where tumors of different genetic backgrounds can be compared *in vivo*. Whereas previous studies of statins in xenografts in mice have used intratumoral (100mg/kg)[36] or intra-peritoneal injection of statins (25mg/kg)[37] to demonstrate an anti-tumor effect, we chose oral administration of statins to more closely mimic their use in humans and we administered doses in line with these previous studies to ensure that we saw an effect in this model.

Mechanistic studies have shown that statins lead to apoptosis in CRC cell lines and that this can be prevented by giving back products of the mevalonate pathway such as mevalonate and geranylgeranylpyrophosphate [9, 14, 38]. These molecules are required for the prenylation and thus normal function of G-proteins such as Ras and Rho, and thus the major current theory is that statins prevent colorectal cancer through their inhibition of isoprenylation of G-proteins. However the mutation status of these G-proteins has no influence on the sensitivity of cell lines to statins [39]. Furthermore, adding back geranylgeranylpyrophosphate or inhibition of protein translation with cyclohexamide completely reverse the effects of statins (both morphological changes and apoptosis)

while neither restore the membrane localization of small GTP-ases such as Rho [14]. Hence it has been suggested that statins may function through an alternative mechanism involving the production of an unidentified protein.

Statins have anabolic effects on bone through specific activation of the BMP2 promoter, increasing the expression of BMP2 mRNA and protein by osteoblast-like cells [15]. Our results show that statins have similar effects on the BMP pathway in CRC cell lines. Presumably this occurs through direct activation of the BMP2 promoter, as in bone cells. This would be supported by the finding that Lovastatin activates a BMP2-promoter luciferase reporter construct. We further show that CRC cell sensitivity to statins is influenced by the presence or absence of SMAD4, a central and critical part of the BMP pathway. 30% of human CRCs express no SMAD4 [40].

In conclusion, statins induce apoptosis in colorectal cancer cells by the induction of BMP. Colorectal cancer cells missing a central element of the BMP pathway, SMAD4, are insensitive to statin treatment and even show a tendency toward increased growth. Our results might help to identify a subgroup of patients that could benefit from statin treatment and subgroup of patients that could respond adversely. While several authors have proposed trials of statins in CRC [2] these data suggest that caution would be advised in trials of statin therapy in unselected CRC. The development of statins or statin-related molecules with enhanced effects on the BMP pathway may offer increased benefit in the treatment of colorectal cancer while maintaining the excellent safety profile of the statins.

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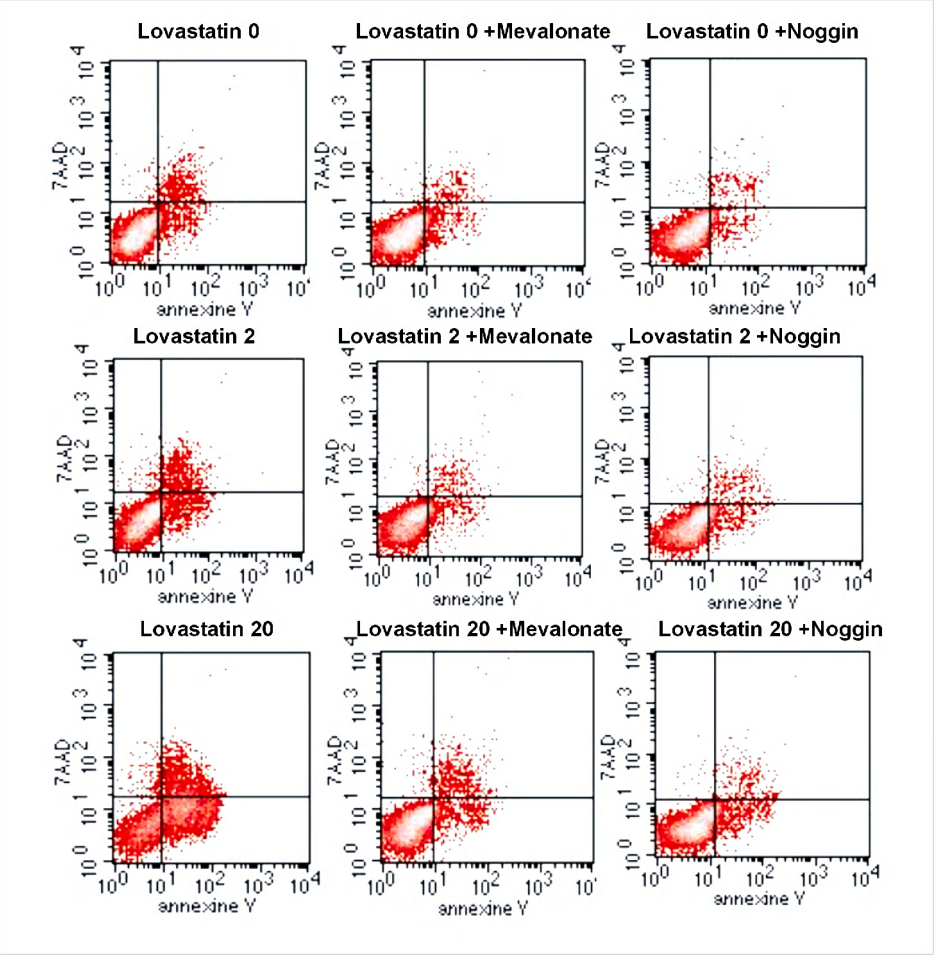
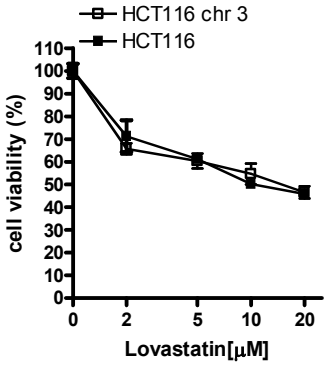
Supplementary Figures



Supplementary Figure 1. HCT116 cells were treated with Lovastatin, Lovastatin in combination with Noggin (0,5µg/mL), or Lovastatin in combination with Mevalonate (100 µM) for 24h and apoptosis was quantified by flow cytometry.



Supplementary Figure 2. The MTT assay was performed in HCT116 or HCT116 chr 3 cells treated for 24h with various concentrations of Lovastatin. Values obtained in the absence of compound have been set at 100. Results represent the mean ± s.e.m.of three experiments.

Chapter 7

Lovastatin induces the demethylation of the Bone Morphogenetic Protein 2 promoter in colorectal cancer cell lines

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Abstract

Background and Aims:

DNA methyltransferase (DNMT) inhibitors have been proposed as a new class of anti-cancer drugs. We have previously shown that Statins induce apoptosis in colorectal cancer (CRC) cells through the induction of Bone Morphogenetic Protein 2 (BMP) expression. The BMP2 promoter is known to be highly methylated in gastric cancers. In this study we evaluated the methylation status of the BMP2 promotor in a panel of CRC cell lines and the effect of Lovastatin treatment on the methylation levels of the BMP2 promoter in order to determine whether Statins act as DNA methyltransferase inhibitors in CRC.

Methods:

We performed methylation specific PCR (MSP), bisulfite sequencing and an in vitro DNMT assay on CRC cell lines. Immunoblot analysis was used to investigate the effects of Lovastatin treatment on BMP2 protein expression.

Results:

The CpG island of the BMP2 promoter region is methylated in 3 out of 8 colon cancer cell lines. In HCT116 cells the promoter is fully methylated, while SW48 and RKO cell lines exhibit partial methylation. Treatment of HCT116 cells with 2 μM of Lovastatin leads to demethylation of the BMP2 promoter region as shown by both MSP and bisulfite sequencing, and to dramatic upregulation of BMP2 protein expression. Lovastatin treatment inhibits DNMT activity in HCT116 cells .

Conclusions:

CRC cell lines show methylation of the BMP2 promoter. Lovastatin treatment induces BMP2 protein expression through inhibition of DNMTs and demethylation of the BMP2 promoter region. Statins could be particularly useful as chemotherapeutic agents in a subgroup of patients with methylation of the BMP2 promoter.

Introduction

Aberrant DNA methylation of CpG islands in the promoter regions of many genes has been widely observed in human colorectal cancer (CRC) and is associated with tumour-suppressor gene silencing. Cancers that show extensive DNA methylation have been described as having the CpG island methylator phenotype (CIMP)[1]. This DNA hypermethylation has been identified as a critical determinant of tumour initiation and progression[2]. As hypermethylation needs to be actively maintained after each cell division and the process of methylation is regulated by DNA methyltransferases (DNMTs), DNMT inhibitors represent promising new drugs for cancer therapy.

DNA methylation is regulated by DNA methyltransferases (DNMT). DNMTs are enzymes that catalyze the addition of methyl groups to cytosine residues in DNA. In mammalian cells three DNMTs are found – DNMT1, DNMT3a and DNMT3b. DNMT1 appears to be responsible for the maintenance of established patterns of DNA methylation [3], while DNMT3a and DNMT3b seems to mediate the establishment of new DNA methylation patterns [4]. However, recent findings suggest a potential role for DNMT1 in the initiation of promoter CpG island hypermethylation in human cancer cells [5]. The activity of DNMT1, DNMT3a and 3b is elevated in colorectal cancer cells, increasing in a linear fashion with tumour progression [6, 7]. It has been established that the inhibition of DNMT activity can strongly inhibit the formation of tumors in vivo [8].

The most widely used DNMT inhibitor, 5-azacytidine has proven to be effective in a phase III clinical trial [9]. However, currently known DNMT inhibitors cause significant cytotoxicity [10, 11] as they become incorporate into RNA and interfere with protein translation [12].

BMPs are the members of the Transforming Growth Factor-β superfamily. They signal by cooperatively binding type I and type II BMP receptors which in turn phosphorylate the BMP-specific SMADs1,5 and 8 which then complex with SMAD4. The complex translocates to the nucleus and regulates gene transcription of BMP target genes. The BMP pathway has recently been implicated in colorectal cancer with the identification of germline mutations in BMPR1a and SMAD4 in families with familial Juvenile Polyposis Syndrome [13]. Affected individuals have a greatly increased risk of developing cancer [14]. BMP2 inhibits cell proliferation and induces differentiation in colorectal cancer cells [15]. A recent study has shown that BMP2 promoter is methylated in a large

proportion of gastric cancers[16].

The Molecular Epidemiology of Colorectal Cancer (MECC) study showed that using Statins for at least 5 years was associated with significant (47%) reduction in the risk for developing CRC [17]. *In vitro* studies show that Statins inhibit cellular proliferation and induce apoptosis in colon cancer cells[18, 19].

Statins inhibit the enzyme HMGCoA reductase and thereby downregulate cholesterol synthesis [20] and prevent the synthesis of other important isoprenoid intermediates of the mevalonate pathway, such as farnesylpyrophosphate and geranylgeranylpyrophosphate. Posttranslational isoprenylation is important in determining the membrane localization and function of many cellular proteins including small GTPases like Ras and Rho [21] and therefore the major current theory is that Statins prevent CRC through their inhibition of isoprenylation of G-proteins. However the mutation status of these G-proteins has no influence on the sensitivity of cell lines to Statins [22] . Furthermore, adding back geranylgeranylpyrophosphate reverses the effects of Statins (both morphological changes and apoptosis) while not restoring the membrane localisation of small GTP-ases such as Rho [23]. Hence it has been suggested that Statins may function through an alternative mechanism involving the production of an unidentified protein[23 24]. We have recently shown that Statins induce apoptosis through the induction of Bone Morphogenetic Protein (BMP) 2, but the exact mechanism by which Statins upregulate BMP2 protein expression is not known.

Here we have investigated the methylation status of CRC cell lines and the influence of Lovastatin treatment on methylation of the BMP2 promoter in HCT116 cells. We show that in a subgroup of CRC cell lines the BMP2 promoter is methylated. Lovastatin treatment inhibits the activity of DNMTs and thereby leads to demethylation of the BMP2 promoter and to upregulation of BMP2 protein expression.

Materials and methods

Cell culture

SW480, HT29, CACO2, RKO, SW48 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 L-glutamine. This was

supplemented with penicillin (50U/ml) and streptomycin (50µg/ml) and, where serum was used, with 10% fetal calf serum (Gibco, Paisley, Scotland).

Immunoblotting

Cells at 60-80% confluence from 6 well plates were washed in ice-cold PBS and scraped into 200µl of 2×sample buffer (125mM Tris/HCl, pH 6,8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, 1mg bromophenol blue). Protein concentration was measured using the RC DC protein assay kit (Biorad, CA, USA) according to the manufacturer's instructions. The lysates were sonicated and then heated at 95°C for 5 min. 50µg of protein from each sample was loaded onto SDS-PAGE and blotted onto PVDF membrane (Millipore, Bedford, MA, USA). The blots were blocked in block buffer (2% low fat milk powder in Tris-buffered saline with 1% Triton (TBST)) for 1 hour at room temperature and washed 3×10 min in TBST before overnight incubation at 4°C with primary antibody in primary antibody buffer (TBST with 0.2% low fat milk powder). Primary antibodies to BMP2 (mouse monoclonal) were from R&D (Abingdon, UK). Blots were then washed 3×10 min in TBST and incubated for 1 hour at room temperature in 1:2000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit, rabbit anti-goat or goat anti-mouse secondary antibody (Dako, Glostrup, Denmark) in block buffer. After a final 3×10 min wash in TBST, blots were incubated for 5 min in Lumilite Plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Lumi-Imager (Boehringer-Mannheim, Mannheim, Germany).

Methylation analysis

DNA was extracted using the DNeasy Tissue Kit (Qiagen, Venlo, The Netherlands).

Bisulfite treatment was performed using the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Bisulfite sequencing and MSP were performed as described previously[25, 26].

The primer sequences for MSP for BMP2 were as used in [16]. PCR was performed with 40 cycles of 94ºC, 62ºC, and 72ºC of 1- min each, preceded by a 5-min denaturing step at 94ºC and followed by a 10-min extension step at 72ºC. The products were electrophoresed on 5% agarose gel. Human genomic DNA from peripheral blood

lymphocytes was used as an unmethylated control. Human genomic DNA treated in vitro with Sss I methyltransferase (New England Biolabs, Beverly, Massachusetts, USA) was used as a positive control for the methylated reaction.

For bisulfite sequencing, cell line derived DNA was treated with sodium bisulfite and amplified by PCR. The primers for bisulfite sequencing of BMP2 were 5'-GTATTTGGTTTTAGGGTTAGGAGAG -3' (forward) and 5'-CCAAATACTAACACACAACAACAAC -3' (reverse). PCR was performed with 35 cycles of 94ºC, 62ºC, and 72ºC of 1- min each, preceded by a 5-min denaturing step at 94ºC and followed by a 10-min extension step at 72ºC. The PCR product was purified using QIAquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands)**.** The purified PCR products were ligated into pCR2.1-TOPO using the TOPO-TA cloning system (Invitrogen, Breda, the Netherlands). Bacteria TOP10 were transformed with plasmids and cultured overnight, and the plasmid DNA was isolated using the Miniprep kit (Qiagen, Venlo, The Netherlands). For each sample, five to 10 separate clones were sequenced on an ABI 377 or 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA) using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the original primers for bisulfite sequencing of BMP2.

In vitro DNA methyltransferase (DNMT) assay

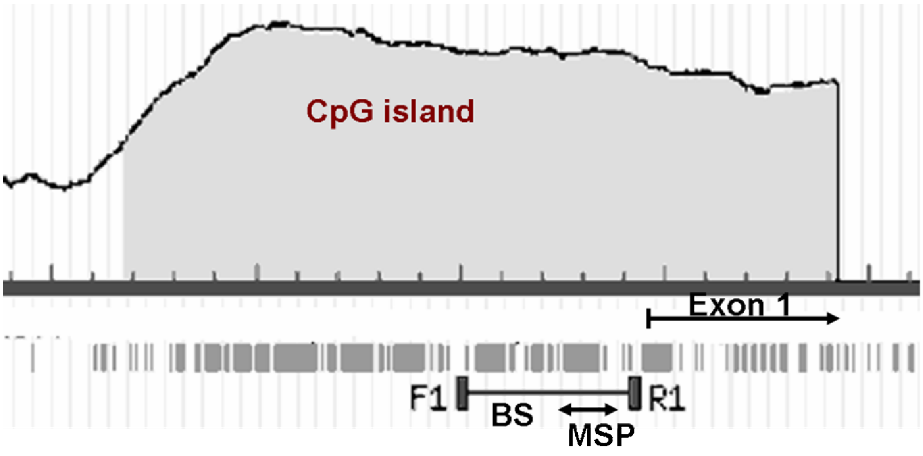
HCT116 cells at 50-70% confluence in 6 well plates were treated with different concentrations of Lovastatin, 5 μM 5-azacytidine (5-AZA) (Sigma, St. Louis, MO) or 100 nM Trichostatin A (TSA) for 48 or 72 hours. Cells were washed in ice-cold PBS and scraped into 200μl ice-cold cell extract buffer (10mM Hepes-KOH, pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM dithiothreitol (DTT) and 0.2mM phenylmethylsulfonyl fluoride (PMSF). The cells were kept on ice for 10 min, vortexed for 10 sec, and centrifuged at 4°C at 14000rpm for 30 sec. The supernatant was discarded and the pellet was resuspended in 30μl of nuclear extraction buffer (20mM HEPES-KOH, pH 7.9, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT and 0.2mM PMSF), placed on ice for 20 min, and centrifuged at 4°C at 14000rpm for 2 min. The supernatant was saved as the nuclear extract and used for measuring of the total Dnmt

activity using EpiQuik DNA Methyltransferase Activity/ Inhibition Assay Kit according to the manufacturer's instructions.

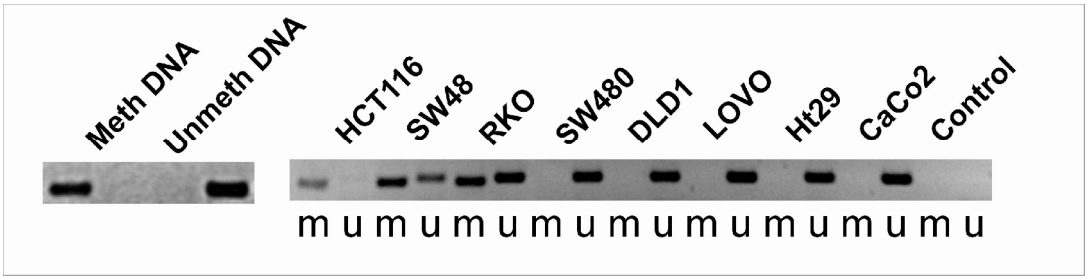
Results

The BMP2 promoter is methylated in CRC cell lines

We performed MSP for the BMP2 promoter region starting -214bp in front of exon 1 (Figure1A). This region contains a CpG island and has been shown to be methylated in gastric cancers [16]. Three out of eight CRC cell lines show CpG island methylation of the BMP2 promoter region. MSP for HCT116 cells contains only signals for methylated alleles of BMP2. RKO and SW48 seem to be partly methylated as they exhibit both methylated and unmethylated signals whereas only unmethylated alleles are found in SW480, DLD1, LOVO, HT29 and CaCo2 cells (Figure1B).



A

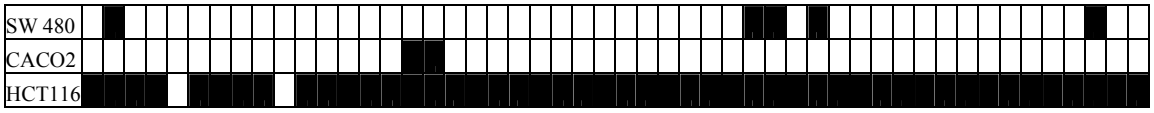


B

Figure 1. A, Schematic representation of the GpG island in the BMP2 promoter region. Vertical bars show CpG sites. Black arrows below the CpG sites indicate the regions subjected to methylation specific PCR analysis (MSP) and bisulfite sequencing (BS). **B,** MSP analysis of the CpG island methylation status of the BMP2 promoter region in colorectal cancer cell lines. PCR products specific for unmethylated (U) and methylated (M) CpG sites were analysed in 2.5% agarose gels. U control – human genomic DNA from peripheral blood lymphocytes. M control - human genomic DNA treated in vitro with Sss I methyltransferase.

We verified the results of MSP by direct bisulfite sequencing (Figure2A). The examined region of the promoter CpG island between positions -453 and -2 contains 50 CpG dinucleotides. HCT116 cells show dense methylation of the BMP2 promoter as 96% of CpGs were methylated. SW480 and CACO2 cells show only scattered methylation within the examined region supporting the results of MSP.

It was not possible to perform direct bisulfite sequencing on SW48 and RKO cell lines probably due to mixture of methylated and unmethylated signals. Therefore we did bisulfite sequencing of multiple independent clones to determine the BMP2 promoter methylation status in these cell lines. As seen in Figure 2B and 2C, the level of BMP2 promoter methylation in SW48 cells is 51% and in RKO is 72%.



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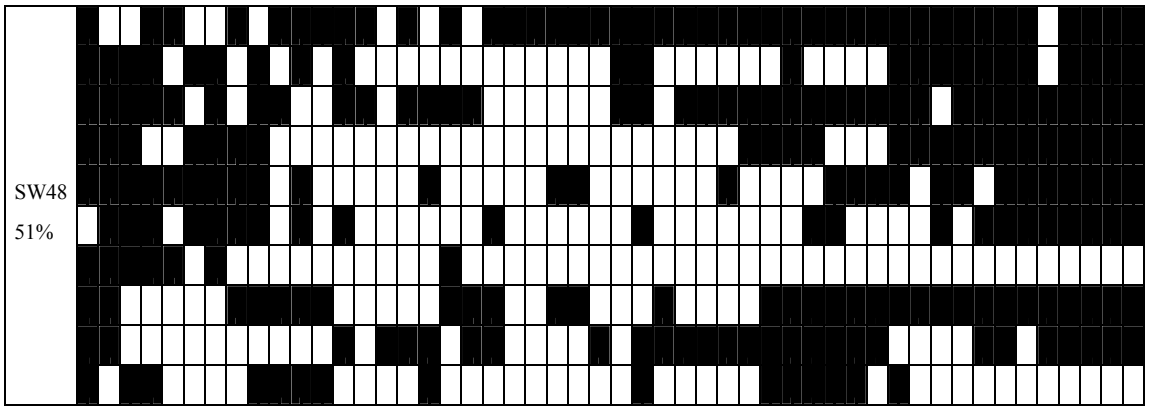
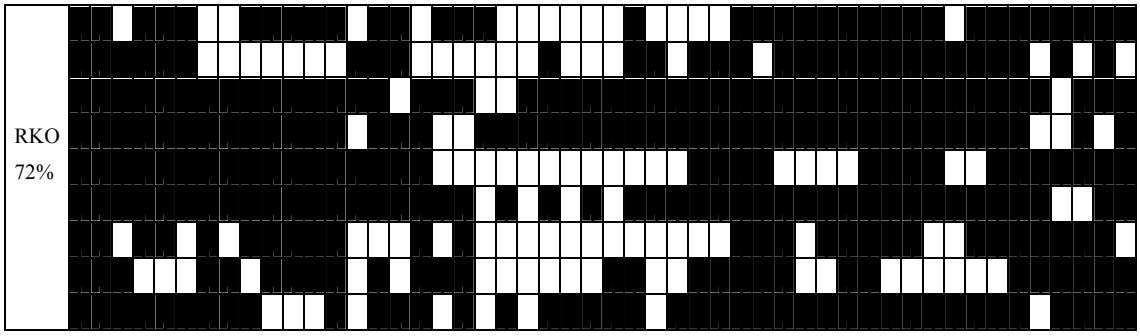


B



C

Figure 2. A, Direct bisulfite sequencing of the BMP2 promoter in SW480, CACO2 and HCT116 colorectal cancer cell lines. **B**, **C**, Bisulfite sequencing of multiple independent clones from SW48 (B) and RKO (C) cell lines. Each horizontal row of squares represents the 50 CpG sites contained in the region. Solid and open squares represent methylated and unmethylated CpG sites, respectively. Percentages indicate the fraction of methylated CpG dinucleotides.

Induction of BMP2 expression and demethylation of BMP2 promoter by Lovastatin treatment

To determine whether BMP2 expression in the HCT116 cell line, which has high degree of BMP2 promoter methylation, could be modulated by treatment with Lovastatin, we treated the cells with Lovastatin and performed immunoblot analysis. Lovastatin treatment leads to a time-dependent upregulation of BMP2 protein expression (Figure 3A).

Next, we evaluated the methylation status of the BMP2 promoter after treatment of HCT116 cells with Lovastatin for 48h and 72h by MSP. As shown in Figure 3B, Lovastatin treatment leads to demethylation of the BMP2 promoter, as MSP for HCT116 cells treated with Lovastatin contains both the methylated and unmethylated signals.

We performed bisulfite sequencing of multiple independent clones from HCT116 cells and HCT116 cells treated for 72h with Lovastatin. The BS results show that the BMP2 promoter methylation decreases from 97% to 40% after treatment with 2 μM of Lovastatin for 72h (Figure 3B and 3C).

Lovastatin inhibits DNMT activity in vitro

To further investigate the mechanism by which Lovastatin leads to BMP2 promoter demethylation, we performed an in vitro DNA methyltransferase (DNMT) assay. Treatment with 5-AZA or TSA results in downregulation of DNMT activity to a background level (Figure 4A). Treatment with different concentrations of Lovastatin leads to a dose-dependent downregulation of DNMT activity. Remarkably, even low concentrations of Lovastatin (0,25 and 0,5 μM) downregulate the DNMT activity after 48h and especially after 72h. Treatment with 10 μM of Lovastatin downregulates the activity of DNMT to a background level (Figure 4B). These data further support the conclusion that Lovastatin acts as a DNMT inhibitor and thus leads to BMP2 promoter demethylation and upregulation of BMP2 protein expression.

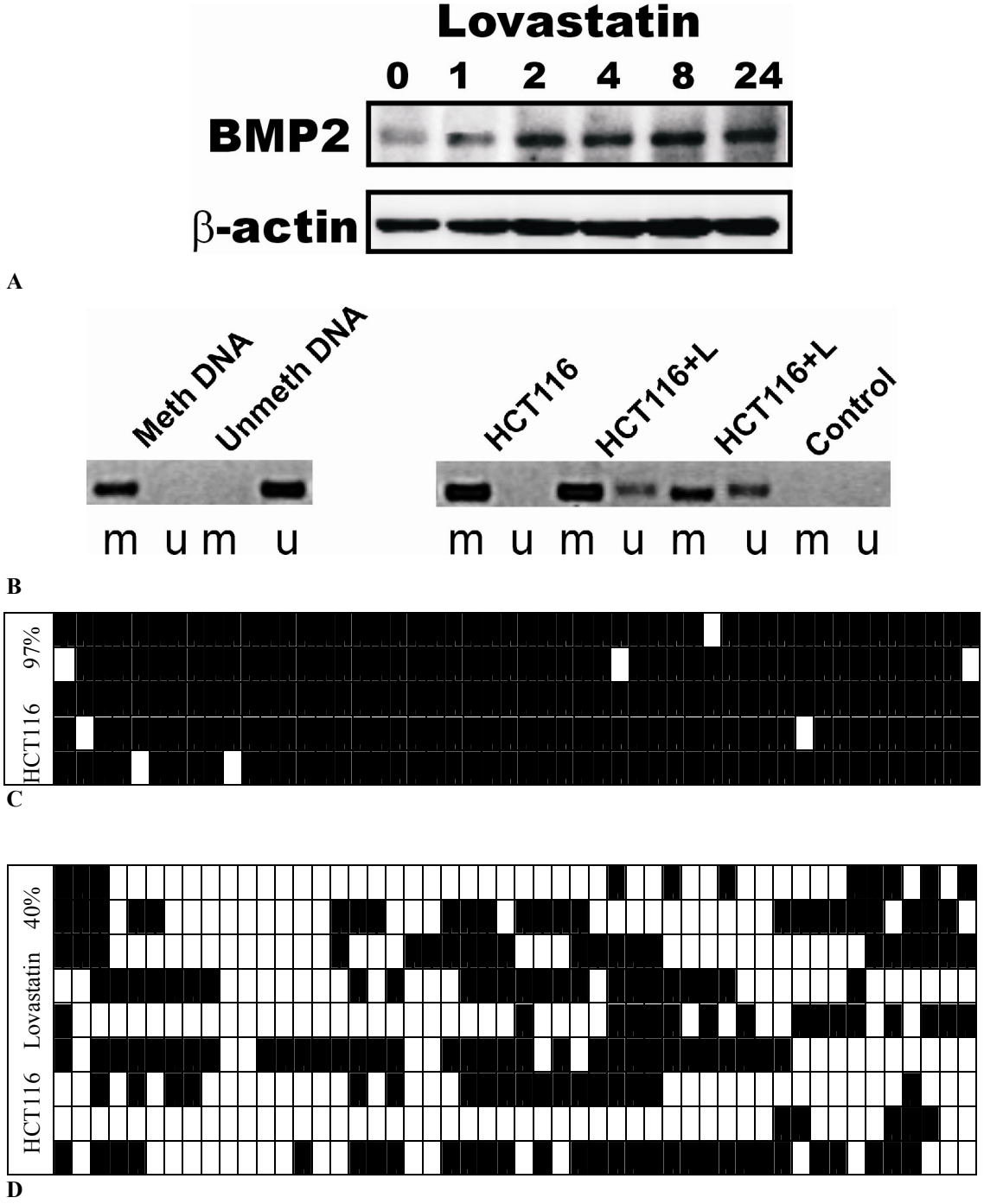


Figure 3. A, Immunoblots of HCT116 cells treated with Lovastatin for different time points (shown in hours). The protein expression was analyzed using the corresponding specific antibody. β-actin served as a loading control. **B**, MSP for HCT116 cells treated with 2 μM of Lovastatin for 48 and 72 h. PCR products specific for unmethylated (U) and methylated (M) CpG sites were analysed in 2.5% agarose gels. U control – human genomic DNA from peripheral blood lymphocytes. M control - human genomic DNA treated in vitro with Sss I methyltransferase. **C**, Bisulfite sequencing of multiple independent clones from HCT116 cells without Lovastatin treatment. **D**, Demethylation of BMP2 by Lovastatin. HCT116 cells were treated with 2 μM of Lovastatin for 72 h and bisulfite sequencing of multiple independent clones was performed. Each horizontal row of squares represents analysis of 50 CpG sites contained in the region. Solid and open squares represent methylated and unmethylated CpG sites, respectively. Percentages indicate the fraction of methylated CpG dinucleotides.

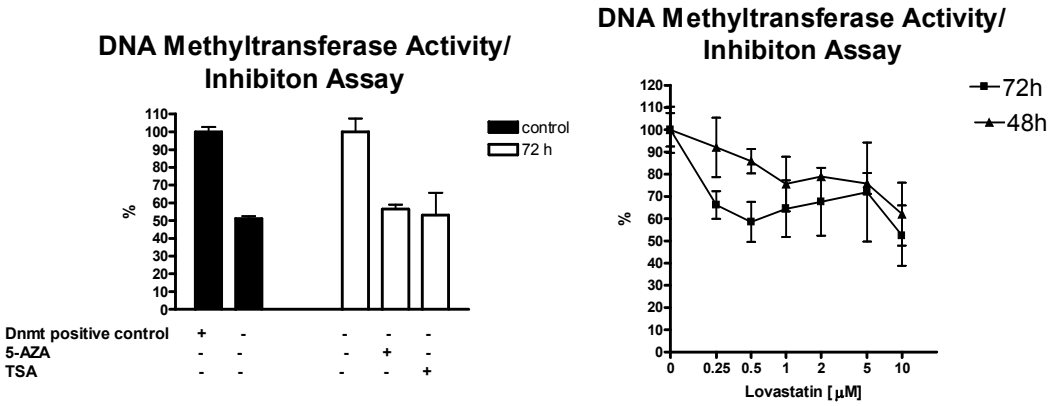


Figure 4. A, HCT116 cells were treated for 72 h with 5-AZA or TSA and subjected to DNA methyltransferase activity/Inhibition assay. 5-AZA and TSA treatment inhibits activity of DNMTs to a background level. **B,** HCT116 cells were treated with 2 μM Lovastatin for 48 and 72 h and then DNA methyltransferase activity/Inhibition assay was performed. Treatment with Lovastatin inhibits DNMTs activity in a time and dose-dependent manner. DNMT positive control is provided by the manufacturer and values obtained with DNMT positive control have been set at 100%. No nuclear extract was added in blank wells and the measured absorbance was considered as a background level.

Discussion

DNA hypermethylation of the promoter region of tumor suppressor genes occurs in most cases of cancer and results in the transcriptional silencing and loss of function of critical tumor suppressor genes[27]. Epigenetic alterations do not involve changes in the DNA sequence and are thus potentially reversible making the use of demethylating agents a promising new option in cancer therapy.

Statins reduce the number and size of tumours in various CRC models in mice [28, 29] and they have antitumoural activity in CRC cell lines[18, 30]. We have previously shown that Lovastatin treatment leads to BMP2 upregulation and to the induction of apoptosis in CRC cell lines through the activation of the BMP pathway, but the mechanism of BMP2 upregulation by Statins remains unknown. The BMP2 promoter is known to be methylated in a large proportion of gastric cancers [16]. In this study we investigated the level of BMP2 promoter region methylation in a panel of CRC cell lines and the influence of Lovastatin treatment on the methylation status of the BMP2 promoter.

First, we evaluated the methylation status of the BMP2 promoter in 8 CRC cell lines and found that HCT116 has a fully methylated BMP2 promoter (as shown previously[16]). SW48 and RKO exhibit partial methylation of the BMP2 promoter, as we have shown by Methylation Specific PCR and Bisulfite Sequencing of multiple independent clones.

This suggests that the BMP2 promoter could be methylated in human CRC. However, further study on DNA isolated from patient material should be performed to test this hypothesis.

Subsequently we examined the influence of Lovastatin treatment on the methylation level of the BMP2 promoter. For this purpose we used HCT116 cells which are completely methylated in the BMP2 promoter region, and treated them with Lovastatin. Treatment with Lovastatin for 48 and 72 h leads to the appearance of the unmethylated-specific product using MSP and to a reduction in methylation from 97% to 40% when we performed BS of multiple independent clones. Lovastatin treatment also upregulates the expression of BMP2 protein in a time dependent manner and downregulates the activity of DNMTs. These data suggest that Lovastatin can induce DNA demethylation and the reactivation of BMP2 gene expression, which is silenced by hypermethylation in CRC cells. Even at low concentrations of Lovastatin (0,25μM), which is comparable with the serum levels of ~ 0.1 μM measured in patients treated with statins at standard doses for hypercholesterolemia[31], inhibition of DNMTs can be seen. These low concentrations of Statins are safe and well tolerated by patients for years. Thus, in contrast to most known DNMT inhibitors, Lovastatin inhibits DNMTs and induces DNA demethylation at non-toxic doses.

One important question not addressed by this study is whether Lovastatin specifically demethylates the BMP2 promoter or whether this effect is universal. The observed effects on DNMTs would suggest the latter but the influence of Lovastatin treatment on a wide panel of tumor-supressor genes know to be methylated in CRC such as CDKN2A (also known as p16), MINT1, MINT2, MINT3, MLH1, CACNA1G, IGF2, NEUROG1, RUNX3 and SOCS1 [32, 33] could be tested to answer this question.

The CpG island methylator phenotype is found not only in colorectal cancers but also in colonic polyps [34, 35] and even in the normal colorectal mucosa in patients with hyperplastic polyposis [36]. This group of patients would greatly benefit from chemopreventive agents exhibiting mild demethylating properties and an excellent safety profile. As Statin treatment also reduces morbidity and mortality associated with cardiovascular disease [37] their demethylating effect could make Statins a particularly valuable chemopreventative agents in a well defined group of patients.

In conclusion, the BMP2 promoter is methylated in a subgroup of CRC cell lines.

Lovastatin demethylates the BMP2 promoter and reactivates proapoptotic BMP2 protein expression. These results reveal the mechanism of BMP2 upregulation by Statins. Our results could help to identify a subgroup of patients where Statins could be effectively used as chemopreventative drugs, patients prone to CpG island methylator phenotype colorectal cancer.

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Chapter 8

Effect of Aspirin on the Wnt/β-catenin pathway is mediated via Protein Phosphatase 2A

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Abstract

Background and Aims

NSAIDs show chemopreventive efficacy in colon cancer but the mechanism behind this remains unclear. Elucidating this mechanism is seen as vital to the development of new chemopreventive agents.

Methods

We studied the effects of aspirin on the oncogenic Wnt/β-catenin pathway activity in colorectal cancer cell lines using immunoblotting, immunoprecipitation, luciferase reporter assay and and PP2A activity determination. Furthermore, we assessed the ubiquitination and cytoplasmic levels of β-catenin and also showed the localization of β-catenin by GFP-tagged β-catenin and time-lapse fluorescent imaging after aspirin treatment.

Results

We observed that aspirin dose-dependently decreased the activity of Wnt/β-catenin pathway, as judged by TCF-driven luciferase activity, reduced Wnt target gene expression and increased phosphorylation of β-catenin by immunoblotting. Importantly, aspirin treatment caused increased phosphorylation of protein phosphatase 2A (PP2A), an event associated with inhibition of PP2A enzymatic activity, which was confirmed by a reduction in enzymatic PP2A activity. Moreover, this inhibition of PP2A enzymatic activity was essential for the effects of aspirin on the Wnt/β-catenin pathway as shown by transient transfection with PP2A constructs.

Conclusions

The findings in this article provide a molecular explanation for the efficacy of aspirin in chemoprevention of colorectal cancer and shows biochemical evidence that PP2A is an important regulator of Wnt/β-catenin pathway activity in these cells.

Introduction

Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce death rates due to colon cancer [1] and to reduce the size of colonic adenomatous polyps [2], both in animal and in human studies ([3] and references therein). These findings suggest that chemoprevention of colon cancer may be a possible way of reducing the impact of this increasingly common and frequently fatal disease. There remain, however, serious problems with the widespread use of NSAIDs in those at risk for the development of colon cancer. The incidence of side effects such as gastrointestinal haemorrhage and renal damage preclude their use in this setting. The development of drugs with similar efficacy in colon cancer with acceptable side effects is in process. Understanding the mechanism of action of NSAIDs in colon cancer is an essential step in this process.

The original and still the leading theory is that NSAIDs act through their ability to block cyclooxygenase (COX) enzymes. However, related compounds with no COX-inhibitory activity retain their antitumour activity [4] and cancer cells lacking COX-2 enzyme are still sensitive to these compounds [5]. Therefore COX-independent effects of NSAIDs in colon cancer have been postulated (reviewed in [6]) such as their ability to block peroxisome proliferation activator delta [7], inhibition [8] or activation [9] of nuclear factor kappa beta and effects on the TGF-beta family member, macrophage inhibitory cytokine [10]. A more direct explanation of the effects of NSAIDs in colon cancer would be that they target elements within the most essential oncogenic pathway in colon cancer, the Wnț/β-catenin pathway. An activating mutation of the Wnt/β-catenin pathway is the first step in almost all colorectal cancers. Here the essential feature is an increase in the levels of free cytoplasmic β-catenin (reviewed in [11]). In most cases Wnt/β-catenin pathway activation is brought about by a mutation in the adenomatous polyposis coli (APC) gene (reviewed in [12]). This leads to a truncated APC protein that cannot perform its function as part of the β-catenin destruction complex, which in turn leads to reduced β-catenin degradation and abnormally high levels of cytoplasmic β-catenin.

This stabilized β-catenin translocates to the nucleus where it binds with members of the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family of transcription factors, and activates the transcription of Wnt target gene expression such as cyclin D and Myc [13]. In many of the remaining cases, mutations in β-catenin itself at sites of Glycogen Synthase

Kinase-3β (GSK3β) phosphorylation leads to its cytoplasmic accumulation and Wnt/β-catenin pathway activation [14].

We set out to further investigate the effects of NSAIDs on the Wnt/β-catenin pathway in colon cancer cells. A reduction in the constitutive activity of this pathway in colon cancer, for example by reducing levels of signalling β-catenin, reverses this earliest and most essential oncogenic signal and has been shown to be an effective anti-tumour strategy *in vitro* and *in vivo* [15, 16].

Evidence has been building that there may be direct effects of NSAIDs on this pathway [17-19]. This would concur with the epidemiological data that suggest NSAIDs act at a very early stage in colon cancer and must be given at this earliest point in the disease process to be effective [20]. Here we provide evidence that NSAIDs inhibit the Wnt/β-catenin pathway by stimulating the phosphorylation and breakdown of β-catenin.

Furthermore, the precise biochemical mechanism by which the β-catenin degradation complex is regulated is not yet known. Alterations in protein phosphorylation states are likely to be central to this regulation, because all the elements of the β-catenin degradation complex are phospho-proteins. The phosphorylation status of a protein is determined by the relative activities of both kinases and phosphatases. A number of protein kinases have been shown to influence Wnt/β-catenin signalling, including GSK3β, protein kinase C, and casein kinase I and II [21]. The protein phosphatases involved have received less attention. However, in *Xenopus laevis* axis formation the catalytic subunit of the serine/threonine protein phosphatase 2A (PP2A) exerts a positive role on Wnt/β-catenin pathway activity whereas the inhibitory regulatory B56 subunit of PP2A exerts a negative influence on Wnt/β-catenin signalling in mammalian cells and Xenopus embryo explants [22, 23]. Mutations in PP2A subunits in various types of cancer, including colon cancer, have also been described, but their precise role in the cancerous process is as yet unknown (reviewed in [24]).

For these reasons we decided to study the effect of aspirin on the Wnt/β-catenin pathway and the role of PP2A in these effects. Our results show that aspirin inhibits Wnt pathway activity at the level of PP2A enzymatic activity, providing a possible molecular mechanism for aspirin effects in chemoprevention.

Materials and Methods

Cell Culture

DLD-1, SW480, and HCT116 colon cancer cell lines were obtained from the ATCC, and cultured in Dulbecco's Modified Eagles Medium (DMEM)(Gibco, Pailsey, Scotland) with 4.5 g/L Glucose and L-glutamine, which was supplemented with penicillin (50 U/ml) and Streptomycin (50 μg/ml) and, were serum was used, with 10% fetal calf serum (Gibco). Cells were grown in monolayers in a humidified atmosphere containing 5% CO_2.

TOPFLASH assay

Figure 1: SW480 cells and DLD-1 cells were seeded in 24-well plates at 1×10^5 cells/well. The following day cells were transiently transfected with 0.4 μg of the TCF reporter, pTOPFLASH (TCF Optimal Motif), or pFOPFLASH ('Far from OPtimal' motif) [25] (pTOPFLASH and pFOPFLASH were gifts from Dr. Hans Clevers (Utrecht University, The Netherlands)), and 0.008 μg of pCMV-*Renilla* luciferase (Promega, Madison, WI, USA) per well, using LipofectAMINE-Plus reagent according to the manufacturers instructions (Invitrogen™ life technologies) for 5 h. Hereafter, cells were incubated for 48 hours in DMEM with 10% serum and no antibiotics containing various concentrations of aspirin.

Figure 6C: DLD-1 cells were seeded in 24-well plates at 1×10^5 cells/well. The following day cells were transiently transfected overnight with 0.4 μg of the TCF reporter, pTOPFLASH, 0.008 μg of pCMV-*Renilla* luciferase (Promega), 0.8 μg $(HA)_3$-C_{Y307F} construct (more information see transient transfection with PP2A constructs) or control per well, using Lipofectamine-2000™ reagent according to the manufacturers instructions (Invitrogen™ life technologies) overnight. Hereafter, cells were incubated for 6 hours in DMEM with 10% serum and no antibiotics containing various concentrations of aspirin.

Samples were further prepared using the dual luciferase reporter kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and luciferase levels measured using a dual injector luminometer.

Preparation of Immunoblot Samples

DLD-1 or SW480 cells were seeded in 6-well plates (Nalge Nunc, Denmark) and left to adhere for 24 hours. The next day, at 60-80% confluence, cells were treated with aspirin

(as indicated). Afterwards, cells were washed in ice cold phosphate buffered saline (PBS) and scraped into 200 µl of 3x SDS-sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, bromphenol blue). Protein concentration was measured using the RC DC protein assay kit (Biorad, CA) according to the manufacturer's instructions. Samples were stored at -20°C.

Immunoblotting

Samples were boiled for 5 minutes at 95ºC followed by SDS-PAGE and blotted on PVDF membrane (Millipore). The blots were blocked with either 1% low fat milk powder (for phospho-β-catenin, β-catenin, β-catenin (both obtained from Cell Signaling Technology™, Beverly, MA, USA), β-actin, phopho-PP2A, ubiquitin (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA), and PP2A (BD transduction laboratories, San Jose, CA) or 0.5% low fat milk powder (for cyclin D1(Neomarkers, Fremont, CA), c-myc, c-met (both obtained from Santa Cruz Biotechnology) in Tris Buffered Saline supplemented with 0.1% Tween-20 (TBST) for one hour at room temperature and washed three times with TBST before overnight incubation at 4ºC with primary antibody in blocking buffer.

Subsequently, blots were washed three times for 10 minutes in TBST and incubated with, depending on used primary antibody, a secondary HRP-linked antibody (Goat-α-Mouse, Swine-α-Rabbit, Rabbit-α-Goat, all from DAKO, Glostrup, Denmark) for one hour at room temperature in blocking buffer. Hereafter, the blots were three times washed in TBST and after chemoluminescence using Lumilight^{+} as a substrate, antibody binding was visualized using a Lumi-Imager (both from Roche).

Cytoplasmic extraction

Cytoplasmic extractions were performed as described previously by Orford *et al*. [26]. Briefly, SW480 cells were seeded in 6-well plates and left to adhere for 24 hours. The next day, at 60-80% confluence, cells were treated with 5 mM aspirin for 16 hours, washed with ice-cold PBS and scraped into ice-cold lysis hypotonic buffer (10 mM Tris pH 7.4, 0.2 mM $MgCl_2$, and 1 mM of Pefablock (Merck, Darmstadt, Germany)). Cell lysates were homogenized gently by passing it 10x through a 27 gauche needle and then centrifuged for one hour at 100,000 *g* at 4°C. The supernatant, designated S100, was removed and added to an equal volume of 3x SDS-sample buffer. The resultant pellets,

designated as P100, were taken up in diluted SDS-sample buffer to the same dilution as the S100 fraction for immunoblotting.

Real-time imaging of GFP-tagged β-catenin

SW480 cells were seeded out overnight onto glass coverslips. The following day cells were transiently transfected with GFP-tagged β-catenin using LipofectAMINE-Plus reagent according to the manufacturers instructions and then grown overnight in DMEM with 10% serum. The following day single GFP-expressing cells were centered under a fluorescent microscope in a chamber containing medium at 37°C. The medium was changed for medium with 10mM Aspirin 37°C after one minute, and the cells were followed for a further 3000 seconds with one image taken every 60 seconds.

Immunoprecipitation for ubiquitinated β-catenin determination

SW480 cells were grown in monolayers and were incubated with 5 mM for one hour. Afterwards, cells were washed in ice-cold PBS and scraped into ice-cold lysis buffer (150 mM NaCl, 0.5% Triton-X-100, 5 mM EDTA, 0.1% SDS, 0.5% sodiumdeoxycholate, 1 mM Pefablock) and incubated at 4°C for 15 minutes. Cell lysates were then centrifuged for 5 minutes at 14000 rpm and supernatants were incubated overnight at 4°C with anti-β-catenin antibodies (1:500). This was followed by incubation with protein-A sepharose beads (Sigma, St. Louis, MO) for 1 hour at 4°C. After centrifugation for 15 minutes at 14000 rpm at 4˚C the beads were washed 5 times in ice-cold PBS before the addition of an equal volume of 3x SDS-sample buffer. Samples were immunoblotted using anti-ubiquitin antibodies.

Immunoprecipitation for PP2A activity determination

DLD-1 and SW480 cells were grown in monolayers and were incubated with 5 mM aspirin for 60 minutes. Afterwards, total cellular proteins were extracted in a phosphate free buffer (10 mM Tris HCl, pH 7.6, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EGTA, 1% Triton-X-100, 0.1 mM $MnCl_2$, 0.1 mg/ml BSA, 10 μg/ml leupeptin (Boehringer-Mannheim, Mannheim, Germany), 1 μg/ml aprotinin (Roche), 1 mM Pefablock) and sonicated for 10 seconds. Cell lysates were then centrifuged for 5 minutes at 14000 rpm at 4°C. A-specific binding was blocked by preincubation for 2 hours with protein-G sepharose beads (Sigma). After 5 minutes centrifugation at 14000 rpm at 4°C, supernatants were incubated overnight at 4˚C with anti PP2A catalytic site

α antibodies (1:2000). This was followed by incubation with protein-G sepharose beads for 2 hours at 4°C. After centrifugation for 5 minutes at 14000 rpm at 4°C the beads were washed 3 times in ice-cold phosphate free buffer before the addition of 200 µl phosphate free buffer. Immunoprecipitates were used for PP2A activity determination.

PP2A activity determination

Phosphatase activity was assessed by using the malachite green-based phosphatase assay kit (Upstate Biotechnology Inc.) that measures phosphate release. Assays were run in 96-well plates according to the manufacturer's instructions. Briefly, 25 µl per sample were incubated with 100 µM of phosphopeptide (amino acid sequence: KRpTIRR, obtained from the kit), in total volume of 50 µl. Reaction was started by addition of the phosphopeptide and conducted for 5 minutes at room temperature. The reaction was stopped by the addition of 100 µl malachite green solution. After 15 minutes of colour development, the release of phosphate was quantified by measuring the absorbance at 650 nm in a microtiterplate reader. The amount of released phosphate (pmol) was then calculated from a standard curve (0-2000 pmol). Percentages of PP2A activity in aspirin treated cells are compared to basal PP2A activity measured in untreated cells.

Transient transfection with PP2A constructs

DLD-1 were transfected with an N-terminal triple (HA)-tagged PP2A catalytic subunit C constructs [27] or GFP-tagged DNA at 6×10^6 cells/transfection on a nucleofector (Amaxa, Cologne, Germany) using a standard protocol from the supplier and solution V and program T-20. The $(HA)_3$-C_{Y307F} construct contains a mutated tyrosine 307 of the catalytic subunit (tyrosine is mutated to a phenylalanine) to eliminate this site of phosphorylation and one wild-type catalytic subunit ($(HA)_3$-C_{WT}) to serve as control [27]. These constructs were kindly provided by Dr D.L. Brautigan from the University of Virginia, Charlottesville, Virgina, USA. The GFP-tagged DNA serves as a transfection efficiency control. After transfection, cells were immediately seeded in a 12-well plate and the cells were allowed to adhere overnight. Subsequently, cells were treated with 5 mM of aspirin for 60 minutes. Afterwards, cells were washed in ice cold phosphate buffered saline (PBS) and scraped into 100 µl of 3x SDS-sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, bromphenol blue). Samples were used for immunoblotting.

Statistical analysis

Statistical analysis was performed using two-tailed Student's t-test, and $p< 0.05$ was considered significant. Data are expressed as the mean and standard deviation.

Results

Aspirin down-regulates constitutively active β-catenin-Tcf signaling shown by TCF reporter assay and the expression of Wnt/β-catenin targets in APC mutant cells

The Wnț/β-catenin pathway acts to control the transcription of genes through the binding of a complex of β-catenin and TCF to specific promoter elements. The activity of this final step in the Wnt/β-catenin pathway can be measured using a luciferase reporter construct [25]. SW480 cells were transiently transfected with the pTOPFLASH or pFOPFLASH (containing a 'far from optimal' TCF binding site) construct together with a pCMV-*Renilla* luciferase construct to act as an internal control for transfection efficiency and potential toxicity of treatments. Figure 1A shows that aspirin down-regulates the constitutively active β-catenin-TCF signaling in SW480 cells in a dose-dependent manner. The specificity of the aspirin effect on TCF reporters was confirmed by the fact that pFOPFLASH was not influenced by aspirin (data not shown). Similar results were obtained in a second colon cancer cell line, DLD-1, and using a second NSAID, indomethacin (data not shown). Comparable data on TOPFLASH/FOPFLASH reporter assays with aspirin and indomethacin were also shown by Dihlmann *et al*. [19]. To confirm that aspirin inhibits Wnt/β-catenin pathway activity, protein levels of various Wnt/β-catenin targets were assessed by immunoblotting.

As shown in Figure 1B and 1C, aspirin treatment induces a decrease of the expression of c-myc, CD44 variant 3, c-met and cyclin D1 in SW480 cells, all proteins known to be in influenced via the Wnt/β-catenin pathway [28, 29]. DLD-1 and SW480 cell lines both harbour APC mutations and express wild-type β-catenin.

To study the effect of aspirin on β-catenin mutated cells we performed a TOPFLASH/FOPFLASH assay and analysed the expression of Wnt targets in HCT116 cells, a cell line expressing wild-type APC and a mutant β-catenin (mutations described in [29]). As shown in Figure 1D, aspirin has no effect on the β-catenin/TCF transcriptional activity and or on the expression of Wnt targets in β-catenin mutated HCT116 cells.

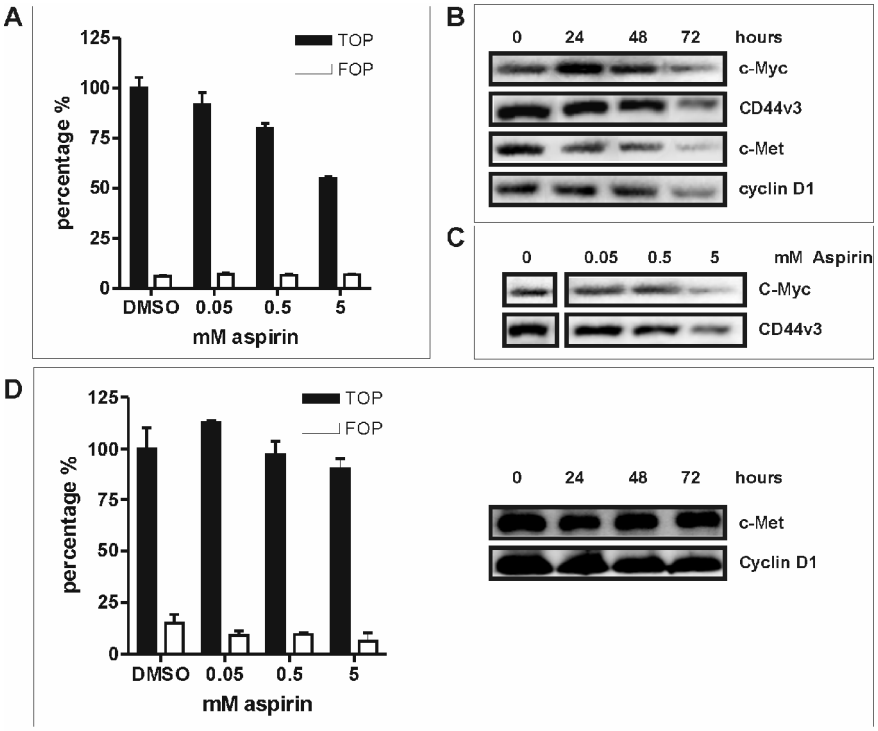

Figure 1. Aspirin down-regulates constitutively active β-catenin-Tcf signaling shown by TCF reporter assay and the expression of Wnt/β-catenin targets. (A) TCF reporter assay in SW480 cells treated with various concentrations of aspirin. Cells were transiently co-transfected with either pTOPFLASH or pFOPFLASH luciferase reporter constructs as well as pCMV-Renilla luciferase construct to serve as an internal control for transfection efficiency and potential toxicity of treatments. Cells were then treated with aspirin for 48 hours, lysed and analyzed using the dual luciferase assay system (Roche). Each treatment was performed in triplicate and experiments were repeated at least three times and normalised to the internal control. Cells treated with the vehicle control (DMSO) are depicted as 100% and the treated cells were correlated to this sample. (B) Western blot analysis of DLD-1 and SW480 cells incubated with 5 mM aspirin for 24, 48 and 72 hours. A time-dependent decrease of c-Met, CD44v3, c-myc, and cyclin D1 is shown. This blot is a representative of three individual experiments. (C) Western blot analysis of DLD-1 and SW480 cells incubated with various concentrations of aspirin (as indicated) for 72 hours. A dose-dependent decrease of c-myc and CD44v3 expression is shown. This blot is representative of three individual experiments. (D) On the left the TCF reporter assay in HCT116 cells treated with various concentrations of aspirin is shown. In the right panel Western blot analysis of HCT116 cells incubated with 5 mM aspirin for 24, 48 and 72 hours are shown. A time-dependent decrease of c-Met, and cyclin D1 is shown. This blot is a representative of three individual experiments.

Aspirin leads to increased the phosphorylation of Serine 41/Threonine 45 of β-catenin

Having shown that Wnt/β-catenin pathway activity is down-regulated by aspirin, we looked further at which elements within this pathway are affected. A central element within this pathway is β-catenin. It is the excess of cytoplasmic β-catenin that leads to the overactivity of the Wnt/β-catenin pathway seen in colon cancer [26, 30]. Control of the size of this pool is regulated by the phosphorylation of β-catenin. This phosphorylation then targets β-catenin for ubiquitination and degradation by the 26S proteasome, and

thus this phosphorylation is the limiting event in Wnt/β-catenin signalling. We assessed the effect of aspirin on β-catenin phosphorylation and as seen in Figure 2 a dose- (Figure 2A) and time-related (Figure 2B) increase in β-catenin phosphorylation is induced after aspirin treatment. The activity of GSK3β is controlled by its phosphorylation. We therefore assessed the activity of this kinase using phosphospecific antibodies in SW480 cells treated with aspirin. No alteration in the phosphorylation levels of GSK3β was observed with aspirin (Figure 2A), suggesting that the increase in phosphorylated β-catenin observed is GSK3β independent.

These experiments were repeated with similar results in the DLD-1 colon cancer cell line suggesting the effect is not cell type specific (not shown). These results back up findings of Dihlman *et al*. [31]. To confirm that the phosphorylation of β-catenin is GSK3β independent, SW480 cells were pretreated with LiCl, a potent and well known inhibitor of GSK3β [32]. Briefly, SW480 cells were pretreated with LiCl before the aspirin treatment. Hereafter the phosphorylation status of β-catenin was assessed by immunoblotting, which showed no change in the effect of aspirin on the phosphorylation of this protein (data not shown). Furthermore, nuclear extracts of SW480 cells pretreated with LiCl before the aspirin treatment showed no differences in downregulation of the nuclear β-catenin expression compared to nuclear extracts of aspirin treated SW480 cells (data not shown). These findings confirm those of Dihlmann and coworkers [31] and are therefore not presented here.

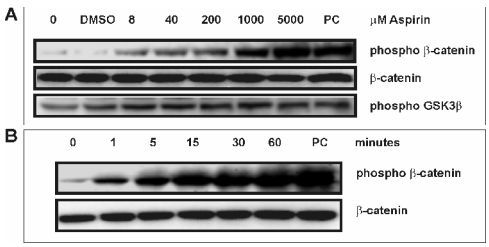

Figure 2. Aspirin leads to increased Serine 41/Threonine 45 phosphorylation of β-catenin. A, Western blot analyses of SW480 cells treated with DMSO (0.5%), various concentrations of aspirin (as indicated) and the phosphatase inhibitor Calyculin A (50nM) as a positive control (PC) for 60 minutes using antibodies raised against phosphorylated β-catenin (Thr41, Ser45), phosphorylated GSK3β (Ser9, Ser21), and β-catenin (as indicated). All were performed on the same blot which was stripped between successive antibodies. These blots are representative of at least three individual experiments. **B**, Western blot analyses of SW480 cells treated with 5 mM aspirin for various times (as indicated). The positive control (PC) was Calyculin A (50nM) for 60 minutes. An increase in phosphorylation of β-catenin is shown.

Aspirin leads to a reduction in cytoplasmic β-catenin levels in APC mutated cells

Phosphorylation of β-catenin classically leads to its ubiquitination and breakdown. We initially tried to confirm this by blotting for total β-catenin in whole cell lysates of SW480 cells treated with aspirin. As shown in Figure 2, total β-catenin levels remained unchanged while there was a convincing increase in β-catenin phosphorylation seen on the same blots. β-catenin exists in two main cellular pools, a structural pool important for the cytoskeleton and a smaller free cytoplasmic pool. It is only this latter pool that plays a part in the Wnt/β-catenin pathway.

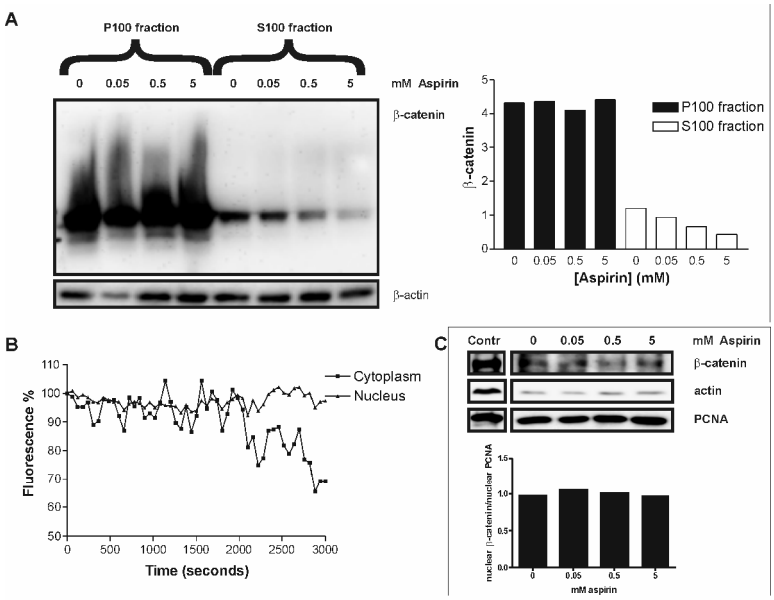

Figure 3. Aspirin leads to a reduction in cytoplasmic β-catenin levels in APC mutant cells. (A) Western blot analysis of cytoplasmic extracts (S100) and the non-cytoplasmic fraction (P100) of SW480 cells treated with aspirin using anti-β-catenin or anţi-β-actin antibodies. The graph shows the relative amounts of β-catenin corrected for β-actin loading controls. The cytoplasmic pool of β-catenin is only one quarter of the size of the non-cytoplasmic pool demonstrating why β-catenin analysis in whole cell lysates may not adequately reflect changes in the cytoplasmic pool. (B) Analysis of the mean fluorescence of the cytoplasm and nucleus of a single SW480 cell transiently transfected with GFP-tagged β-catenin followed over time after treatment with aspirin (10mM) at time=0. Cells were grown to 80% confluence on glass coverslips and then transiently transfected with GFP-tagged β-catenin and further cultured overnight. The following day single GFP-expressing cells were centred under a fluorescent microscope and a series of images taken automatically using time-lapse fluorescent imaging. The mean fluorescence of a selected area within the nucleus or cytoplasm was analysed using image analysis software for the series of images. (C) Western blot analysis of nuclear fractions of HCT116 cells treated with aspirin using anti-β-catenin antibodies. No effect on β-catenin expression in the nuclear fraction is shown. The presence of β-actin and PCNA in the nuclear samples were assayed as a control to assess purity of the isolation (β-actin is not expressed in the nuclear fraction) and as a control for equal loading. As a further control, the expression of untreated, whole cell lysate of HCT116 cells for all three proteins is shown (indicated with 'contr'). These blots are representative of two individual experiments. The bar graph shows the relative amounts of nuclear β-catenin corrected for nuclear PCNA loading controls. These blots are representative of two separate experiments.

We therefore analyzed the effect of aspirin on the cytoplasmic pool of β-catenin (S100 cellular fraction), according to the method of Orford *et al*. [26], and as shown in Figure 3A, the cytoplasmic pool decreases in a dose dependent manner upon the addition of aspirin. This effect is also seen with indomethacin in the DLD-1 cell line (not shown). To confirm this effect of reduction of β-catenin in the cytoplasmic pool, we used GFP-tagged β-catenin and visualized the effect of aspirin in real time using a fluorescent microscope and time-lapse imaging in a single living colon cancer cell. Addition of aspirin led to a 40% reduction in cytoplasmic β-catenin levels as seen in Figure 3B. To investigate whether the effects seen on β-catenin are dependent on intact β-catenin we investigated the β-catenin mutant HCT116 cell line. As shown in Figure 3C aspirin treatment did not lead to a reduction in cytoplasmic β-catenin levels. This confirms the unchanged activity of the Wnt/β-catenin pathway as shown by the TOPFLASH assay (Figure 1A) and Wnt/β-catenin target protein levels (Figure 2C).

Aspirin leads to an increase in ubiquitinated β-catenin

β-catenin is marked for degradation by the 26S proteasome by the covalent addition of ubiquitin. This task is performed by the SCF ubiquitin ligase complex, which contains a component, β-TrCP that binds specifically to phosphorylated β-catenin. To confirm that the reductions in cytoplasmic β-catenin levels are indeed brought about by this mechanism we assessed the effect of aspirin treatment on the appearance of higher molecular weight ubiquitinated β-catenin protein. We immunoprecipitated β-catenin from SW480 cells (untreated or treated with 5 mM aspirin for one hour) and then probed the blotted immunoprecipitate with anti-ubiquitin antibodies according to the method of Kitagawa *et al*. [33]. Figure 4 shows the appearance of an ubiquitin immunoreactive ladder extending upwards from the 97 kD height of non-ubiquitinated β-catenin, induced by the addition of aspirin. Furthermore, when proteasomal activity is inhibited by a proteasome inhibitor MG132, the inhibiting effect of aspirin on TCF/β-catenin transactivation is abolished, supporting the concept that aspirin acts on the Wnt/β-catenin pathway via a proteosome-dependent mechanism (data not shown).

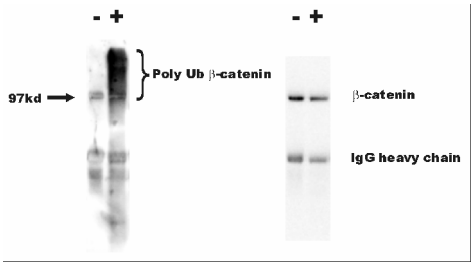

Figure 4. Aspirin leads to an increase in ubiquitinated β-catenin. Cell lysates of SW480 cells with or without 5 mM aspirin treatment for one hour were immunoprecipitated with anti-β-catenin antibodies and then immunoblotted using anti-ubiquitin antibodies. The blot was then stripped and reprobed using anti-β-catenin antibodies to show that equal amounts of protein had been immunoprecipitated

Aspirin leads to increased phosphorylation of Tyrosine 307 of PP2A thereby inhibiting the PP2A enzymatic activity

While trying to identify the cause of aspirin-induced phosphorylation of β-catenin we found no evidence for increased activity of the kinase GSK3β with aspirin. This led us to think that aspirin might act as a phosphatase inhibitor. In support of this we see high levels of phosphorylation of β-catenin when cells are treated with a known phosphatase inhibitor, calyculin A (Figure 2). This suggests that aspirin inhibits the activity of a phosphatase rather than stimulating a kinase to stabilize the phosphorylation of β-catenin. Taking this together with new evidence that has emerged that protein phosphatase 2A (PP2A) is a possible regulator of the Wnt/β-catenin pathway in colon cancer cells [22, 23], we were prompted to investigate a possible effect of aspirin on PP2A activity. To this end we evaluated the phosphorylation status of the inhibitory tyrosine 307 in PP2A by immunoblotting. As evident from Figure 5A, aspirin treatment resulted in a time- and dose-dependent increase of phosphorylation of PP2A at this amino acid residue. These results suggest that aspirin negatively influences PP2A enzymatic activity.

To establish whether increased tyrosine 307 phosphorylation actually corresponds with reduced PP2A activity we performed the malachite green assay. In this assay PP2A activity is measured by the release of P_i from the synthetic substrate KIpTIRR.

Since this assay cannot fully differentiate between PP1 and PP2A activity, PP2A was purified by immunoprecipitation and these samples were used for the malachite green assay. As evident from Figure 5B, in both DLD-1 and SW480 cells, treatment with 5 mM aspirin in a time-course of 60 minutes resulted in reduced PP2A enzymatic activity that is consistent with the effects of aspirin on the β-catenin phosphorylation.

We also investigated the effect of aspirin on PP2A activity in the β-catenin mutant cell line HCT116. As shown in Figure 5C, aspirin treatment results in a decreased activity of PP2A in HCT116 cells. Together these data show that aspirin reduces PP2A enzymatic activity in all the cell lines tested but that this only leads to reduced Wnt/β-catenin-pathway activity in cells with wild-type β-catenin.

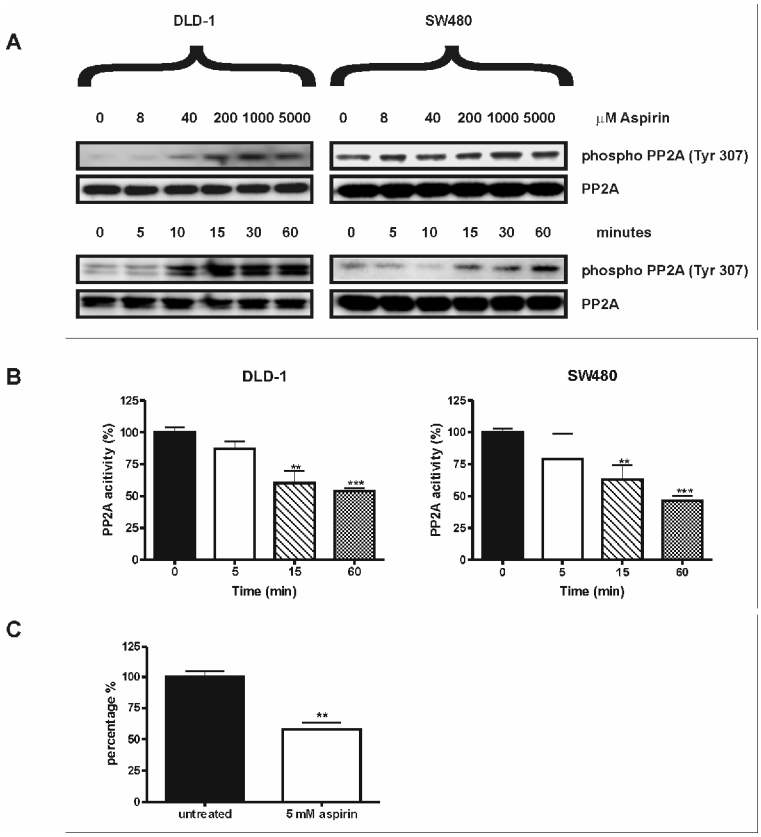

Figure 5. Aspirin leads to increased phosphorylation of Tyrosine 307 of PP2A thereby inhibiting the PP2A enzymatic activity. (A) Western blot analysis of DLD-1 and SW480 cells were incubated with 5 mM aspirin over a time course (as indicated), or with various concentrations of aspirin (as indicated) for 60 minutes. A time- and dose-dependent increase in phosphorylation of PP2A is shown. The blots probed with an antibody against phosphorylated PP2A (Tyr307) were re-incubated with total PP2A antibody (directed against the catalytic alpha subunit). These blots are representative of three individual experiments. (B) Malachite green assay using DLD-1 and SW480 cells after treatment with 5 mM aspirin for various time-points (as indicated). Samples were immunoprecipitated with PP2A antibodies directed against the catalytic alpha subunit and the immunoprecipitate used for the malachite green assay. Error bars are represented as SD. Total amounts, in picomoles, of formed phosphate were calculated from a phosphate standard curve. Untreated cells are depicted as 100% and the treated cells were correlated to this sample (** - p-value < 0.01, *** - p-value < 0.001). Incubations were performed in duplicate and experiments were repeated three times. (C) Malachite green assay of HCT116 cells after treatment with aspirin (5 mM) for 60 minutes. Untreated cells are depicted as 100% and the treated cells were correlated to this sample (** - p-value < 0.01). Incubations were performed in duplicate and experiments were repeated two times.

PP2A enzymatic activity is essential for the effect of aspirin on β-catenin phosphorylation

To investigate whether PP2A is an essential mediator of aspirin effects on the Wnt/β-catenin pathway we employed okadaic acid, a well-known inhibitor of PP2A. Briefly, DLD-1 and SW480 cells were preincubated with 100 nM Okadaic Acid (concentration commonly used in cell-based assays [34, 35], which efficiently inhibits PP2A without affecting protein phosphatase 1 (PP1) [36-38]), or with vehicle control. Cells were subsequently incubated with aspirin (5 mM) in the presence of Okadaic Acid or vehicle control during a time course (as indicated). In agreement with a role for PP2A in controlling β-catenin phosphorylation, we observed that treatment with okadaic acid alone increases the phosphorylation of β-catenin (Figure 6A). As already shown in Figure 2A and 2B, a time- and dose-dependent increase of phosphorylation of β-catenin is shown in cells treated with aspirin and vehicle control (left panels of Figure 6A). This time- and dose-dependent increase of phosphorylated β-catenin is no longer seen after preincubation with okadaic acid and the subsequent incubation with aspirin (in the presence of okadaic acid) (right panels Figure 6A). This suggests that aspirin leads to increased phosphorylation of β-catenin by inhibiting PP2A.

To further investigate whether the increased phosphorylation of β-catenin is directly linked to the increased phosphorylation of tyrosine 307 and thus inhibition of PP2A, DLD-1 cells were transiently transfected with a N-terminal triple (HA)-tagged PP2A catalytic subunit C construct [27]. A wild-type catalytic subunit ($(HA)_3$-C_{WT}) construct served as a control and a construct in which tyrosine 307 of the catalytic subunit is mutated to a phenylalanine ($(HA)_3$-C_{Y307F}) to eliminate this site of phosphorylation. As expected, we observed an increase of phosphorylation of β-catenin after one hour incubation with 5 mM aspirin in the non-transfected cells (Figure 6B).

The same effect is seen in cells that were transiently transfected with the wild-type construct. In the cells that were transiently transfected with the mutated construct, phosphorylated β-catenin is seen in untreated cells, as expected. Furthermore, in these cells transfected with the mutated PP2A construct, no effect of aspirin was observed (Figure 6B), suggesting that tyrosine 307 is the target of aspirin.

In addition, to test the effect of PP2A inhibition in a functional assay, we performed a TOPFLASH assay in DLD-1 cells. DLD-1 cells were transiently transfected with

pTOPFLASH, pCMV-*Renilla* (to serve as an internal control) and either with a wild-type or mutant PP2A construct (the same constructs were used in Figure 6B) and after treatment with aspirin for 6 hours the β-catenin/TCF transcriptional activity was assessed.

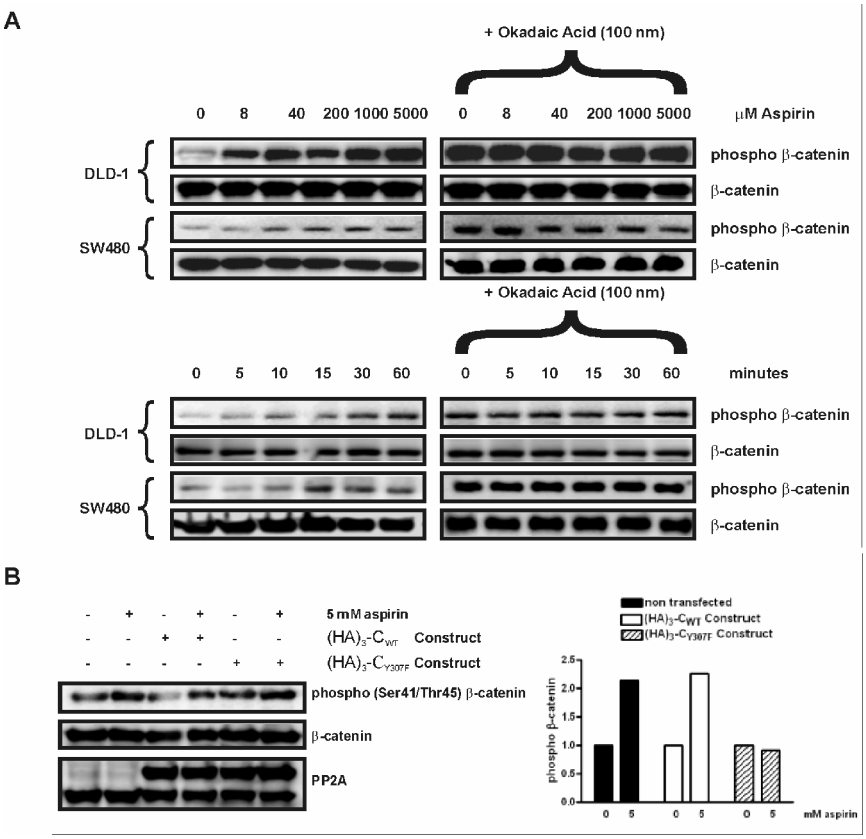

Figure 6: PP2A enzymatic activity is essential for the effect of aspirin on β-catenin phosphorylation. (A) Western blot analysis of DLD-1 and SW480 cells preincubated with 100 nM okadaic acid or with vehicle control and subsequently incubated with aspirin (5 mM) in the presence of okadaic acid or vehicle control during a time course (as indicated). Treatment with okadaic acid only results in an increase of phosphorylation of β-catenin. A time- and dose-dependent increase of phosphorylation of β-catenin is shown in cells treated with aspirin and vehicle control (left panels of Figure). This time- and dose-dependent increase of phosphorylated β-catenin is no longer seen after preincubation with okadaic acid and subsequent incubation with aspirin (in the presence of okadaic acid)(right panels Figure 6A). These blots are representative of three individual experiments. (B) DLD-1 cells were transiently transfected with either wild-type or mutant PP2A constructs for 24 hours (more information in experimental procedures). Cells were subsequently incubated with aspirin (5 mM) for 60 minutes. Western blot analysis shows an induction of phosphorylation of β-catenin after treatment with aspirin in the non-transfected cells and in the cells transiently transfected with a PP2A construct containing a wild-type catalytic subunit. No effect of aspirin is seen in the cells transfected with constitutively active PP2A. The bar graph shows the relative amounts of phospho β-catenin corrected for total β-catenin loading controls. These blots are representative of two separate experiments.

As shown in Figure 6C, aspirin reduced the β-catenin/TCF transcriptional activity in cells transfected with the wildtype construct. Transfection with the mutant construct reduced TCF/β-catenin transcriptional activity compared to the cells transfected with the wildtype PP2A construct.

Transfection with the mutant construct reduced β-catenin/TCF transcriptional activity to the same level as wildtype transfected cells treated with aspirin. Furthermore, the β-catenin/TCF activity in the cells transfected with the mutant construct was not affected by aspirin treatment. We conclude that treatment of colon cancer cell lines with aspirin promotes β-catenin phosphorylation by inactivating PP2A. This is achieved by promoting the phosphorylation of Tyr307 on the catalytic subunit of PP2A thereby inactivating it.

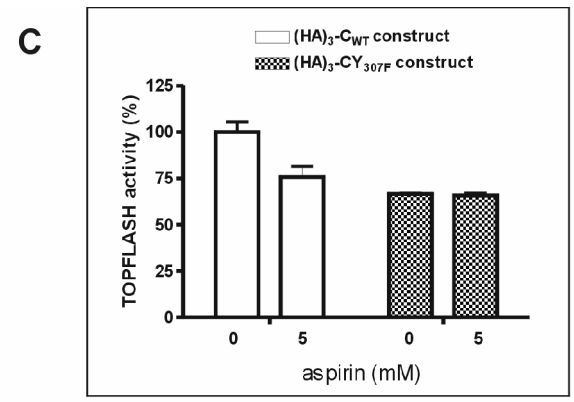

Figure 6C: PP2A enzymatic activity is essential for the effect of aspirin on β-catenin phosphorylation. (C) DLD-1 cells were transiently co-transfected with pTOPFLASH, pCMV-*Renilla* and wildtype or mutant PP2A constructs overnight (more information in experimental procedures). The transfection was followed by 5 mM aspirin treatment for 6 hours. Afterwards, cells were lysed and analyzed using the luciferase assay system (Roche). Incubations were performed in triplicate and experiments were repeated two times. The presented results are represented as percentages compared to the untreated cells transfected with pTOPFLASH, pCMV-*Renilla* (to serve as an internal control) and wildtype PP2A construct, normalized to the internal control.

Discussion

Understanding of the mechanism of action of NSAIDs in colon cancer is seen as the key to developing new more effective chemopreventive treatments with fewer side effects. Most of the investigation has focused on COX enzymes 1 and 2 with the accumulation of genetic and pharmacologically derived data to support the importance of COX-2 in the progression of colon cancer [39, 40]. However, more and more evidence emerges that NSAIDs have a wide range of targets other than COX [6] and this coupled with evidence mitigating against an essential role for COX-2 has led several investigators to propose COX-independent mechanisms for the action of NSAIDs in colon cancer. In the present

study we provide evidence that aspirin acts through PP2A inhibition to increase β-catenin phosphorylation thereby reducing Wnt/β-catenin pathway activity.

We set out to investigate whether NSAIDs have direct effects on the Wnt/β-catenin pathway and elements within it. Constitutive activation of this pathway is found in almost all colon cancers. Mutations leading to truncation of the APC protein or in β-catenin itself lead to disruption of normal β-catenin breakdown, increasing free cytoplasmic β-catenin, which translocates to the nucleus to bind with TCF and switch on Wnt/β-catenin target gene expression. The level of free cytoplasmic β-catenin is controlled largely by its ubiquitination and breakdown by the proteasome [26]. We first used a TCF reporter assay to assess the effects of NSAIDs on the activity of the pathway as a whole. We then used immunoblotting using phospho-specific antibodies to detect changes in the level of phosphorylated β-catenin and GSK3β. We found a time- and concentration-dependent increase in β-catenin phosphorylation. Importantly, this effect was seen at micromolar concentrations of aspirin. Proponents of the COX-2 theory have voiced doubts over the relevance of COX-independent actions of NSAIDs which require high doses (millimolar) that they feel are not achievable *in vivo* [41, 42]. However, human subjects given a short analgesic dose (600 mg qid) attain millimolar (0.05-1.13 mM) concentrations of salicylate [9] and up to 10 mM Aspirin has been used by these investigators in cell culture. The increase in phosphorylation of β-catenin we show here was also reported by Dihlmann and coworkers [31]. Furthermore, they suggest that inactivation of a phosphatase, rather than stimulation of a kinase is a possible cause of the increased phosphorylation seen. Indeed, we show here that aspirin reduces the enzymatic activity of PP2A.

An association between PP2A and the Wnt/β-catenin pathway in colorectal cancer has been made earlier, although the evidence presented to date has been mainly on a genetic level [22-24]. This led us to investigate the effect of aspirin on PP2A as an explanation for the effects of aspirin on the Wnt/β-catenin pathway. In this study we directly show that PP2A enzymatic activity influences the phosphorylation status of β-catenin and that reduced PP2A activity may well explain the effects of aspirin on the Wnt/β-catenin pathway activity.

In summary, in the present study we provide evidence that aspirin acts through PP2A inhibition to increase β-catenin phosphorylation thereby reducing Wnt/β-catenin pathway activity.

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Chapter 9

Violacein Synergistically Increases 5-Fluorouracil Cytotoxicity, Induces Apoptosis and Inhibits Akt-mediated Signal Transduction in Human Colorectal Cancer Cells

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Abstract

Background and Aims:

Despite recent additions to the armory of chemotherapeutic agents for colorectal cancer treatment, the results of chemotherapy remain unsatisfactory. 5-fluorouracil (5-FU) still represents the cornerstone of treatment and resistance to its actions is a major obstacle to successful chemotherapy. Therefore new active agents in colorectal cancer and agents that increase the chemosensitivity of cancer cells to 5-FU are still urgently required. Violacein, a pigment isolated from *Chromobacterium violaceum* in the Amazon River, has a diverse spectrum of biological activities, and represents a novel cytotoxic drug with known antileukemic properties.

Methods:

To assess the suitability of violacein as a chemotherapeutic agent in colorectal cancer its cytotoxic effects were evaluated both as a single agent and in combination with 5-FU. Its underlying mechanisms of action were further investigated by studying its effects on the cell cycle, apoptosis and cell survival pathways (PI3 kinase/Akt, p44/42 MAP kinase and nuclear factor kappa-B) in colon cancer cell lines. We investigated the effects of vilacein on colorectal cancer cell lines using immunoblotting, measurements of apoptosis and cell proliferation, and PI3 kinase assay**.**

Results:

Violacein inhibits the growth of all four colon cancer cell lines tested. It induces apoptosis, and potentiates the cytotoxic effect of 5-FU in a poorly differentiated microsatellite unstable cell line (HCT116). Violacein causes cell cycle block at G1, upregulates p53, p27 and p21 levels and decreases the expression of cyclin-D1. Violacein leads to dephosphorylation of Rb and activation of caspases and a pancaspase inhibitor abrogates its biological activity. Our data provide evidence that violacein acts through the inhibition of Akt phosphorylation with subsequent activation of the apoptotic pathway and downregulation of NF-κB signaling. This leads to the increase of chemosensitivity to 5-FU in HCT116 colon cancer cells.

Conclusions:

Taken together, our findings suggest that violacein will be active in the treatment of colorectal tumors and offers new prospects for overcoming 5-FU resistance.

Introduction

Violacein, a purple-colored pigment produced by one of the strains of *Chromobacterium violaceum* found in the Amazon River, Brazil, is an indole derivative characterized as 3-(1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene)-1,3-dihydro-2H-indol-2-one[1] (Figure 1A). The biosynthesis and biological properties of violacein[1, 2] have attracted attention due to its antitumoral[3, 4], antiulcerogenic[5], antileishmanial[6], antibacterial and antiviral[7] activities. Animal experiments during the evaluation of the ability of violacein to prevent gastric ulceration in mice show that the oral administration of violacein in doses up to 10 mg/kg does not result in any toxic effect in the animals[1]. The cytotoxicity of violacein to V79 fibroblasts was determined in previous experiments (IC_{50}=7 μM) when cancer cells (leukemia cells, lung cancer and colorectal cancer cell lines) were found to be very sensitive to violacein treatment[8]. A very recent study has shown a potent antileukemic effect of violacein in HL60 cells, while cytotoxic effects in relevant concentrations in untransformed cells have not been found[9].

Colorectal cancer is the second leading cause of cancer death in the United States and Europe[10]. Conventional chemotherapy of colorectal cancer with 5-FU in combination with other agents improves overall and disease-free survival of patients after surgery[11]. When given alone in advanced disease, 5-FU produces response rates of between 11% and 17% and a median survival time of approximately 1 year[12]. Combination with newer medicines such as irinotecan or oxaliplatin improves the response rates for advanced colorectal cancers to 40-50% and increases the median survival time to 15-20 months[13, 14]. Despite advances in therapy, the prognosis of advanced colorectal cancers remains poor due to resistance of cancer cells to conventional chemotherapeutic drugs, so the search for new alternatives is needed.

Self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis are the hallmarks of cancer[15]. Defects in apoptosis are an important aspect not only for tumorigenesis but for the development of resistance to anticancer drugs[16]. Reactivation of the apoptotic cascade in apoptosis-reluctant cancer cells via selective inhibition of survival pathways (Raf,

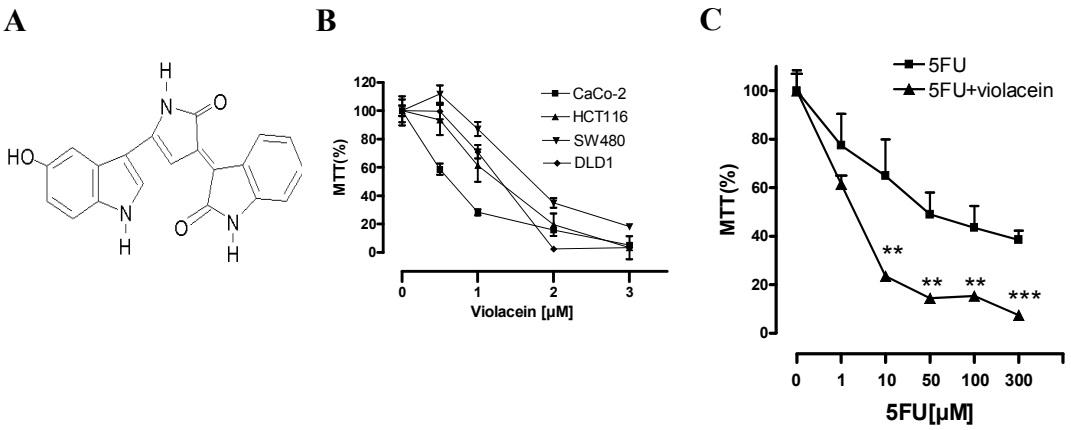

Figure 1. Cytotoxicity of violacein in colorectal cancer cell lines and synergistic cytotoxic interaction of 5-FU and violacein. A, Chemical structure of violacein. B, The MTT assay was performed in four colon cancer cell lines treated for 48 hours with various concentrations of violacein. Values obtained in the absence of compound have been set at 100%. Results represent the mean ± standard deviation (n=10) of three independent experiments. C, HCT116 cells were seeded treated with 5-FU (1-300 μM) or 5-FU in combination with violacein (1 μM), and the MTT assay was performed. Control cells were treated with DMSO (5-FU experiment) or with violacein (1 μM) (combined treatment experiment). Values are expressed as percentage of living cells relative to the control with control values set at 100%. Results represent the mean ± standard deviation (n=10) of three independent experiments.

MAP kinase, PI3 kinase, Akt, NF-κB) and activation of extrinsic and intrinsic apoptotic pathways can be approached through synergistic combination of two agents[17].

Colorectal cancer also vary in their initial response to 5-FU. For example, microsatellite unstable colorectal cancer cell lines show growth advantage and tolerance to 5-FU treatment in comparison with mismatch repair (MMR) proficient cell lines, and these results suggest that deficient MMR system reduces 5-FU cytotoxicity[18]. A recent clinical study on hereditary nonpolyposis colorectal cancer patients showed no differences between patients treated with and without adjuvant 5-FU[19]. The importance of microsatellite instability status of patients with colorectal cancer for outcome of chemotherapy with 5-FU has been suggested by other clinical trials showing improved survival in patients with microsatellite stable tumors compared with untreated patients and no survival benefits in patients with microsatellite unstable tumors[20].

The above-mentioned considerations prompted us to investigate the effect of violacein on colon cancer cell lines. The development of violacein as a prospective new therapeutic agent depends on its ability to target colon cancer cells, its ability to work in combination with established compounds perhaps sensitizing drug-resistant malignant cells, and the establishment of its molecular mechanisms of action. In this study we firstly examined the cytotoxic effect of violacein as a single agent on four colon cancer

cell lines. Subsequently we studied the combination of violacein and 5-FU on a poorly differentiated microsatellite unstable human colon cancer cell line (HCT116). Finally, we investigated the mechanism of action of violacein by assessing its effects on the cell cycle, apoptosis and survival pathways. Our results suggest that violacein has potential as a novel and active chemotherapeutic agent in colorectal cancer. The ability of violacein to restore apoptosis of cancer cells, block cell cycle progression, inhibit survival pathways and increase the sensitivity of cells to 5-FU could provide the basis for the incorporation of this compound into the combined therapy of colorectal cancer.

Materials and Methods

Reagents and antibodies

Antibodies against p21 (rabbit polyclonal), p27 (mouse polyclonal), p53 (mouse monoclonal), NF-κB p50 (goat polyclonal), p65 (rabbit polyclonal), IkBα (rabbit polyclonal) and β-actin (goat polyclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cyclin D1 antibodies (mouse monoclonal) were purchased from NeoMarkers (Fremont, CA, USA). Antibodies against cleaved caspase 3 (rabbit polyclonal), pGSK3 α/β (Ser21/9) (rabbit polyclonal), phospho-Rb (rabbit polyclonal), Bad (rabbit polyclonal), phospho-Bad (Ser136) (rabbit polyclonal), FADD (goat polyclonal), phospho-Akt (Thr308) (rabbit polyclonal), phospho-p44/42 MAP Kinase (Thr202/Tyr204) (E10) (mouse monoclonal), phospho-IkBα (Ser32/36) (mouse monoclonal), phospho-PTEN (Ser380) (rabbit polyclonal) were all purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-TCF4 antibodies (mouse monoclonal) were purchased from Upstate (Lake Placid, NY, USA). Horseradish peroxidase-linked secondary goat anti-rabbit, rabbit anti-goat, goat anti-mouse antibodies were bought from Dako (Glostrup, Denmark). 5-fluorouracil was from Sigma (Schnelldorf, Germany). Stock solution of 5-FU was prepared in DMSO and stored at -20°C. Z-VAD(OMe)-FMK was from Instruchemie (Delfzijl, The Netherlands), Z-LE(OMe)HD(OMe)-FMK and Z-IE(OMe)TD(OMe)-FMK were from Kordia Life Sciences (Leiden, The Netherlands). Stock solutions of 20mM of all caspase inhibitors were prepared in DMSO and stored at -20°C. Violacein was extracted from Chromobacterium violaceum (CCT 3496), purified according to Rettori and Durán and dissolved in DMSO[2].

Cell Culture

CACO-2, DLD-1, SW480, 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 4.5 g/l glucose and L-glutamine. This was supplemented with penicillin (50 U/ml) and streptomycin (50 μg/ml) and, when serum was used, with 10% fetal calf serum (Gibco). Cells were grown in monolayer in a humidified atmosphere containing 5% CO2. In all experiments the concentration of DMSO in control and treated samples was equal (0,1%).

Immunoblotting

Cells at 60-80% confluence from 6 well plates were washed in ice-cold PBS and scraped into 200μl of 2×sample buffer (125mM Tris/HCl, pH 6,8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, 1mg bromophenol blue). Protein concentration was measured using the RC DC protein assay kit (Biorad, CA) according to the manufacturer's instructions. The lysates were sonicated and then heated at 95°C 5 min. 50μg of protein from each sample was loaded onto SDS-PAGE and blotted onto PVDF membrane (Millipore, Bedford, MA, USA). The blots were blocked with 2% low fat milk powder in Tris-buffered saline with 1% Triton (TBST) for 1 hour at room temperature and washed 3×10 minutes in TBST before overnight incubation at 4°C with primary antibody in primary antibody buffer (TBST with 0.2% low fat milk powder). Blots were then washed 3×10 minutes in TBST and incubated for 1 hour at room temperature in 1/2000 horseradish peroxidase (HRP)-conjugated secondary antibody in block buffer. After a final 3×10 minute wash in TBST, blots were incubated for 5 minutes in Lumilite plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Lumi-Imager (Boehringer-Mannheim).

NF-κB Translocation

Cells at 60-80% confluence from 6 well plates were washed in ice-cold PBS and scraped into 200μl ice-cold cell extract buffer (10mM Hepes-KOH, pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM dithiothreitol (DTT) and 0.2mM phenylmethylsulfonyl fluoride (PMSF). The cells were kept on ice for 10 minutes, vortexed for 10 seconds, and centrifuged at 4°C at 14000rpm for 30 seconds. The supernatant was discarded and the pellet was resuspended in 30μl of nuclear extraction buffer (20mM HEPES-KOH, pH 7.9, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT and

0.2mM PMSF), placed on ice for 20 minutes, and centrifuged at 4°C at 14000rpm for 2 minutes. The supernatant was saved as the nuclear extract and used in western blotting assay.

PI3 kinase Assay

HCT116 cells at 60-80% confluence from 6 well plates were washed in ice-cold PBS and lysed in 500µl ice-cold lysis buffer (137mM NaCl, 20mM Tris-HCl, pH 7.4, 1mM $CaCl_2$, 1mM $MgCl_2$, 0.1mM sodium orthovanadate, 1% NP-40 and 1mM PMSF). PI3 kinase was immunoprecipitated with 5µl of rabbit antibody against full-length PI3 kinase (which coprecipitates the p110 catalytic subunit of PI3 kinase) and 60µl of protein A-sepharose beads (Amersham Pharmacia Biotechnology). PI3 kinase activity in the immunoprecipitates was analyzed with PI3 kinase ELISA (from Echelon biosciences incorporated, Salt Lake City, Utah, USA) according to the manufacturer's instructions. Briefly, immunoprecipitated enzyme and PI(4,5)P2 substrate were incubated for 1 hour at room temperature in the reaction buffer. Kinase reaction was stopped by pelleting the beads by centrifugation and transferring the reaction mixture to the incubation plate and incubated overnight at 4ºC with a PI(3,4,5)P3 detector protein, then added to the PI(3,4,5)P3-coated microplate for 1 hour for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric detection (absorbance was measured at 450nm) is used to detect PI(3,4,5)P3 detector protein binding to the plate. The colorimetric signal is inversely proportional to the amount PI(3,4,5)P3 produced by PI3 kinase. The expression levels of the PI3 kinase components p85 and p110 for each time points were detected by western blot analysis of pelleted beads.

MTT assay and cytotoxicity analysis

Cells were trypsinized and taken up in DMEM with 0.5 % fetal calf serum. 5×10^3 cells were seeded in flat-bottomed tissue culture treated 96-well plates (Falcon) and allowed to adhere for 12 hours. Cells were then stimulated with different concentrations of violacein or 5-FU for 24 and 48 hours. After treatment, MTT solution was added (final concentration 0.5 mg/ml, stock solution 5 mg/ml MTT in PBS), for 3 hours. Medium was discarded and cells were lysed in acidified 2-propanol. Absorbance was measured at 550-560nm. The combined cytotoxic effect of 5-FU with violacein was determined by CI-isibologram using CalcuSyn software (Biosoft, Cambridge, UK).

Annexin V and 7-amino-actinomycin D assays

Control and violacein-treated cells were collected and resuspended in 1×binding buffer (0.01M Hepes/NaOH, pH 7.4, 0.14mM NaCl and 2.5mM $CaCl_2$) at a concentration of $2x10^7$ cells/ml. Subsequently, 100µl of cell suspension was transferred to a 5ml tube and Annexin V-APC (5µl) and 7-amino-actinomycin D (7-AAD) - (5µl) was added. The cells were incubated at room temperature for 15 minutes, after which 400µl of 1×binding buffer was added, and apoptosis analyzed by flow cytometry (Becton Dickinson FACSCalibur, Rockville, MD, USA) and data were analyzed using the software Cell Quest Pro (BD Biosciences Pharmingen).

Flow cytometry analysis

Cells were plated at a density of $5x10^5$ cells/plate in 60mm tissue culture dishes in culture media (described above) 1 day before the agent treatment. At the indicated time, control and treated cells were washed once with PBS, harvested by trypsinization, collected, and then resuspended in 300µl of PBS and fixed with 5 ml of ice-cold 75% ethanol. After fixation for at least 3 hours at 4°C, the cells were sedimented by centrifugation and resuspended in PBS containing 1mg/ml glucose and 1mg/ml RNase A up to $1×10^6$ cells/ml and incubated at room temperature for 30 minutes. Subsequently propidium iodide solution (1mg/ml in distilled water) was added to each sample and incubated in the dark for an additional 30 minutes. The samples were analyzed with flow cytometry (Becton Dickinson FACScan). Analysis was performed after 10,000 counting events. Histograms were analyzed using Modifit software (Veriety Software House Inc., Topsham, ME).

Plasmids and transfection procedure

HCT116 cells were transiently transfected either with pSG-Gag-Akt (kindly provided by Professor P.J. Coffer, University Hospital Utrecht, The Netherlands) a plasmid vector encoding a version of Akt that is constitutively activated, or pmaxGFP control vector (from amaxa GmbH, Cologne, Germany)) using Lipofectamine Plus (Invitrogen, Breda, The Netherlands) according to manufacturer's instructions. Efficiency of transfection was determined by the measurement of GFP-positive cells and was at least 90%.

5-FU(μM)	violacein(μM)	CI
1	1	1,302
10	1	0,861
50	1	0,776
100	1	0,794
300	1	0,682

Table 1. Combination index (CI) of 5-FU+violacein for HCT116 cell line. The combined cytotoxic effect of 5-FU with violacein was determined by CI-isibologram using CalcuSyn software. CI: CI>1, =1 and <1 indicate antagonistic, additive and synergistic effects, respectively.

Statistical analysis

Statistical analysis was performed using two-tailed Student's *t*-test and $p<0.05$ was considered significant. Data are expressed as the mean and standard deviation.

Results

Effect of violacein on the growth of colorectal cancer cell lines and combined growth inhibitory synergy of 5-FU and violacein in HCT116 cells

In order to establish the cytotoxicity of violacein on colorectal cancer cells four cell lines were tested and cell viability was assessed using the MTT assay. Violacein exerts a growth inhibitory effect in all colon cancer cell lines tested. Figure 1B illustrates that violacein inhibits the growth of CACO-2, HCT116, SW480 and DLD1 cells after 48 hours of incubation and displayed an IC_{50} value around 1-2 μM.

To test the combined effect of 5-FU and violacein on cell growth, HCT116 cells were cultured with 5-FU (1-300 μM) with or without violacein (1 μM) for 48 hours before MTT analysis. Figure 1C demonstrates that the growth inhibitory effect of 5-FU on HCT116 cell line was significantly enhanced by violacein. To determine if the combined effect of violacein and 5-FU is synergistic, the effect of these two drugs on HCT116 cells was subjected to Combination Index-isobologram analysis[21]. The results indicate a synergistic cytotoxic effect between violacein and 5-FU at all concentrations of 5-FU above 1μM (Table 1).

Activation of apoptosis and enhancement of 5-FU induced apoptosis by violacein

To further investigate the underlying mechanism for observed reduction in cell viability using the MTT assay, we determined the sensitivity of HCT116 cells to violacein-induced apoptosis and evaluated whether violacein could increase the apoptosis induced

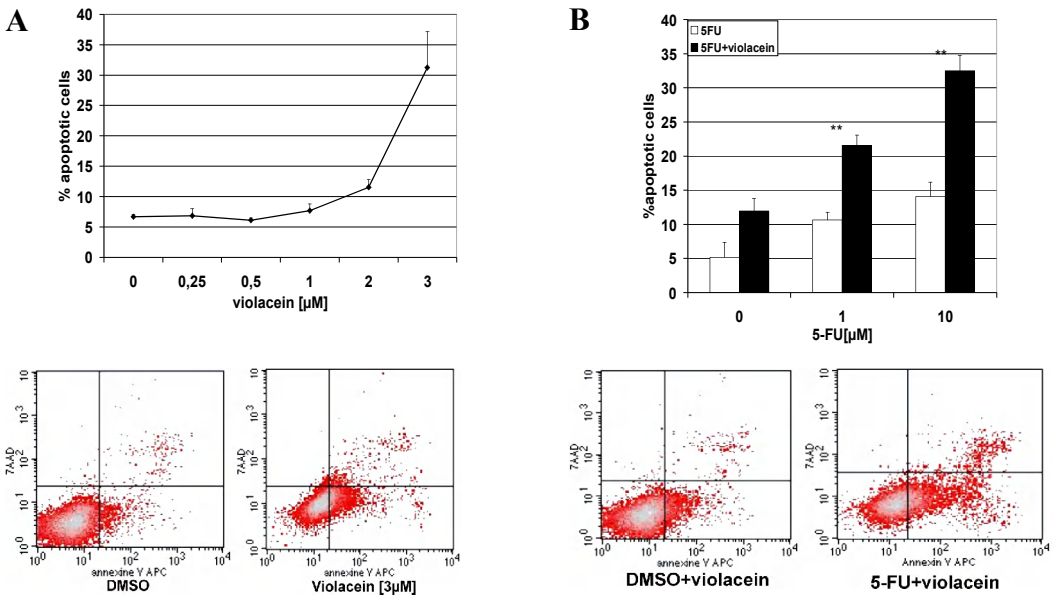

Figure 2. Effect of violacein on HCT116 cells apoptosis induction HCT116 cells were treated for 24 hours either with violacein (0.25-3 μM), or with 5-FU (1-10 μM), or with 5-FU (1-10 μM) + violacein (1 μM) and harvested for quantification of apoptosis by flow cytometry measured as the percentage of Annexin V-APC positive cells. Control cells were treated with DMSO (5-FU experiment and violacein experiments) or with violacein (1μM) (combined treatment experiment). A, violacein treatment results in dose-dependent augmentation of apoptosis. B, violacein enhances 5-FU induced apoptosis (**- p-value < 0.01) experiments is presented.

by 5-FU. Cells were incubated either with violacein (0.25-3 μM), or with 5-FU (1-10 μM), or with 5-FU (1-10 μM) + violacein (1 μM). Apoptosis was then quantified by flow cytometry. Violacein causes a dose-dependent increase in apoptosis in HCT116 cells (Figure 2A) and enhances 5-FU-induced apoptosis (Figure 2B). Importantly, while a concentration of 1μMviolacein does not cause apoptosis after 24 hours of incubation and 5-FU 10μM leads to 14% apoptosis induction, combined treatment results in 33% apoptotic HCT116 cells (Figure 2B).

Effects of violacein and combined violacein/5-FU treatment on the cell cycle

To determine the effect of violacein on the cell cycle progression of HCT116 cells, flow cytometry analysis was performed on cells treated with various concentrations of violacein, 5-FU, or a combination of both. Administration of violacein results in a concentration-dependent increase of around 10% in the proportion of the cells in G_1 phase with a corresponding decrease in the proportion of the cells in S and G_2/M phases in comparison with control cells (Figure 3A) while cells treated with 5-FU alone do not show any changes in the proportions of the cells in G_1 phase (Figure B). Combined administration of violacein with 5-FU increases the proportion of cells in G_1 phase to

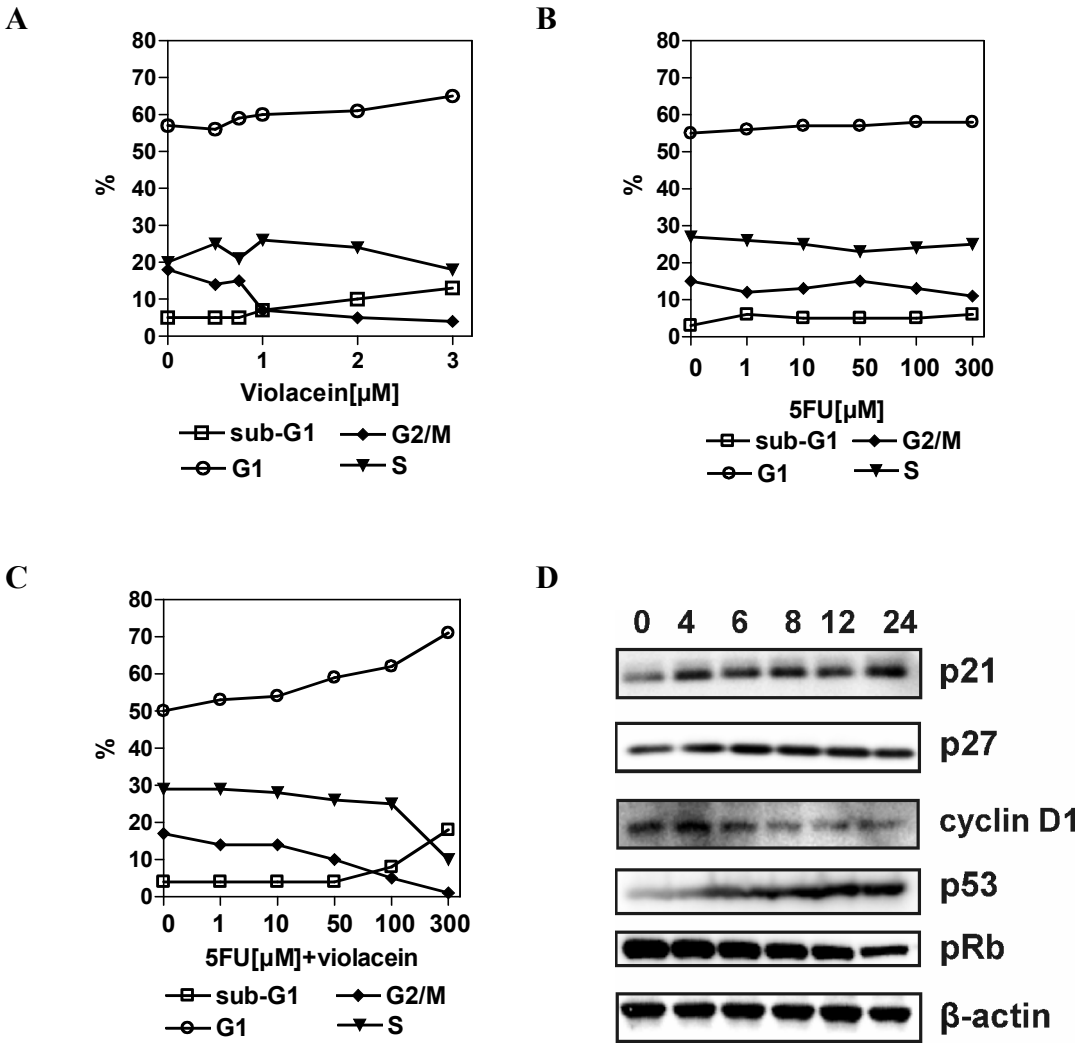

Figure 3. Analysis of the effects of 5-FU and violacein on the cell cycle HCT116 cells were grown to 60% confluence, treated for 24 hours either with violacein (0.25-3 μM), or with 5-FU (1-10 μM), or with 5-FU (1-10 μM) + violacein (1 μM), harvested, fixed with ethanol, and stained with propidium iodide. The stained cells of each sample were analyzed by flow cytometry. Control cells were treated with DMSO (5-FU experiment and violacein experiments) or with violacein (1 μM) (combined treatment experiment). (A) Violacein induces a concentration-dependent increase in the proportion of the cells in G_1 phase and decrease in the S and G_2/M phases. (B, C) combined administration of violacein with 5-FU synergistically induces cell cycle arrest in G_1 phase and decreases the amount of the cells in S and G_2/M phases compared with violacein and 5-FU given alone. (D) Regulation of G_1-related proteins by violacein. Western blot analysis was performed on HCT116 cells treated with violacein (1 μM) for various times (shown in hours). 50 μg of protein from cell lysates was loaded per line, and expression of p21, p27, p53, cyclin D1 and Rb were analysed by blotting with the corresponding specific antibody. Results representative of 3 independent experiments are presented.

around 20% (Figure 3B, C) and decreases the amount of the cells in S and G_2/M phases compared with violacein and 5-FU given alone. In combined treatment experiments the sub-G1 fraction, representing apoptotic cells, increases to 18% compared with 4% in controls (sub-G1 fraction increases to 10% with violacein treatment and no changes are

observed with 5-FU alone). The above data show that violacein and 5-FU have combined cytostatic and cytotoxic effects in HCT116 cells.

To elucidate the specific cell cycle regulatory proteins responsible for the G_1 block mediated by violacein in HCT116 cells, we performed western blot analysis on protein extracts from HCT116 cells treated with 1 μM violacein at different time points. In agreement with the observed cell cycle arrest induced by violacein, we found a time-dependent upregulation of p21, p27 and p53 protein expression (Figure 3D). Furthermore, a significant reduction of cyclin D1 expression and downregulation of phospho-Rb were detected (Figure 3D). Our results imply that violacein-dependent cell cycle arrest in G_1 is induced via upregulation of Kip/Cip cyclin-dependent kinase inhibitors with consistent downregulation of cyclin D1 and dephosphorylation of pRb.

The level of cyclin D1 as well as p53, p21 and p27 could be regulated by the PI3 kinase/Akt pathway, suggesting that violacein may inhibit the Akt survival pathway with subsequent cell cycle block.

Effects of violacein on the activation of apoptotic pathways

To obtain further insight into the mechanism of violacein-induced apoptosis, we first assessed the involvement of caspase 3. We performed western blotting on HCT116 cells treated with violacein at different time points and used anti-cleaved caspase 3 antibodies. The activation of caspase 3 can already be observed after 45 minutes (Figure 4A).

In order to confirm that activation of caspases culminates in the apoptosis of HCT116 cells, we treated these cells with violacein or violacein in combination with a pancaspase inhibitor. Subsequently cell viability was determined using the MTT assay. A pancaspase inhibitor significantly reduces the effect of violacein on cell viability, confirming that violacein-induced cell death is a caspase dependent process (Figure 4B).

In order to gain more information about the apoptotic mechanism induced by violacein, we assessed the involvement of caspase 8 and caspase 9 in the death process. For this purpose specific caspase 8 and caspase 9 inhibitors were used. Each inhibitor was added to HCT116 cells 30 minutes before violacein treatment and cell viability was determined using the MTT assay. Both caspase 8 and caspase 9 inhibitors reduce the

effect of violacein on cell growth suggesting that these caspases are both involved in violacein-induced cell death (Figure 4C).

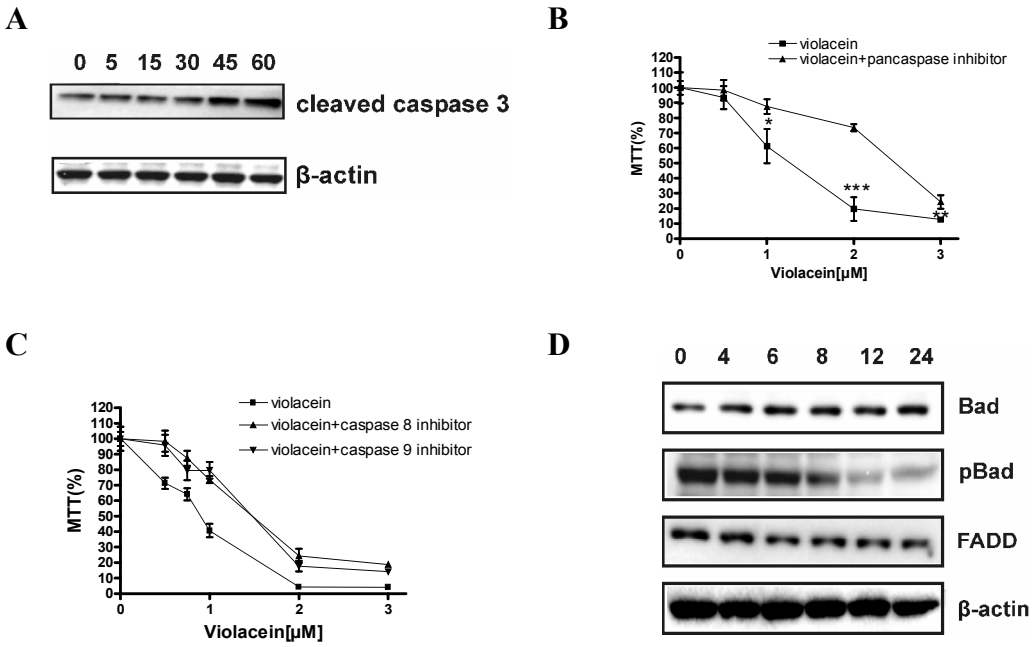

Figure 4. Effect of violacein on the activation of apoptotic pathways. A, Immunoblot of HCT116 cells treated with violacein (1 μM) for various times (shown in minutes). 50 μg of total protein from cell lysates was loaded per lane and blotted with anti-cleaved caspase 3 antibodies. Equal loading was confirmed by showing equal β-actin levels. B, HCT 116 cells were treated with different concentrations of violacein (0.5-3 μM) for 24 hours with and without caspases inhibitor Z-VAD(OMe)-FMK. Cell viability was assessed by the MTT assay (* - p-value < 0.05, ** - p-value < 0.01,*** - p-value < 0.001). C, HCT116 cells were treated with different concentrations of violacein (0.5-3 μM) for 24 hours with and without caspase 8 inhibitor Z-IE(OMe)TD(OMe)-FMK or caspase 9 inhibitor Z-LE(OMe)HD(OMe)-FMK. Inhibitors were added to HCT116 culture plates at 20 μM, 30 minutes before violacein treatment. Cell viability was assessed using the MTT assay. D, Immunoblot of HCT116 cells treated with violacein (1μM) for various times (shown in hours). 50 μg of total protein from cell lysates was loaded per lane and blotted with antibodies against Bad, phospho-Bad and FADD. Equal loading was confirmed by showing equal β-actin levels.

To elucidate the upstream pathway leading to caspase 3 activation, western blotanalysis was performed. Violacein treatment results in increased levels of pro-apoptotic Bad protein and a decrease in phosphorylated Bad^{Ser136}, thereby promoting apoptosis. These results indicate that Bcl-2 family proteins, members of the intrinsic apoptotic pathway, are involved in violacein-induced apoptosis. As phosphorylation of the pro-apoptotic protein Bad at Ser136 occurs via the serine-threonine kinase Akt, our findings suggest the involvement of Akt in the mechanism of violacein action. The level of FADD was reduced by violacein treatment implying that extrinsic apoptotic pathway is not primarily involved in cell death caused by violacein (Figure 4D).

Effects of violacein on survival pathways

The PI3 kinase/Akt pathway is an important regulatory pathway governing the apoptotic response. We therefore investigated the effect of violacein on this pathway. As the activity of Akt is regulated by phosphorylation, we examined the level of phosphorylated Akt^{Thr308} in HCT116 cells treated with violacein at different time points and found time dependent downregulation of phosphorylated AktThr308 (Figure 5A). As confirmation we also show a decrease in the phosphorylation of GSK-3$\alpha/\beta^{Ser21/9}$ a known downstream target of Akt (Figure 5A). In order to examine the influence of violacein on PI3 kinase, we performed immunoprecipitation of PI3 kinase from HCT116 cells treated with violacein and measured the PI3 kinase activity by competitive ELISA. Compared with PI3 kinase activity from control cells, violacein does not affect the PI3 kinase activity or the expression of the PI3 kinase components (p85α and p110α) (Figure 5B). We evaluated the level of phosphorylated $PTEN^{Ser380}$ (phosphatase and tensin homologue deleted on chromosome ten) as PTEN is a major negative regulator of the PI3 kinase/Akt pathway and found no changes after violacein treatment (Figure 5A). These results suggest that violacein inhibits Akt activity in a PI3 kinase and PTEN independent manner.

We assessed the activity of the p44/42 MAP kinase pathway, another major pathway involved in cellular proliferation and apoptosis. The level of phosphorylated P44/42 MAP $kinase^{Thr202/Tyr204}$ is not affected by violacein treatment suggesting that the MAP kinase pathway is not affected by violacein (Figure 5A).

To further test the hypothesis that violacein-induced apoptosis is mediated through the inhibition of the Akt pathway, we transiently transfected HCT116 cells with a plasmid expressing a constitutively active form of Akt (pSG-Gag-Akt). Control cells were transfected with pmaxGFP plasmids. 24 hours after transfection, cells were treated with DMSO or different concentrations of violacein (0,5-3μM) for 48 hours and then the MTT assay and Annexine V measurements were performed. HCT116 cells transfected with pSG-Gag-Akt show an increase in cell survival, relative to control cells (Figure 5C). While violacein strongly induces apoptosis in HCT116 cells transfected with pmaxGFP (9% apoptosis with 0,5 μM violacein up to 84% apoptosis with 3μM violacein) cells transfected with pSG-Gag-Akt were far more resistant to apoptosis with 3 μM violacein) as shown in Figure 5D. In all tested concentrations the percentage of

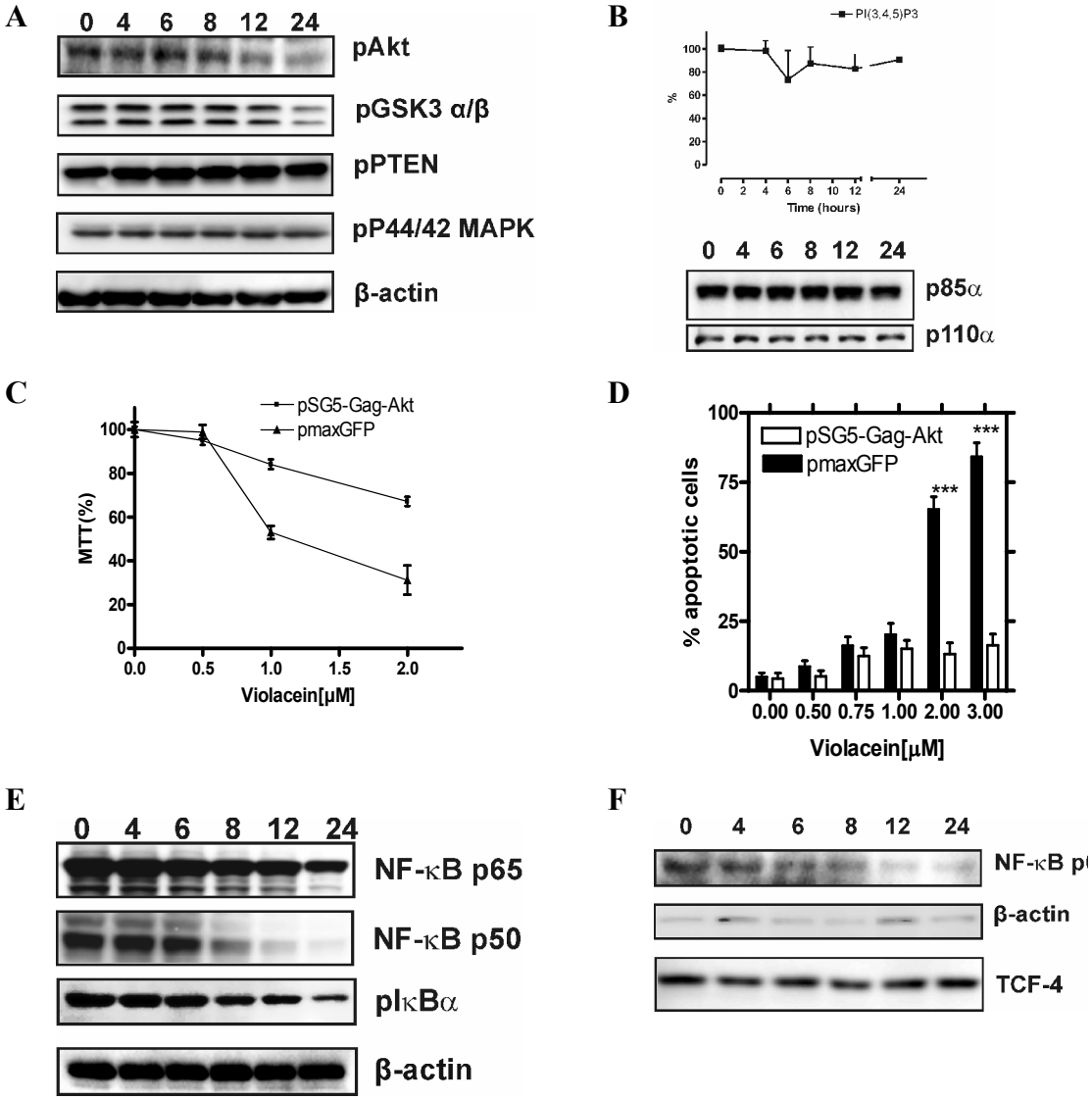

Figure 5 Violacein inhibits the activity of Akt and NF-κB signalling pathways. HCT116 cells were treated with violacein (1 μM) for various times (shown in hours). A, 50 μg of total protein from cell lysates was loaded per line and western blot analysis was performed. Violacein treatment caused time dependent downregulation of phosphorylated Akt, decreased the phosphorylation of its downstream target GSK-3α/β, but had no effect on phosphorylation of PTEN and phosphorylation of P44/42 MAP kinase. B, PI3 kinase was immunoprecipitated and its activity was analyzed as described in materials and methods. The data were quantified and expressed as a percentage of control. The expression levels of the PI3 kinase components p85α and p110α for each time points were detected by western blot analysis. C, HCT116 cells were transfected either with pmaxGFP or pSG5-Gag-Akt, and after 24 hours, were treated with DMSO or different concentrations of violacein for 48 hours. D, HCT116 cells were transfected either with pmaxGFP or pSG5-Gag-Akt, and after 24 hours, were treated with DMSO or different concentrations of violacein for 48 hours. Apoptosis was measured by flow cytometry as the percentage of Annexin V-APC positive cells (*** - p-value < 0.001). E, 50 μg of total protein from cell lysates was loaded per lane and western blot was performed. A time dependent decrease of the expression of p50 and p65 subunits of NF-kB and decreased phosphorylation of IkBα were observed. F, nuclear extracts of HCT116 cells were subjected to immunoblot analysis with NF-kB p65 antibody. Lack of β-actin shows effective nuclear fractionation, and TCF4 levels were used to prove equal loading.

apoptotic cells was lower in pSG-Gag-Akt transfected cells compared with pmaxGFP transfected cells, and with concentrations of 2 μM and 3 μM of violacein the difference was significant (*p*-value < 0.001), suggesting that the induction of apoptosis by violacein in HCT116 cells results, at least in part, from an inhibition of the Akt pathway.

Finally we investigated the influence of violacein on the activity of the NF-κB pathway as activation of NF-κB blocks apoptosis and promotes cell proliferation in colon cancer. We show a time-dependent decrease of expression of p50 and p65 subunits of NF-κB with violacein treatment (Figure 5E). Inactivation of NF-κB is confirmed by direct observation of the decrease in nuclear translocation of NF-κB as determined by western blot analysis of nuclear extracts of HCT116 cells (Figure 5F). NF-κB is present in the cytosol in an inactive state complexed with the inhibitory IκB proteins. Activation occurs via phosphorylation of IκBα at Ser32/Ser36, resulting in the release and nuclear translocation of NF-κB. To further confirm violacein-induced downregulation of the activity of NF-κB pathway, we performed western blot for phosphorylated $I\kappa B\alpha^{Ser32/36}$. Violacein treatment leads to a decrease in IkBα phosphorylation (Figure 5E) suggesting that violacein blocks IκBα phosphorylation preventing its ubiquitination and further degradation thus preventing nuclear translocation of NF-κB.

Discussion

To combat the problem of chemoresistance new alternative effective agents are urgently needed in the treatment of colorectal cancer. Natural products are an important source of potential chemotherapeutic agents[21, 22]. Violacein has attracted attention as a possible candidate for cancer treatment due to its high cytotoxicity in transformed cells and low toxic effect in relevant concentrations in untransformed cells[5, 8, 9]. Earlier studies screening a large panel of cancer cell lines have already shown that violacein is promising with respect to leukemia, lung cancer and colorectal cancer[8]. Our work has specifically concentrated on colorectal cancer, a major malignancy with insufficient treatment options and one of the leading causes of cancer death in Western countries.

We find that violacein is highly effective cytotoxic compound for colorectal cancer cells. Combined treatment with violacein and 5-FU synergistically potentiates the cytotoxic effect of 5-FU in a microsatellite unstable cell line, HCT116. We further investigated the mechanisms involved in this effect by studying the effects of violacein on apoptosis, the cell cycle and major cellular survival pathways. We find that violacein reactivates the apoptotic cascade in colon cancer cells, which we have confirmed using multiple methodologies. We tried to elucidate whether this occurs primarily through the intrinsic mitochondrial pathway or through the extrinsic apoptotic pathway using specific inhibitors of caspase 8 and 9 and by western blot analysis of components of both pathways. While both the caspase 8 and 9 inhibitors seem to block the effects of violacein to a similar extent this can perhaps be explained by the extensive crosstalk between these two apoptotic pathways. Our findings of reduced levels of FADD and increases in the levels and activity of Bad lead us to suggest that the mitochondrial pathway is primarily affected although our data are not conclusive.

Violacein treatment alters the expression of proteins involved in the cell cycle such as p21 and p27, downregulates the level of cyclin D1 and blocks pRb phosphorylation, thus leading to the arrest of cancer cells in G_1 that we observe using flow cytometry.

Analysis of components of the MAP kinase, NF-κB and Akt and survival pathways show that violacein has no effect on the MAP kinase pathway but reduces the activity of both the NF-κB and Akt pathways. In the MAP kinase pathway levels of phosphorylated p44/42 MAP kinase$^{Thr202/Tyr204}$ remain unchanged with violacein treatment. Violacein decreases the expression of p50 and p65 subunits of NF-κB, reduces IκBα$^{Ser32/36}$ phosphorylation and inhibits nuclear translocation of NF-κB consistent with downregulation of the NF-κB pathway and violacein reduces levels of phosphorylated Akt^{Thr308} suggesting that violacein blocks Akt signaling. This last action of violacein is particularly interesting when trying to explain the data as a whole as Akt inhibition could explain most of the various effects of violacein observed.

The apoptotic response to conventional chemotherapy may be augmented by the inhibition of the Akt pathway. Akt directly phosphorylates several components of the cell death machinery and in this way protects cells from death and inhibits apoptosis[23]. Phosphorylation of the pro-apoptotic protein Bad by Akt, inhibiting Bad function, has

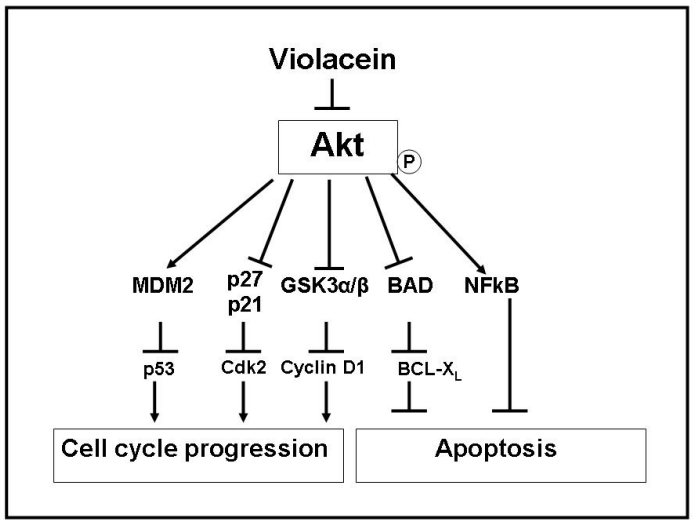

Figure 6. Schematic representation of the proposed molecular mechanism of violacein action.

previously been described[24]. The observed effects of violacein on Bad and phosphorylated Bad are therefore consistent with inhibition of Akt.

Akt is also known to modulate the NF-κB pathway. Akt leads to the activation of the NF-κB pathway via phosphorylation and activation of IκB kinase, which induces degradation of the NF-κB inhibitor, IκB ultimately leading to increase nuclear translocation of NF-κB[25]. A variety of human cancers have demonstrated constitutively high levels of NF-κB activation[26]. Exposure of cancer cells to anticancer drugs can induce the activation of the NF-κB pathway leading to the expression of anti-apoptotic genes [27] and resistance to chemotherapy. Colorectal cancer cell lines resistant to thymidylate synthase inhibitors, such as 5-FU, demonstrate NF-κB mRNA and protein overexpression and high NF-κB nuclear accumulation. Inhibition of NF-κB activity can enhance the cytotoxic effect of irinotecan and TNFα [28, 29]. Our observation of inhibition of the NF-κB pathway, possibly through Akt downregulation, could also be responsible for the proapoptotic effect of violacein and for sensitizing of HCT116 cells to 5-FU.

Akt is also known to have effects on various components of the cell cycle. Akt affects levels of cyclin D through its actions on GSK-3 α/β[30] and effects on both p21 and p27 are also described[31, 32]. Thus the effects of violacein on the cell cycle are also consistent with Akt inhibition.

To provide further evidence for Akt being a crucial target of violacein we further investigated the effects of violacein on another Akt target, p53. Akt is known to promote cell survival through activation of the p53-binding protein MDM2, resulting in enhanced p53 degradation[33]. We find that the level of p53 is increased due to violacein treatment. We show that transfection with constitutively active Akt blocks the effects of violacein and finally we show that two upstream modulators of Akt activity, PI3 kinase and PTEN are not affected by violacein.

Our data provide evidence that violacein is a promising chemotherapeutic agent that acts by blocking Akt activation and inducing apoptosis thus increasing the chemosensitivity of colon cancer cells to 5-FU treatment (Figure 6).

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Chapter 10

Summary

Summary in Dutch

General Discussion

Future Perspectives

Acknowledgements

List of Publications

Summary

During the last decade important progress has been made in our understanding of the role of molecular pathways in gastrointestinal (GI) tract polyp formation and colorectal carcinogenesis. The main goal of this thesis was to study the role of the one of the most important morphogenetic pathway, the Bone Morphogenetic Protein (BMP) pathway, in colorectal cancer (CRC), and the use of modulators of the BMP pathway for therapeutic intervention in GI tumors.

In **chapter 2** we aimed to answer the question whether and where during the adenoma-carcinoma sequence of CRC formation BMP signaling is disrupted. We report that the expression of BMPRs is intact and the pathway is active at the adenoma stage of colon cancer progression. Conversely, BMPR2 and SMAD4 are frequently lost in cancer specimens and BMP signaling is lost in the majority of CRCs. Results presented in **chapter 2** indicate that loss of BMP signaling correlates tightly with progression of adenomas to cancer, and occurs relatively early during cancer progression.

Further, in **chapter 3** we investigated the expression of elements of the BMP pathway in CRC cell lines and in a large group of patient specimens using a tissue microarray and immunohistochemistry. We tried to find the relationship between the expression of components of the BMP pathway and the MSI status of CRCs. In **chapter 3** we demonstrate that BMPR2 protein expression is abrogated in microsatellite unstable (MSI) cell lines while SMAD4 protein expression is abrogated in microsatellite stable (MSS) cell lines and BMPR2 is mutated in all MSI and in none of the MSS cell lines. We observe that BMPR2 expression is impaired more frequently in MSI CRCs than MSS and shows a mutually exclusive pattern of impaired expression compared to SMAD4 as seen in cell lines. The BMP pathway is inactivated in 70% of CRCs and this correlates with BMPR and SMAD4 loss. Therefore, our data suggest that the BMP pathway is inactivated in the majority of sporadic CRCs. In MSI CRC this is associated predominantly with impaired BMPR2 expression and in MSS CRC with impaired SMAD4 expression.

In **chapter 4** of this thesis we studied the interaction between the proproliferative Wnt pathway and proapoptotic Bone Morphogenetic Protein pathway. Based on results of our study, we hypothesize that there is a reciprocal interaction between BMP and Wnt signaling. The proapoptotic BMP signaling counterbalances proproliferative Wnt and if BMP signaling

is disrupted, as in case of JP adenomas, increased accumulation of nuclear β-catenin takes place even without mutations in APC or β-catenin. We show that the Wnt pathway is overactive in FAP adenomas and this leads to downregulation of BMP pathway and to further imbalance between proliferation and apoptosis, giving survival advantage to these cells and playing an important role in colon cancer initiation and progression.

In **chapter 5** we evaluated BMP signaling in the Helicobacter pylori infected gastric mucosa. The observations in this chapter show that the influx of inflammatory cells in the mesenchyme is a source of BMP2 protein in the chronic inflamed stomach. The increased BMP protein production in the stomach mesenchyme correlates with an increased BMP signaling activity and with apoptotic cell death in the stomach epithelium. This suggests that morphogens produced by cells in the inflammatory infiltrate may influence epithelial cell fate and may be responsible for the histological epithelial changes that are associated with inflammatory conditions of the gut.

Epidemiological evidence suggests that statins prevent CRC but the biological mechanism remains obscure. In **chapters 6 and 7** we show a novel mechanism of action of Statins in CRC. In **chapter 6** we investigated the effects of statins on CRC cell lines and xenografts in order to test the hypothesis that statins act through their actions on the BMP pathway. We show that statins induce BMP2 in colorectal cancer cells and that Noggin (a BMP inhibitor) specifically blocks statin-induced apoptosis in colon cancer cell lines. Knock-out of SMAD4, a central and critical part of the BMP pathway, leads to the loss of statin sensitivity and reconstitution with SMAD4, to the restoration of statin sensitivity. In a xenograft mouse model, tumors from SMAD4 proficient and SMAD4 deficient cell lines were treated with oral Simvastatin. Significant inhibition of tumor growth using SMAD4 proficient cells but increased tumor growth when using SMAD4 deficient cells was observed. Our results suggest that loss of SMAD4 confers resistance to statins and leads to growth promotional effects. This implies that statin therapy may only be effective in a subgroup of SMAD4-expressing colorectal cancers.

In **chapter 7** we evaluated the methylation status of BMP2 promotor in a panel of CRC cell lines and the effect of Lovastatin treatment on the methylation levels of the BMP2 promoter in order to determine whether Statins act as DNA methyltransferase inhibitors in CRC. We demonstrate that CRC cell lines show methylation of the BMP2 promoter. Lovastatin treatment induces BMP2 protein expression through inhibition of DNMTs and demethylation

of the BMP2 promoter region. Our results could help to identify a subgroup of patients where statins could be effectively used as chemopreventative drugs, patients with extensive CpG island methylation. Statins could be particularly useful as chemotherapeutic agents in a subgroup of patients with a methylated BMP2 promoter.

We investigated the effect of aspirin on the most important signal transduction pathway in CRC, the Wnt pathway in **chapter 8**. We show that aspirin decreases the activity of this pathway. Aspirin treatment inhibits the activity of the protein phosphatase 2A (PP2A), what is essential for the effects of aspirin on the Wnt pathway. The findings in this chapter provide a molecular explanation for the efficacy of aspirin in chemoprevention of CRC and show biochemical evidence that PP2A is an important regulator of Wnt pathway activity in CRC cells.

Natural products have been regarded as important sources of potential chemotherapeutic agents. In the **chapter 9** of this thesis we evaluated the effect of violacein, a pigment isolated from the Chromobacterium violaceum in the Amazon River, on CRC cells. Our data provide evidence that violacein acts in CRC cells through the inhibition of Akt phosphorylation with subsequent activation of apoptotic pathways and downregulation of NFkB signaling. This leads to increased 5-FU chemosensitivity in HCT116 colon cancer cells. Taken together, our findings suggest that violacein is a promising chemotherapeutic agent and offers new prospects for overcoming 5-FU resistance.

In conclusion, this thesis provides novel insight into the role of the Bone Morphogenetic Protein pathway in colorectal cancer. Modulation of the activity of morphogenetic pathway signaling by known chemopreventive agents is a promising approach for successful prevention therapies for GI malignancies.

Summary in Dutch

Gedurende de laatste decennia zijn er belangrijke vorderingen gemaakt in het verkrijgen van inzicht met betrekking tot de moleculaire processen die betrokken zijn bij de ontwikkeling van poliepen en van colorectale carcinogenese in de gastro-intestinale (GI) tractus. Het belangrijkste doel van dit proefschrift was het bestuderen van de rol van één van de belangrijkste morphogenetische signaal transductie paden, het Bone Morphogenetic Protein (BMP) pad in colorectale kanker (CRK). Tevens werd de potentie van modulatoren van deze BMP signaal transductie weg voor therapeutische interventie in GI kanker onderzocht.

In **hoofdstuk 2** proberen we de vraag te beantwoorden of en –zo ja- waar gedurende de adenomacarcinoma sequentie in het ontstaan van CRK de BMP signaalroute wordt verstoord. Wij rapporteren dat de expressie van BMPRs intact is en dat de signaalroute actief is gedurende de adenomafase tijdens colonkankerprogressie. Daarentegen, BMPR2 en SMAD4 gaan frequent verloren in kankerweefsels en BMP signalering vertoond een sterke correlatie met de progressie van adenomafase naar de tumor fase.

Vervolgens onderzoeken we in **hoofdstuk 3** de expressie van componenten uit de BMP signaalroute in colonkanker cellijnen en in een grote groep patiëntenbiopten met behulp van weefsel microarray en immunohistochemie. We probeerden een relatie te vinden tussen de expressie van componenten uit de BMP pathway en de MSI status van CRK. In **hoofdstuk 3** rapporteren we dat BMPR2 eiwitexpressie verdwijnd in cellijnen met microsatelliet onstabiliteit (MSI), terwijl SMAD4 eiwit expressie verdwijnt in cellijnen die microstatelliet stabiel zijn (MSS). Verder observeren we BMPR2 gemutaties is in alle MSI cellijnen die heel wel verdwijnen van de eiwitexpressie in de cellijnen kan verklaren. Ook konden we vaststellen dat BMPR2 expressie vaker verdwijnt in monsters van MSI CRK patiënten dan in monsters verkregen van MSS CRK patiënten. Omgekeerd is het verdewijnen van SMAD4 expressie weer gereflecteerd in materiaal van MSS CRK patiënten. Er is dus sprake van specifieke van inhibitie van expressie van SMAD4 of BMPR2 afhankelijk van de genomische stabiliteit van de kanker. De BMP pathway wordt geïnactiveerd in 70% van CRK en dit correleert nauw met BMPR en SMAD4 verlies. Daarom suggereren onze data dat de BMP pathway wordt geïnactiveerd in de meeste gevallen van sporadisch CRK. In MSI CRK wordt dit hoofdzakelijk geassocieerd met onderbroken BMPR2 expressie en in MSS CRK met onderbroken SMAD4 expressie.

In **hoofdstuk 4** van dit proefschrift bestuderen wij de interactie tussen de pro-proliferatieve WNT pathway en de pro-apoptotische Bone Morphogenetic Protein pathway. Gebaseerd op resultaten van ons onderzoek, veronderstellen wij een reciproque interactie tussen BMP en WNT signaling. De pro-apoptotische BMP signaling antagoneert de proliferatieve Wnt signalering en zodra BMP signalering wordt onderbroken, zoals dat het geval is met JP adenomen, vindt er een steeds groter opstapeling van nuclear β-catenin plaats, zelfs zonder APC of β-catenin mutaties. Wij tonen aan dat de Wnt signalerings weg overactief is in FAP adenomen en dat dit leidt tot de downregulatie van de BMP pathway en dat een verdere verstoring van het evenwicht tussen proliferatie en apoptosis, een overlevingsvoordeel oplevert voor deze cellen en dit speelt een belangrijke rol in colonkanker initiatie en progressie.

In **hoofdstuk 5** evalueren we BMP signaling in Helicobacter Pylori geïnfecteerde maagmucosa's. De observaties beschreven in dit hoofdstuk tonen dat de infiltratie van ontstekingscellen in het mesenchym een bron is van BMP2 eiwit in de chronisch ontstoken maag. De toename van BMP eiwit productie in het maagmesenchym correleert met de toename van BMP signaling activiteit en met de apoptotische celdood in het maagepitheel. Dit suggereert dat morfogenen, geproduceerd door cellen in het ontstekingsinfiltraat, het lot van de epitheelcellen kunnen bepalen en verantwoordelijk kunnen zijn voor de histologische veranderingen in het epitheel, die geassocieerd zijn met ontstekingen van de maag.

Epidemiologische gegevens wekken de suggestie dat statinen CRK kunnen voorkomen, maar het biologische mechanisme blijft duister. In **hoofdstuk 6 en 7** tonen wij een nieuw werkingsmechanisme aan van statinen in CRK. In **hoofdstuk 6** onderzoeken wij de effecten van statinen op CRK cellijnen en xenografts om de hypothese te toetsen dat statinen hun preventieve werkingen uitvoeren via de BMP pathway. We laten zien dat statinen BMP2 induceren in colorectale kankercellen en dat Noggin, (een BMP inhibitor) specifieke statine-geïnduceerde apoptosis blokkeert in colonkanker cellijnen. Knock-out voor SMAD4, een centrale en essentieel onderdeel van de BMP pathway, leidt tot het verlies van gevoeligheid voor statines en reconstitutie met SMAD4, leidt tot herstel van de gevoeligheid voor statines. In een xenograft muismodel, werden tumoren van SMAD4 positieve en SMAD4 deficiënte cellijnen oraal behandeld met Simvastatine. Er werd een significante tumorgroei remming waargenomen waneer er gebruik werd gemaakt van SMAD4 positieve cellen en tumorgroei,

wanneer gebruik werd gemaakt van SMAD4 deficiënte cellen. Onze resultaten suggereren dat het verlies van SMAD4 resistentie genereerd tegen statines en leidt tot groei-stimulerende effecten. Dit suggereert dat statine therapie alleen effectief zal zijn in de subgroep colorectale kanker, die SMAD4 tot expressie brengen.

In **hoofdstuk 7** wordt de methylatie status van BMP2 promotor onderzocht in een panel van CRK cellijnen en het effect van Lovastatine behandeling op de methylatie levels van de BMP2 promotor om vast te stellen of statinen werken als DNA methyltransferase inhibitoren in CRK. Wij laten zien dat de BMP2 promotor in CRK cellijnen methyleringen bevatten. Lovastatine behandeling induceert BMP2 eiwitexpressie door de inhibitie van DNMTs en demethylatie van de BMP2 promotor region. Onze resultaten kunnen een subgroep patiënten helpen identificeren, waarbij statines effectief gebruik kunnen worden als chemopreventieve geneesmiddelen, namelijk patiënten met een uitgebreide CpG island methylatie. Statines kunnen in het bijzonder nuttig zijn als chemopreventieve geneesmiddelen in een subgroep van patiënten met een gemethyleerd BMP2 promotor.

Wij onderzochten de effecten van Aspirine op een van de meest belangrijkste signaal transductie routes in CRK, the Wnt pathway in **hoofdstuk 8**. Wij tonen aan dat Aspirine de activiteit van deze pathway verlaagd. Aspirine behandeling remt de activiteit van de protein fosfatase 2A (PP2A), dat essentieel is voor de effecten van Aspirine op de Wnt pathway. De bevindingen in dit hoofdstuk geven een moleculaire verklaring voor de effectiviteit van Aspirine in de chemopreventie van CRK en leveren biochemisch bewijs dat PP2A een belangrijke regulator is van Wnt pathway activiteit in CRK cellen.

Natuurlijke producten worden vaak beschouwd als belangrijke bronnen voor potentiële chemotherapeutische middelen. In **hoofdstuk 9** van dit proefschrift worden de effecten van violacein, een pigment geïsoleerd uit de Chromobacterium violaceum in de Amazone rivier onderzocht op CRK cellen. Onze data bewijzen dat violacein Akt fosforylering remt met daarop volgend activatie van apoptose pathways en downregulatie van NFkB signaling in CRK cellen. Dit leidt tot een verhoging van 5-FU chemosensitiviteit in HCT116 colonkanker cellen. Samengevat, suggereren onze bevindingen dat violacein een veelbelovend chemotherapeuticum is en nieuwe mogelijkheden biedt om 5-FU resistentie te voorkomen.

Geconcludeerd kan worden, dat dit proefschrift nieuwe inzichten verschaft in de rol van Bone Morphogenetic Protein pathway in colonrectale kanker. Veranderingen in de activiteit van de morphogentische pathway signaling door bestaande chemopreventieve geneesmiddelen is een veelbelovende ontwikkeling voor een succesvolle preventietherapie voor GI maligniteiten.

General Discussion and Future Perspectives

In the experimental studies described in this thesis, we tried to improve understanding of the role of the Bone Morphogenetic Protein pathway in colorectal cancer. Despite several recent findings regarding the physiological function of BMPs in normal colon and studies on patients with Juvenile Polyposis suggesting the involvement of BMPs in CRC [1, 2,3, 4] the role of BMP signaling in carcinogenesis and the expression of BMP components in sporadic CRC have not been investigated. We found that the BMP pathway is active at the adenoma stage of colon cancer progression, and loss of BMP signaling correlates tightly with progression of adenomas to cancer, but occurs relatively early during cancer progression. The BMP pathway is inactivated in the majority of sporadic CRCs. In MSI CRC this is associated predominantly with impaired BMPR2 expression and in MSS CRC with impaired SMAD4 expression. These are important findings supporting the results of previous studies obtained in transgenic mouse models suggesting a tumor suppressor function of BMP pathway in the bowel: the villin-noggin mouse in which BMP expression is completely abrogated and develops neoplasia[5] and BMPR1a mutant mice which develop intestinal polyposis [6].

Further, we investigated the interaction between the BMP pathway and the main pathway involved in CRC – the Wnt pathway. Using archival material from patients with rare inherited CRC syndromes, we found that the BMP pathway is not active in patients with Familial Adenomatous Polyposis (FAP), suggesting the interaction of these two important morphogenetic pathways in humans. Previously, BMP-Wnt interaction has been studied only in mice models. Villin-noggin transgenic mice develop polyps with high levels of nuclear ß-catenin [5]. He et al. showed that BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-ß-catenin signaling and BMPR1a mutant mice develop intestinal polyposis with nuclear accumulation of ß-catenin [6]. Further investigation will help to identify the basis for these interactions.

Based on the results obtained in the first part of this thesis we hypothesize that the activation of the BMP pathway could be a valuable target for the development of cancer therapies and

chemoprevention. Lovastatin and Simvastatin are the most potent compounds (from 30000 compounds tested) that induce BMP2 gene expression in bone cells[7], thus we investigated the influence of statin treatment on the BMP pathway in CRC cell lines and the mechanism by which statins upregulate BMP2. In chapter 6 and 7 we showed that statins upregulate BMP2 expression in colon cancer cells by demethylating the BMP2 promoter in HCT116 cells and activate BMP signaling in SMAD4 expressing CRC cell lines, thus resulting in apoptosis of these cells. Interestingly, SMAD4 defective cells and xenografts show an increased growth in response to Statin treatment. This is an important finding which on the one hand could help to explain why the epidemiological evidence for a beneficial effect of statins in CRC is conflicting [8, 9 10 11, 12 13], and on the other would help to select a group of patients for safe and effective chemoprevention with statins. The activity of statins as demethylating agents demonstrated in chapter 7 of this thesis also offers new prospects for the further research and clinical application of statins.

Lovastatin augments NSAID-induced apoptosis in colon cancer cells and potentiates the chemopreventive effects of sulindac[14]. Low doses of Atorvastatin, Aspirin, and Celecoxib in combination inhibit colon carcinogenesis more effectively than when they are given individually at higher doses in rats[15]. In chapter 4 we show the Wnt-BMP pathway interaction, then in chapter 6 we activate proapoptotic BMP pathway with statins and in chapter 8 we inhibit the proproliferative Wnt pathway with aspirin. Therefore, our findings help to explain why statins and NSAIDS synergistically suppress the growth of CRC. These observations are of clinical significance because these results could pave the way for the use of combinations of these agents in small doses as a chemopreventive strategy against colon cancer.

We know already about the modulation of the BMP pathway in brain malignancies [16]. Piccirillo et al. show that the BMP–BMPR signaling system may act as a key inhibitory regulator of tumour-initiating stem-like cells from human glioblastomas and their results identify BMP4 as a novel, non-cytotoxic therapeutic effector which may be used to prevent the growth and recurrence of glioblastomas in humans. In the view of the findings described in the first part of this book supporting the tumor suppressor role of BMPs in CRC, research directed at the possible use of BMPs to treat tumour-initiating, stem-like cells from human colon cancer, would be an exiting and promising perspective.

In conclusion, The Bone Morphogenetic Pathway plays an important role as a tumor suppressor in human colon, and inactivation of BMP signaling contributes to colorectal

cancer initiation and progression. The activation of BMP pathway in well defined groups of patients could be a promising approach for successful colorectal cancer prevention therapies.

Algemene discussie en toekomstige perspectieven

In experimentele studies beschreven in dit proefschrift, proberen wij de inzichten te verbeteren in de rol van de Bone Morphogenetic Protein signeleringsweg in colorectale kanker. Ondanks sommige recente ontdekkingen over de fysiologische functie van BMPs in de normale dikkedarm en patiëntstudies met Juvenile Polyposis, die de suggestie wekken dat BMP betrokken is in CRC[1,2,3,4] is de rol van BMP signalering in de carcinogenesis en de expressie van BMP componenten in sporadische CRK nog niet onderzocht. We hebben ontdekt dat BMP signalering actief is gedurende de adenomafase van colonkanker progressie en dat het verlies van BMP signaling nauw correleert met de progressie van adenoma naar kanker, maar dit gebeurt relatief vroeg in de kanker progressie. De BMP pathway is inactief in het merendeel van sporadische CRKs. In MSI CRK wordt dit hoofdzakelijk geassocieerd met verhindering van de BMPR2 expressie en in MSS CRK met verhindering van de SMAD4 expressie. Dit zijn belangrijke ontdekkingen, die eerdere verkregen resultaten ondersteunen uit onderzoeken met transgene muizen, die een tumor suppressie functie van BMP pathway in de darm veronderstelden: the villin/noggin muis, waarin de BMP expressie compleet is onderdrukt en die neoplasieën ontwikkelt en de BMPR1a mutante muis, die poliepen ontwikkeld in de darm.[6]

Verder onderzochten wij de interactie tussen BMP signalering en de belangrijkstesignaal transductie weg betrokken bij CRK – de Wnt pathway. Door gebruik te maken van archief materiaal van patiënten met een zeldzame overerfelijke CRK syndroom, ontdekten wij dat de BMP signalering niet actief is in patiënten met Familiale Adenomateuze Polyposis (FAP). Dit suggereert een interactie tussen deze twee belangrijke morphogenetische pathways in mensen. In het verleden zijn BMP-Wnt interacties alleen bestudeerd met muismodellen. Villin-noggin transgene muizen ontwikkelen poliepen met hoge nuclear β-catenin levels.[5] He et al. toonden aan dat BMP signaling, intestinale stamcel regeneratie verhinderde door suppressie van Wnt-β-catenin signaling en dat BMPR1a gemuteerde muizen intestinale polyposis ontwikkelden met nucleaire accumulatie van β-catenin[6]. Verder onderzoek zullen bijdragen aan de identificatie van het mechanisme van deze interacties.

Gebaseerd op de resultaten verkregen in het eerste deel van dit proefschrift, veronderstellen wij dat de activatie van de BMP pathway een belangrijke target is voor de ontwikkeling van kankertherapieën en chemopreventie. Lovastatine en Simvastatine zijn de meest actieve verbindingen (van 3000 onderzochte verbindingen), die BMP2 gen expressie induceren in bot cellen[7], daarom hebben wij de invloed onderzocht van statine therapie op de BMP pathway in CRK cellen alsmede het mechanisme verantwoordelijk voor de upregulatie van BMP2. In hoofdstuk 6 en 7 tonen wij aan dat statines BMP2 expressie in colonkanker cellen opregelen door middel van demethylering van de BMP2 promotor in HCT116 cellen en via het activeren van BMP signalering in CRK cellijnen, die SMAD4 tot expressie brengen, uiteindelijk resulterend in apoptosis van deze cellen. Interessant is dat SMAD 4 defecte cellen en xenografts een toegenomen groei vertonen als reactie op statines behandeling. Dit is een belangrijke ontdekking dat aan de enerzijds helpt te verklaren waarom de epidemiologische bewijs voor het gunstige effect van statines in CRK tegenstrijdig is.[8, 9, 10, 12, 13], en anderzijds zou kunnen helpen om een subgroep patiënten te selecteren voor een veilige en effectieve chemopreventieve behandeling met statinen. De activiteit van statinen als demethylerende geneesmiddelen, beschreven in hoofdstuk 7 van dit proefschrift, biedt nieuwe vooruitzichten voor verder onderzoek en klinische toepassing van statinen.

Lovastatine verhoogt NSAID-geïnduceerde apoptosis in colonkanker cellen en versterkt de chemopreventieve effecten van Sulindac[14]. Lage dosering van een combinatie bestaande uit Atorvastine, Aspirine en Celecoxib remmen colonkanker carcinogenesis meer effectief dan waneer ze apart worden gegeven in ratten.[15] In hoofdstuk 4 laten we de Wnt-BMP pathway interactie zien, vervolgens in hoofdstuk 6 activeren we de proapoptotische BMP pathway met statines en in hoofdstuk 8 remmen we de preproliferatieve Wnt pathway met Aspirine. Daarom helpen onze bevindingen te verklaren waarom statines en NSAIDs samen de groei van CRK remmen. Deze bevindingen bezitten tevens klinische relevantie omdat deze resultaten de eerste stap zijn voor het gebruik van laag gedoseerde combinaties van deze geneesmiddelen als een chemopreventieve strategie tegen colon kanker.

We weten al dat maligniteiten in de hersenen BMP signalering veranderd is[16]. Piccirillo et al toonden aan dat de BMP-BMPR signaling systeem kan optreden als een alles bepalende remmende regulator van beginnende stamcelachtige tumorcellen van het humane glioblastoma's en hun resultaten identificeren BMP4 als een nieuwe, niet-cytotoxische therapeutische effector, die gebruikt kan worden om groei en recidivering te voorkomen van glioblastoma's in mensen. Gezien de ontdekkingen, beschreven in het eerste deel van dit boek die de tumor suppressor rol van BMP's in CRK ondersteunen, zou onderzoek naar het

gebruik van BMPs in de behandeling van beginnende tumorachtige, stamcelachtige cellen van humane colonkanker, een veelbelovend en opwindend perspectief bieden.

Samengevat, de Bone Morphogenetic Pathway speelt een belangrijke rol als een tumor suppressor in het humane colon en inactivatie van BMP signaling draagt bij aan de initatie en progressie van colorectale kanker. De activatie van BMP pathway in een goed gedefinieerde patiëntgroepen zou een veelbelovende benadering kunnen zijn voor succesvolle preventieve therapieën voor colonrectale kanker.

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