CHAPTER SIX

STATINS AUGMENT THE CHEMOSENSITIVITY OF COLORECTAL CANCER CELLS INDUCING EPIGENETIC REPROGRAMMING AND REDUCING COLORECTAL 'STEMNESS' VIA THE BONE MORPHOGENETIC PROTEIN PATHWAY

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ABSTRACT

Background: Promoter hypermethylation is an important and potentially reversible mechanism of tumor suppressor gene silencing in cancer. Compounds which demethylate tumor suppressor genes and induce differentiation of cancer cells, but do not have toxic side-effects would represent an exciting option in cancer therapy. Statins are cholesterol lowering drugs with an excellent safety profile and associated with a reduced incidence of various cancers including colorectal cancer (CRC). We have previously shown that Statins act by the activation of tumor suppressive Bone Morphogenetic Protein (BMP) signaling in CRC, increasing expression of BMP2. *BMP2* is silenced by hypermethylation in gastric cancer.
Aim: To investigate whether *BMP2* is methylated in CRC, whether Statins can reverse this and what implications this has for the use of Statins in CRC.
Methods: We performed methylation specific PCR, bisulfite sequencing, immunoblotting, RT-PCR, qPCR, FACS analysis, an in vitro DNA methyltransferase assay and cell viability studies on CRC cells. The effect of Statins was confirmed in a xenograft mouse model.
Results: *BMP2* is silenced by promoter hypermethylation in cell lines with the hypermethylator phenotype and in primary tumors. Treatment with Lovastatin downregulates DNA methyltransferase (DNMT) activity, leading to *BMP2* promoter demethylation and to upregulation of BMP2 expression, as well as other genes methylated in CRC. Statins alter gene expression indicative of a shift from a stem-like state to a more differentiated state, thereby sensitizing cells to the effects of 5-FU. In a xenograft mouse model Simvastatin treatment induces BMP2 expression, leads to differentiation and reduced proliferation of CRC cells.
Conclusions: Statins acts as DNMT inhibitors, demethylating the *BMP2* promoter, activating BMP signaling, inducing differentiation of CRC cells and reducing stemness. Our study may indicate that Statins could be used as a differentiating agent in combined or adjuvant therapy in CRC with the CpG Island Methylator Phenotype (CIMP).

SUMMARY

What is already known about this subject?

- The Bone Morphogenetic Protein (BMP) pathway is an important tumor-suppressor pathway in colorectal cancer (CRC)
- Statins are cholesterol lowering drugs with an excellent safety profile and associated with a reduced incidence of various cancers including colorectal cancer
- Statins induce apoptosis in colorectal cancer cells by activating the BMP pathway
- the *BMP2* promoter is methylated in a large proportion of gastric cancers

What are the new findings?

- *BMP2* is silenced by promoter hypermethylation in a subgroup of colorectal cancers
- Statin treatment inhibits DNMT activity, demethylates the promoters of *BMP2* and other tumor-suppressor genes methylated in CRC
- Statins induce differentiation in CRC cells in vitro and in a xenograft model in vivo and reduce stemness.
- Statins increase the chemosensitivity of cancer cells to 5-FU

How might it impact on clinical practice in the foreseeable future?

- Statins could be used as part of combined or adjuvant therqpy in CRC with a CpG Island Methylator Phenotype (CIMP).

INTRODUCTION

Aberrant DNA methylation of CpG islands in the promoter regions of many genes has been observed in human colorectal cancer (CRC) and is associated with tumor-suppressor gene silencing. Cancers that show extensive DNA methylation in the promoter regions of specific genes have been described as having the CpG Island Methylator Phenotype (CIMP) (1) and these cancers are resistant to current chemotherapy (2). Epigenetic alterations do not involve changes in the DNA sequence and are thus potentially reversible. This has already found clinical application in cancer therapy where demethylating agents have proven to be a valuable option in selected malignancies.

DNA methylation is regulated by DNA methyltransferases (DNMTs). DNMTs are enzymes that catalyze the addition of methyl groups to cytosine residues in DNA. The activity of DNMTs is elevated in CRC cell (3) and the inhibition of DNMT activity can strongly inhibit the formation of tumors in vivo (4). DNMT inhibitors have been intensively studied as promising new drugs for cancer therapy. 5-azacytidine and Decitabine have already entered the clinic (5) but currently known DNMT inhibitors cause significant toxicity (6;7) as they

become incorporated into RNA and interfere with protein translation (8). For this reason, compounds acting as DNMT inhibitors but not having toxic side-effects would open up new opportunities in cancer therapy.

Epigenetic reprogramming involving changes in promoter methylation is also the mechanism underlying cell differentiation. Differentiating agents are already successfully used in combined chemotherapy where they are thought to force relatively poorly differentiated cancer stem cells to differentiate, making them more sensitive to the chemotherapeutic agents that are administered at the same time (9). Bone Morphogenetic Proteins (BMPs) have also been used for this same purpose by intratumoral injection of recombinant protein in animal models (10). This methodology is unattractive for clinical application in humans but small molecular compounds that activate the BMP pathway could pose an attractive alternative strategy.

The BMP pathway plays an important role in intestinal epithelial homeostasis (11) and CRC (12;13). BMP signaling promotes intestinal differentiation and inhibits stem cell activation (14;15). Germline mutations in *BMPR1a* and *SMAD4* are the cause of familial Juvenile Polyposis Syndrome (16), a syndrome with a high lifetime risk of developing CRC. Genome Wide Association Studies have identified gene alterations within multiple members of the BMP pathway as being associated with an increased risk of CRC, namely *BMP2, BMP4, Gremlin1* and *Smad7* (17;18). This makes the study of compounds that specifically modify the BMP pathway even more relevant.

HMGCoA reductase inhibitors, better known as the Statins, not only reduce serum cholesterol and decrease the incidence of cardiovascular and cerebrovascular events (19;20), but reduce the risk of developing CRC (21;22). In vitro studies show that Statins inhibit cellular proliferation and induce apoptosis in CRC cells and in animal models (23;24). A screen of 30,000 compounds for their ability to enhance BMP2 expression for eventual use to enhance bone formation, identified two Statins as the two most active compounds (25). We have subsequently shown that the actions of Statins in CRC are dependent on their ability to upregulate BMP2 expression and activate the BMP pathway (24). Interestingly the *BMP2* gene is silenced by promoter hypermethylation in a large proportion of gastric cancers (26), but whether this also occurs in CRCs is unknown.

In this study we initially set out to investigate whether the *BMP2* promoter is hypermethylated in CRC. Having previously shown that Statins upregulate BMP2 expression, we then investigated whether Statin treatment influences the methylation status of the *BMP2* promoter and whether Statins can also alter the methylation of promoters of other tumor suppressor genes methylated in CRC. We then assessed whether Statins inhibit DNMT activity. To look at the overall effect of these epigenetic changes on CRC cell phenotype we then investigated the effects of Statins on differentiation markers and stem cell markers both in CRC cells in vitro and in a xenograft model in vivo. To establish whether the observed differentiation and stemness changes are BMP pathway specific, we activated BMP

signaling in CRC cells by transfection with *BMPR2* and observed the same effects seen in Statin treated cells. Finally, to investigate whether the differentiating effects of Statins have potential clinical application by modifying CRC chemosensitivity we assessed the effect of pretreatment with low doses of Statins, too low to have cytotoxic effects on their own, on the sensitivity of CRC cells to 5-Fluorouracil. Our findings support the possible use of Statins as agents to potentiate standard cytotoxic cancer therapy acting as demethylating agents that activate the BMP pathway.

MATERIALS AND METHODS

(Cell culture, Immunoblotting, DNA Extraction and Bisulfite Modification, Transfection and luciferase reporter assay, Immunohistochemistry, RNA isolation and Real-Time RT-PCR are described in supplementary data section)

Methylation Specific PCR (MSP).

The primer sequences for MSP for *BMP2*, *HIC1* and *TIMP3* were described previously (26;27). PCR was performed with 40 cycles of 94°C, 62°C(*for BMP2*), 65°C (for *HIC1* and *TIMP3*) and 72°C of 1- min each, preceded by a 5-min denaturing step at 94°C and followed by a 10-min extension step at 72°C. 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) was used as a positive control for the methylated reaction.

Bisulfite sequencing (BS).

For BS, cell line-derived DNA was treated with sodium bisulfite and amplified by PCR. The primers for bisulfite sequencing of *BMP2* were 5'- GTATTTGGTTTTAGGGTTAG-GAGAG -3' (forward) and 5'- CCAAATACTAACACACAACAACAAC -3' (reverse). PCR was performed with 35 cycles of 94°C, 62°C, and 72°C of 1-min each, preceded by a 5-min denaturing step at 94°C and followed by a 10-min extension step at 72°C. 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) using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the original primers for bisulfite sequencing of *BMP2*.

In vitro DNA methyltransferase (DNMT) assay.

HCT116 cells were treated with different concentrations of Lovastatin or 5μM 5-azacytidine (5-AZA) (Sigma, St. Louis, MO) 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 14000 rpm 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 14000 rpm for 2 min. The supernatant was saved as the nuclear extract and used for measuring the total DNMT activity using the EpiQuik DNA Methyltransferase Activity/ Inhibition Assay Kit according to the manufacturer's instructions.

Xenograft mouse model.

8 female NMRI nu/nu mice were injected subcutaneously in the flank with 1×10^6 HCT116 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 three weeks, initiated when the tumor volume reached 100-200 mm^3. After the mice were sacrificed the tumors were harvested, and either frozen in liquid nitrogen and later homogenized in Trizol (Invitrogen, Breda, The Netherlands), or embedded into paraffin blocks. We chose Simvastatin as being the second most potent Statin in our *in vitro* experiments (24) and because Simvastatin is licensed for use in humans in the Netherlands while Lovastatin is not.

Chemosensitivity assay.

Cells were plated in 96-well plates and treated with low dose Lovastatin (Sigma) (0.2μM) or vehicle control in DMEM with 0.5 % FCS. This low concentration of FCS allowed us to perform 5-days long exposure without reaching confluence. Most studies use doses between 10 and 30μM in vitro (28). After 5 days of Statin exposure cells were incubated in fresh DMEM with 10% FCS for 24 hours. Subsequently cells were treated with 5-FU (Sigma) (1-50 μM) for 48 hours in DMEM with 0.5% FCS. After treatment, MTT solution was added (final concentration 0.5 mg/ml, stock solution 5 mg/ml MTT in PBS), for 3 hours. The medium was discarded and cells were lysed in acidified 2-propanol. Absorbance was measured at 550-560nm.

FACS Analysis.

Cells were treated with 0.2μM Lovastatin or vehicle control for 72 hours, then harvested, washed, and stained in FACS buffer (PBS containing 1% BSA) on ice with anti-CD166-PE (Becton Dickinson, Breda, The Netherlands). Cells were washed again and CD166 cell

surface levels were analyzed by flowcytometry using a FACSCalibur (BD Bioscience) and FlowJo Software (Treestar, Ashland, OR).

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

BMP2 expression is frequently impaired due to promoter hypermethylation in colorectal cancer.

We performed RT-PCR for *BMP2* in six CRC cell lines. HCT116 cells do not express *BMP2* at the mRNA level. RKO and SW48 (not shown) express very low levels of *BMP2* mRNA (Figure 1A). These 3 cell lines are known to exhibit the CIMP phenotype with hypermethylation of the promoters of several tumor suppressor genes. The main genetic characteristics of the cell lines used in this study are presented in Supplementary table1.

We performed Methylation Specific PCR (MSP) for the *BMP2* promoter region starting 214bp in front of exon 1 (Figure1B). This region contains a CpG island and has been shown to be methylated in gastric cancers (26). The same cell lines that express reduced levels of *BMP2* mRNA, show CpG island methylation of the *BMP2* promoter region. MSP for HCT116 cells reveals 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 (Figure1C). We verified the results of MSP by direct bisulfite sequencing (Figure1D). 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 with 96% of CpGs methylated. SW480 and CACO2 cells show only minimal 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 a 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 1E, the level of *BMP2* promoter methylation in SW48 cells is 51% and in RKO is 72%.

To determine whether *BMP2* is methylated in a subgroup of CRC patients, we performed MSP on bisulfate modified DNA from 54 CRC tumors. Thirteen out of 54 tumors (23%) showed a methylated signal on MSP, confirming the relevance of our in vitro findings for patients (Figure1F). Further characterization of these tumors by BRAF V600E mutation analysis was informative in 40 tumors and revealed 9 BRAF V600E mutations. 3 of the

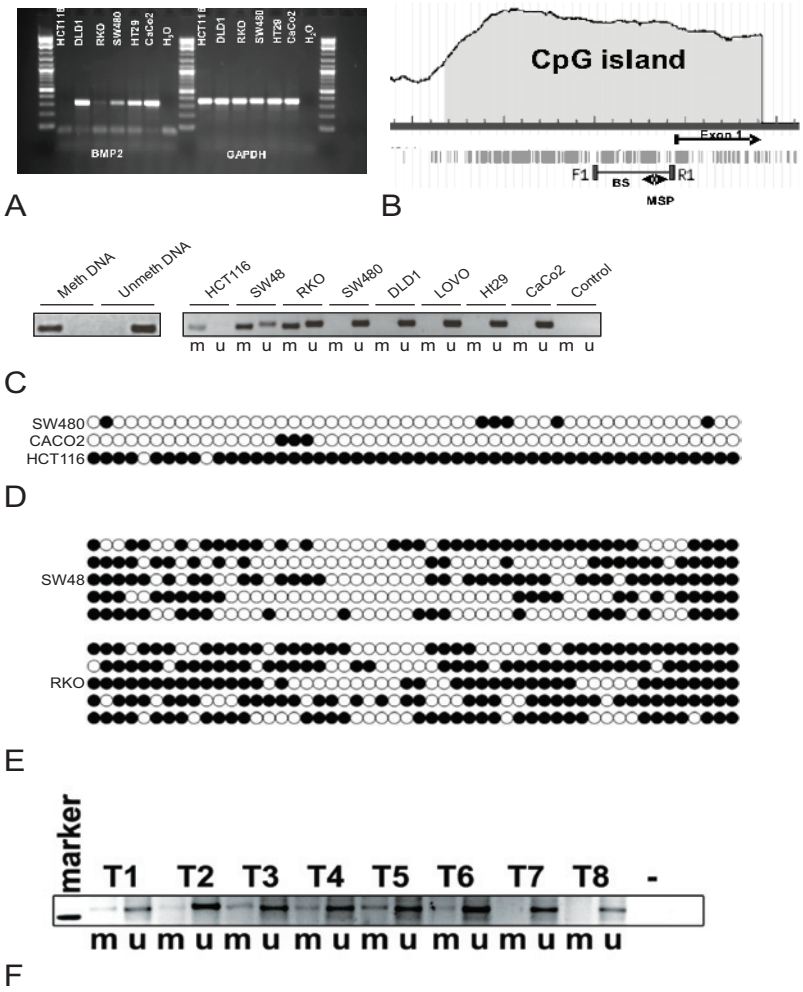

Figure 1. A, RT-PCR analysis of *BMP2* expression in colon cancer cell lines (left) and corresponding *GAPDH* controls (right). HCT116 and RKO cell lines show absent *BMP2* expression. B, Schematic representation of the large CpG island in the *BMP2* promoter region extending into exon 1. The double headed black arrow indicates the region subjected to methylation specific PCR analysis (MSP). C, MSP analysis of the *BMP2* promoter region in colorectal cancer cell lines. PCR products specific for unmethylated (u) and methylated (m) CpG sites were analyzed in 2.5% agarose gels. In the positive controls completely methylated and completely unmethylated DNA show only a methylated and unmethylated band respectively. The same cell lines with absent *BMP2* expression by RT-PCR show full (HCT116) or partial methylation (RKO) of the *BMP2* promoter by MSP. Control is MSP performed without DNA. D, Direct bisulfite sequencing of the *BMP2* promoter in SW480, CACO2 and HCT116 colorectal cancer cell lines. Solid and open circles represent methylated and unmethylated CpG sites, respectively. E, Bisulfite sequencing of multiple independent clones from SW48 (B) and RKO (C) cell lines. Each horizontal row of circles represents the 52 CpG sites contained in the region. Solid and open circles represent methylated and unmethylated CpG sites, respectively. F, MSP in human colorectal cancer tissue. The *BMP2* promoter is methylated in a subgroup of CRC patients. T1-T6 show bands in the 'm' lanes representing *BMP2* promoter methylation. T7 and T8 show no *BMP2* promoter methylation. '-' is MSP without DNA.

13 cancers with BMP2 methylation have BRAF mutations, 1 was uninformative for BRAF status and 9 were wild type (Supplementary table2).

Lovastatin treatment leads to demethylation of the BMP2 promoter region, upregulation of BMP2 expression and to demethylation of the promoters of other genes methylated in CRC

We evaluated the methylation status of the *BMP2* promoter after treatment of HCT116 cells with 2μM of Lovastatin for 48 hours and 72 hours by MSP. As shown in Figure 2A, Lovastatin treatment leads to demethylation of the *BMP2* promoter, with the appearance of the unmethylated signal using MSP. To confirm this finding and to quantify the extent of demethylation, we performed bisulfite sequencing of multiple independent clones from HCT116 cells and HCT116 cells treated for 72 hours with Lovastatin. The bisulfite sequencing results show that *BMP2* promoter methylation decreases from 97% to 40% after treatment with 2μM of Lovastatin for 72 hours (Figure 2B). We performed RT-PCR for *BMP2* to evaluate the differences in the expression of *BMP2* on mRNA level after treatment of HCT116 cells with 2μM of Lovastatin for 3, 5 and 7 days. As shown in Figure 2C, Lovastatin treatment, as well as treatment with a strong demethylating agent 5-deoxy AZA-C, upregulates the mRNA level of *BMP2* in a time dependent manner.

We performed MSP analysis of 3 other genes methylated in HCT116 cells (27) before and after treatment with 2μM of Lovastatin for 72 hours. Lovastatin treatment leads to demethylation of the *Hypermethylated In Cancer 1* (*HIC1*) and *Tissue Inhibitor of Metalloproteinase 3* (*TIMP3*) promoters, with reappearance of the unmethylated band after Lovastatin treatment (Figure 2D). We also performed MSP for *Death-Associated Protein Kinase* (*DAPK*) before and after Lovastatin treatment, but did not see any difference in the methylation level of its promoter (data not shown). These 3 genes belong to a panel of genes hypermethylated in HCT116 cells and in colorectal cancers (27).

Lovastatin inhibits DNMT activity in vitro

To further investigate the mechanism by which Lovastatin leads to promoter demethylation, we performed an *in vitro* DNA methyltransferase (DNMT) assay. The DNMT assay shows that treatment with different concentrations of Lovastatin leads to a dose-dependent downregulation of DNMT activity in HCT 116 cells. Remarkably, even low concentrations of Lovastatin (0.25μM and 0.5μM), concentrations approximating those found in the serum of patients taking standard doses of Statins, downregulate DNMT activity implying a specific effect on DNMT and not a consequence of a general toxicity (Figure 2E). The effect seems to be through inhibiting DNMT function since protein and mRNA levels of DNMTs are not influenced by Lovastatin treatment (Supplementary figure 1). These data further support the conclusion that Lovastatin acts as a DNMT inhibitor and thus leads to promoter demethylation and re-expression of putative tumor suppressors such as BMP2.

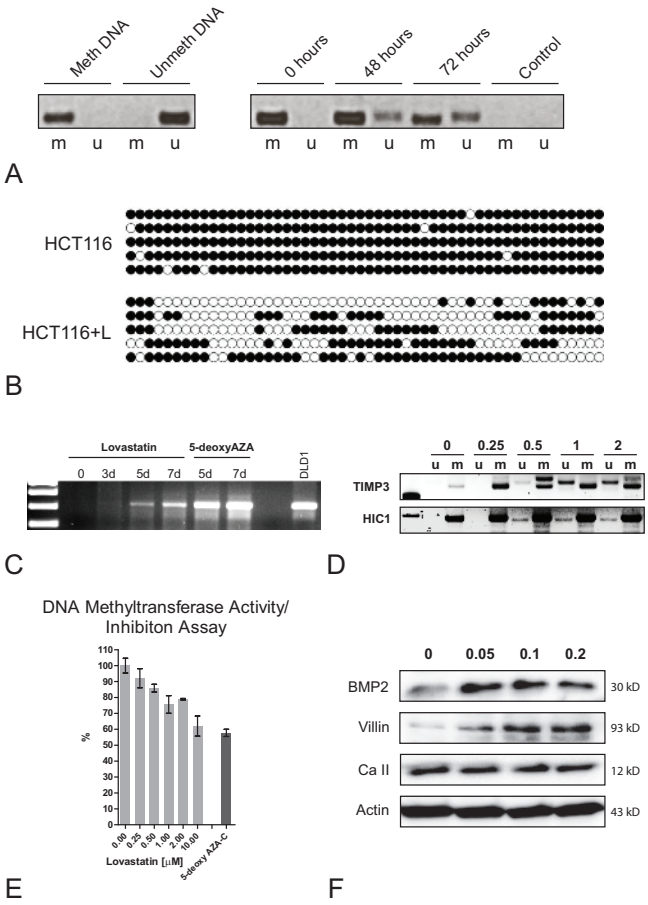

Figure 2. A, MSP of the *BMP2* promoter region for HCT116 cells treated with 2 μM of Lovastatin for 48 and 72 hours. PCR products specific for unmethylated (u) and methylated (m) CpG sites were analyzed in 2.5% agarose gels. Unmethylated DNA control – human genomic DNA from peripheral blood lymphocytes. Meth DNA control - human genomic DNA treated in vitro with Sss I methyltransferase. B, Demethylation of *BMP2* by Lovastatin. HCT116 cells were treated with vehicle (HCT116) or 2μM of Lovastatin (HCT116+L) for 72 hours and bisulfite sequencing of multiple independent clones was performed. Each horizontal row of circles represents analysis of 52 CpG sites contained in the region. Solid and open circles represent methylated and unmethylated CpG sites, respectively. C, RT-PCR analysis of *BMP2* expression in HCT116 cells treated at different time points with 2μM Lovastatin or the demethylating agent 5-deoxy AZA-C. Lovastatin and 5-deoxy AZA-C both lead to re-expression of *BMP2* mRNA. D, MSP of the *TIMP3* and *HIC1* promoter regions for HCT116 cells treated with different concentrations of Lovastatin for 72 hours. PCR products specific for unmethylated (u) and methylated (m) CpG sites were analyzed in 2.5% agarose gels. E, HCT116 cells were treated with 2μM Lovastatin for 48 hours 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. F, Immunoblots for BMP2, Villin and CAII of HCT116 cells treated with different concentration of Lovastatin for 7 days. The protein expression was analyzed using the corresponding specific antibody. Actin served as a loading control.

Lovastatin induces differentiation and reduces 'stemness' in HCT116 cells

The BMP pathway is thought to induce differentiation of normal intestinal epithelial cells counteracting signals such as WNT which impose a more stem-like phenotype (29);(30). BMP2 expression is upregulated by Lovastatin, therefore we investigated whether Lovastatin induces differentiation in CRC cells. We performed immunoblotting on HCT 116 cells treated with Lovastatin for markers of absorptive cell differentiation and qRT-PCR for markers of goblet cell differentiation. We show that Lovastatin induces dose dependent upregulation of BMP2 and Villin, but not CAII expression, suggesting that the induced enterocyte differentiation is partial (Figure 2F). Lovastatin also pushes CRC cells towards goblet cell differentiation as judged by upregulation of the goblet cell markers *Mucin2* and *Galectin4* (Figure 3A). Goblet cell differentiation is controlled by Notch signaling (31). We see downregulation of the Notch pathway target *HES1* and upregulation of *KLF4*, a goblet cell-specific differentiation factor in the colon (32) regulated by Notch signaling (33) in HCT116 cells treated with Lovastatin. An important Wnt-target and oncogene *c-Myc* and gene inducing stemness (34) is also downregulated by Lovastatin treatment (Figure 3A), as well as the expression of two markers of cancer stem cells *CD166* and *EpCAM* (35-37) (Figures 3B, C) further evidence that Statins push CRC cells towards a more differentiated phenotype and away from a stem-like, crypt cell phenotype. The widely used marker of stem cells CD133 is not an optimal marker in HCT116 cells as the *CD133* promoter is densely methylated in HCT116 cell line and was therefore not used in this study (38).

Lovastatin induces differentiation in a xenograft model

Our previous work showed that Simvastatin inhibited the growth of HCT116 xenografts in mice (24), but the effect of oral administration of Statins on the differentiation of cells within the xenografts had not yet been studied. We performed IHC analysis on HCT116 xenografts in mice for the proliferation marker Ki-67, and the differentiation markers Villin and BMP2. After 3 weeks of oral administration of Simvastatin (50 mg/kg/day) HCT116 xenografts show significant downregulation of Ki-67 and upregulation of BMP2 and Villin protein expression (Figure 4A,B). Simvastatin also induces differentiation of HCT xenografts towards the goblet cell lineage as revealed by staining with periodic acid-Schiff (PAS) (Figure 4A). Simvastatin treatment also results in downregulation of *c-Myc* and *HES1* mRNA in xenografts (Figure 4C).

Activation of BMP signaling leads to the differentiation of CRC cells.

In order to test the hypothesis that Statin induced differentiation in CRC cells is due to the activation of BMP signaling, we transfected HCT116 cells with *BMPR2* and activated BMP signaling 6 fold (Figure 5A). This results in upregulation of Villin and *Mucin2* expression, downregulation of Notch pathway activity and downregulation in expression of cancer stem cells markers *CD166* and *EpCAM* as seen with statin treatment (Figure 5B, C). These

data suggest that activation of BMP pathway induces differentiation and reduce stemness of CRC cells and implies that Statins may induce the shift from a stem-like state to a more differentiated state of CRC cells by demethylation of the *BMP2* promoter and activation of BMP signaling.

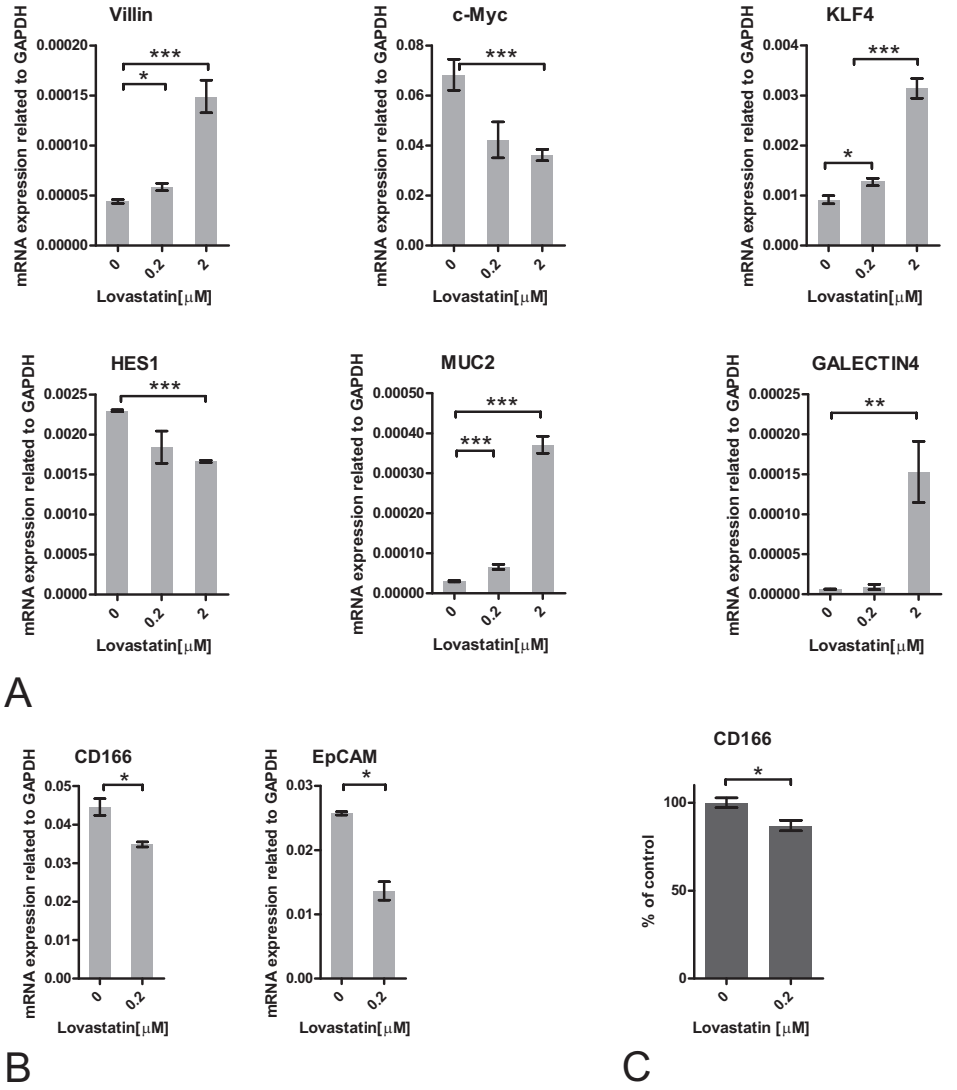

Figure 3. A, Quantitative RT-PCR analysis of *Villin*, *c-Myc*, *KLF4*, *HES1*, *Mucin2* and *Galectin4* expression in HCT116 cells treated with 0.2 or 2 μM of Lovastatin for 72 hours. B, Quantitative RT-PCR analysis of stem cell markers expression in HCT116 cells treated with Lovastatin. C, FACS analysis of CD166 expression in HCT116 cells treated with Lovastatin. Data are from three experiments ±s.e.m.(n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

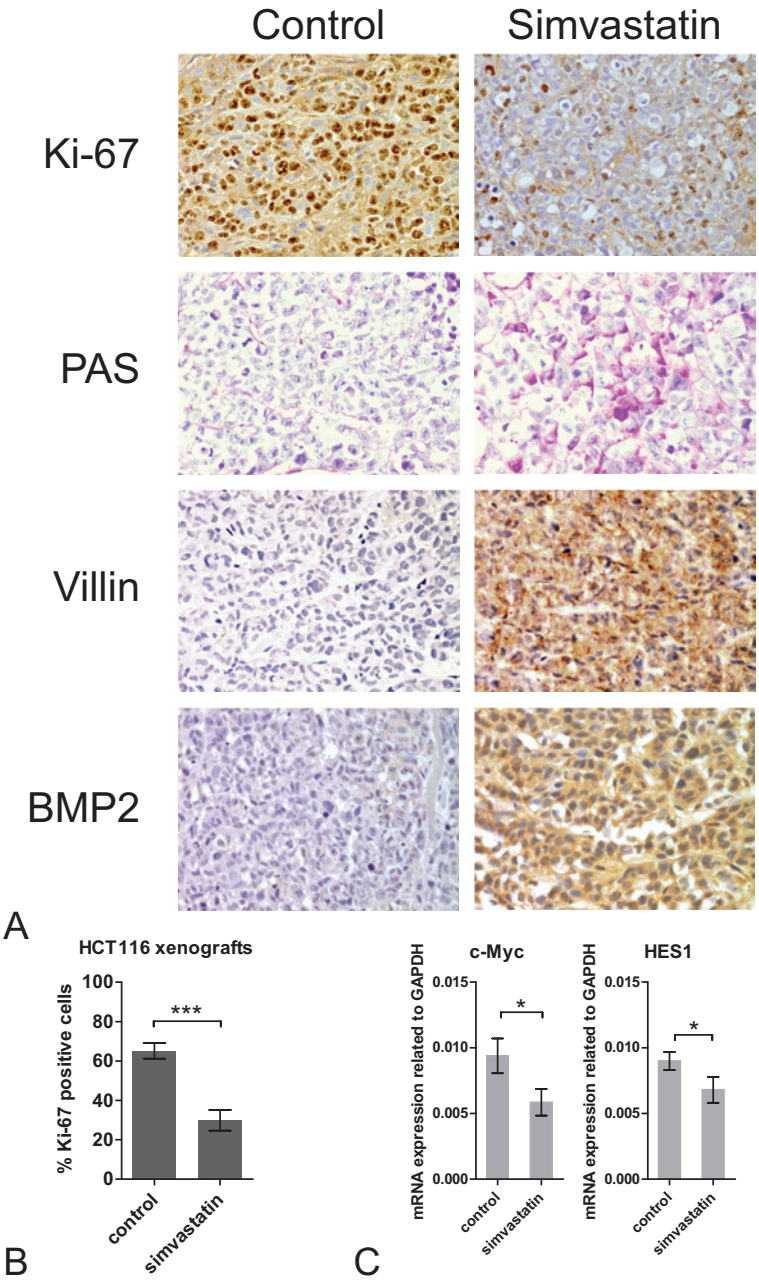

Figure 4. A, Immunohistochemistry for Ki-67, Villin, BMP2 and periodic acid-Schiff (PAS) staining for goblet cells of HCT116 xenografts from mice treated with Simvastatin and from controls. Original magnifications: 20x. B, The percentage of Ki-67-positive cells to all cells per 20x field (for every xenograft 3 images were scored). Error bars represent s.e.m. (n=8). *** $p<0.001$. C, Quantitative RT-PCR analysis of *c-Myc* and *HES1* in HCT116 xenografts from mice treated with Simvastatin and from controls. * $p<0.05$. Error bars represent s.e.m. (n=8).

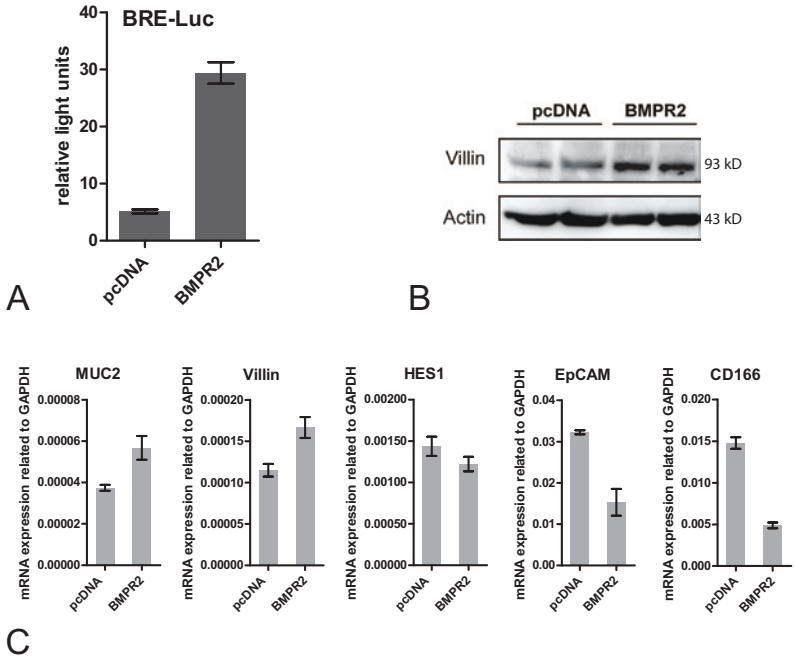

Figure 5. A, HCT116 cells were transiently co-transfected either with pcDNA4/TO/BMPR2 plasmid or pcDNA4/TO control vector and BRE-Luc vectors and Dual Luciferase assay was performed 48 hours after transfection. Data were normalized to Renilla luciferase activity. Data are from three experiments ±s.e.m (n=3). B, Immunoblots for Villin of HCT116 cells transfected with BMPR2 or pcDNA. The protein expression was analyzed using the corresponding specific antibody. Actin served as a loading control. C, Quantitative RT-PCR analysis of *Villin, HES1 Mucin2, CD166* and *EpCAM* of HCT116 cells transfected with pcDNA4/TO/BMPR2 plasmid or pcDNA4/TO control vector.

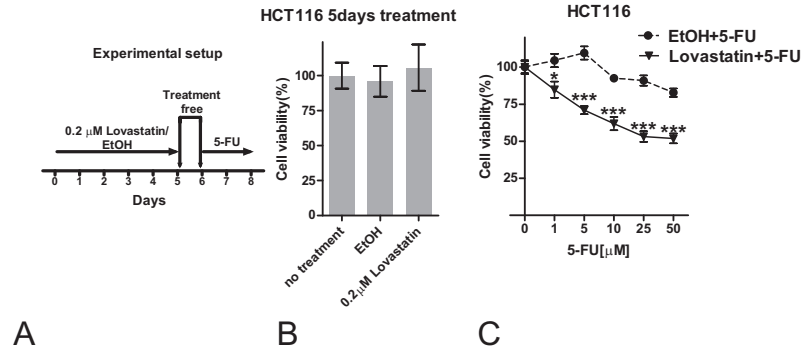

Figure 6. A, Schematic representation of the experimental setup. B, HCT116 cells were plated in 96well plates and either not treated, or treated with EtOH or 0.2 μM Lovastatin (diluted in EtOH) for 5 days and the MTT assay was performed. Values are expressed as percentage of living cells relative to the control with control values set at 100%. Results represent the mean ± s.e.m. (n=10). C, After 5 days of treatment with Lovastatin or EtOH and 1 day of no treatment, cells were treated with different concentrations of 5-FU and the MTT assay was performed. Values are expressed as a percentage of living cells relative to the control with control values set at 100%. Results represent the mean ± s.e.m. (n=10). * $p<0.05$, *** $p<0.001$.

Lovastatin enhances the cytotoxic effect of 5-FU

We tested the effects of Lovastatin treatment on the chemosensitivity of CRC cells to the conventional chemotherapeutic drug 5-FU (experimental setup is depicted in Figure 6A). We treated HCT116 cells with a low concentration of Lovastatin for 5 days in order to differentiate CRC cells. At this time point no effects of this low concentration of Lovastatin on cell viability was observed compared to untreated cells or EtOH treated control cells (Figure 6B). After the removal of Lovastatin cells were then treated with different concentrations of 5-FU for 48 hours. Lovastatin treatment significantly increases sensitivity of cancer cells to 5-FU (Figure 6C). Even at low concentrations of 5-FU which do not inhibit the viability of HCT116 cells when used alone, Lovastatin pre-treatment sensitizes CRC cells and leads to significant inhibition of cell viability. Importantly, concentrations of 5-FU between 1-10μM are clinically relevant as they approximate serum (6-12μM) (39) and tissue (2-5μM) (40) levels in patients.

DISCUSSION

DNA hypermethylation of the promoter region of tumor suppressor genes occurs frequently in cancer and results in the transcriptional silencing and loss of function of critical tumor suppressor genes (41). In this study we show that the *BMP2* promoter is methylated in a subgroup of CRC cell lines and primary cancer specimens. We use HCT116 cells for a large proportion of our studies as BMP2 is fully methylated in these cells. These cells are frequently used by others for methylation studies (42;43) and are a heavily methylated cell line as seen in CIMP (44). However, MLH1 is mutated in HCT116, rather than methylated, suggestive of Lynch syndrome origins.

We have analyzed BRAF V600E mutation status in the same primary cancer specimens in which we analyzed BMP2 methylation. BRAF V600E mutations are frequently used as a marker of CIMP status but may underestimate the prevalence of CIMP by approximately 50% (45). 25% of the cancers we tested show concomitant BMP2 methylation and BRAF mutation. While in cell lines BMP2 promoter methylation is seen exclusively in cell lines with the CIMP phenotype, analysis of BRAF only as a marker of CIMP is insufficient to confirm or refute this in tumor specimens.

We report that Statins exhibit demethylating properties. Inhibition of DNMTs can be seen even at low concentrations of Statins (0.25μM), which are comparable with the serum levels of ~ 0.1 μM measured in patients treated with standard doses for hypercholesterolemia (46) and far lower than the maximum safely achievable levels in humans. These low concentrations of Statins are safe and well tolerated by patients for years. Thus, in contrast to most known DNMT inhibitors, Statins inhibit DNMTs and induce DNA demethylation at non-toxic doses. Our experiments in xenografts investigated whether the demethylating

and differentiating properties of Statins *in vitro* translate into the same differences *in vivo*. We chose oral administration of Statins to more closely mimic their use in humans. We show that oral treatment with Simvastatin induces BMP2 expression, leads to differentiation and to downregulation of proliferation of colon cancer cells in a xenograft mouse model.

Lovastatin treatment induces DNA demethylation and the reactivation of *BMP2* gene expression, which is silenced by hypermethylation in CRC cells. Importantly, we find demethylation of two other methylated in CRC genes *TIMP3* and *HIC1* after treatment with Lovastatin, implying a more general effect on gene hypermethylation. We do not see demethylation of the *DAPK* promoter region. One possible explanation for this could be that other epigenetic factors, for example histone modification, that are not influenced by Statin treatment, regulate methylation more prominently in this gene (47).

From these studies it is unclear how Statins inhibit DNMTs. We show that Statins have little or no influence on expression levels of DNMTs and further studies are needed to determine the mechanism by which Statins inhibit DNMTs. It is also unclear from these studies what the influence of Statins is on global methylation levels. While CIMP CRCs exhibit widespread promoter hypermethylation, global levels of DNA methylation are often found to be lower in colorectal cancer. However the importance and mechanism of action of global hypomethylation are less well established and the influence of Statins, if any, on this phenomenon is a subject for future investigation.

Lovastatin treatment leads to increased expression of markers of intestinal epithelial cell differentiation and decreased expression of cancer stem cell markers. This could be due to demethylation of multiple genes, however, activation of the BMP pathway alone is sufficient to lead to the same effects on differentiation and stem cell markers as Statin treatment, suggesting that the action of Statins is primarily due to their effects on the BMP pathway. Low dose Statin treatment increases the sensitivity of CRC cells to the conventional chemotherapeutic drug 5-FU. Increases in sensitivity to conventional chemotherapy have been seen with the use of other differentiation-inducing agents in CRC cells, such as the γ-secretase inhibitors (48), PPAR- γ agonists (49) and with multiple interventions as aimed at differentiating CRC stem cells and thus improving their chemosensitivity including the use of BMPs. Delivery of BMP protein to tumors is highly challenging and at present has only been successfully performed by intratumoral injection in mouse models of cancer. It is questionable whether delivery of sufficient amounts of BMPs at the required site can be achieved in human cancer therapy. Statins have been shown to increase levels of BMP2 in bone cells (25), and we have shown that this also holds true for CRC cells. While in bone cells the mechanism appears to be direct activation of the *BMP2* promoter, the promoter is frequently silenced in colorectal cancer, as we show here, but this can be reversed by Statin therapy.

The CpG island methylator phenotype (CIMP) is found not only in colorectal cancers but also in colonic polyps (50;51) and even in the normal colorectal mucosa in patients

with hyperplastic polyposis (52). It has been observed that cigarette smoking is strongly associated with CIMP CRC with a dose-response relationship with respect to the amount smoked (53). These groups of patients could potentially benefit from a chemopreventive agent exhibiting mild demethylating properties and an excellent safety profile. As Statin treatment also reduces morbidity and mortality associated with cardiovascular disease their demethylating effect could make them a particularly valuable chemopreventive agent in a well-defined group of patients.

In conclusion, the *BMP2* promoter is methylated in a subgroup of CRC cell lines and in CRC patients. Lovastatin acts as a DNMT inhibitor and demethylates the *BMP2*, *TIMP3* and *HIC1* promoters. Statins decrease stemness and induce differentiation of CRC cells *in vitro* and *in vivo* sensitizing cells to 5-FU chemotherapy. Our study suggests a potential role for Statins as chemopreventive or therapeutic agents in a subgroup of colorectal cancer patients, those with or prone to develop, colorectal cancer with the CpG island methylator phenotype.

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SUPPLEMENTARY DATA

Cell culture. SW480, HT29, CACO2, RKO, SW48, DLD1, LOVO and HCT116 colon cancer cell lines were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland) with 4.5g/l glucose and with 580mg/l L-glutamine. This was supplemented with penicillin (50U/ml) and streptomycin (50μg/ml) and with 10% fetal calf serum (FCS) (Gibco, Paisley, Scotland) unless otherwise stated. All *in vitro* experiments were performed on cells growing exponentially.

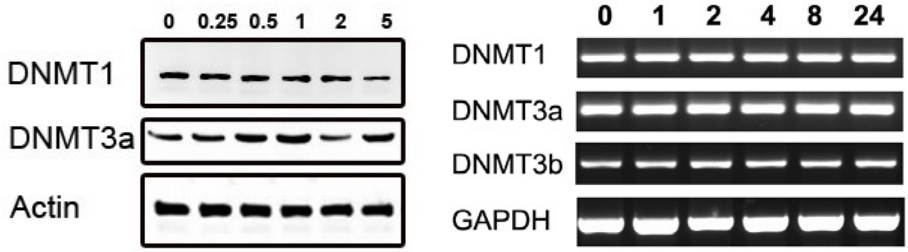
Immunoblotting. Lysates were processed as described previously (27). Primary antibodies to BMP2 (goat polyclonal), Villin (goat polyclonal), CAII (mouse monoclonal) and Actin (rabbit polyclonal) were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary (HRP)-conjugated goat anti-rabbit, rabbit anti-goat or goat anti-mouse antibody were from Dako (Glostrup, Denmark).

DNA Extraction and Bisulfite Modification. 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) according to the manufacturer's instructions.

Transfection and luciferase reporter assay. Cells were transiently transfected with either a pcDNA4/TO/BMPR2 plasmid or pcDNA4/TO control vector (Invitrogen, Breda, the Netherlands) in combination with the BRE-Luc vector and a CMV promoter-driven Renilla luciferase vector (Promega, Leiden, The Netherlands) using Lipofectamine Plus (Invitrogen, Breda, The Netherlands). After 48 hours after transfection luciferase activity was assayed using the Dual-Glo Luciferase Assay System (Promega, Leiden, The Netherlands) on a Luminometer (Berthold Technologies, Bad Wildbad, Germany). Each firefly luciferase value was corrected for its cotransfected Renilla Luciferase value.

Immunohistochemistry. Slides were processed as described previously (12). Slides were incubated with primary antibodies to Villin (1:100) and BMP2 (1:250) (both from Santa Cruz Biotechnology) and Ki-67 (1:200) (Dako) overnight at 4°C in PBS with 0.1% Triton and 1% bovine serum albumin.

RNA isolation and Real-Time RT-PCR. For the RNA isolation, frozen tissue from 54 CRC patients from archive of Gastroenterology and Hepatology Department of Leiden University Medical Center was used. The study was performed according to the instructions and guidelines of the Leiden University Medical Center Medical Ethics Committees. Total RNA was isolated using Trizol. cDNA was synthesized from 1 μg of total RNA using Random primers (Promega, Leiden, The Netherlands) and MMLV-reverse transcriptase (Invitrogen, Breda, The Netherlands). PCR was performed with primers available upon request using iCycler Thermal Cycler and iQ5 Multicolor Real Time PCR Detection System (Bio-Rad). *GAPDH* expression was used to normalize for variance.



Supplementary Figure:

A: Immunoblots for DNMT1 and DNMT3a of HCT116 cells treated with different concentration of Lovastatin for 24 hours. The protein expression was analyzed using the corresponding specific antibody. Actin served as a loading control

B, RT-PCR analysis of *DNMT1, DNMT3a and DNMT3b* expression in HCT116 cells treated at different time points with 2μM Lovastatin and corresponding *GAPDH* controls. Primer sequences are available upon request.

Supplementary table 1. Main genetic characteristics of the cell lines used in this study.

Cell line	MMR genes	B-RAF	K-RAS (codons 12 and 13)
HCT116	hMLH1/hMSH3 mutation	WT	Mutant
RKO	hMLH1 methylation	V600E	WT
SW48	hMLH1 methylation	V600E	WT

Supplementary table 2. BRAF mutation and BMP2 methylation status of patients in this study.

Patient	BRAF	BMP2 methylation
1	WT	+
2	WT	+
3	WT	+
4	WT	+
5	WT	+
6	V600E	+
7	WT	-
8	WT	-
9	WT	-
10	unknown	-
11	WT	-
12	unknown	-
13	WT	-
14	WT	-
15	WT	-
16	unknown	-
17	unknown	-

Supplementary table 2. (Continued)

Patient	BRAF	BMP2 methylation
18	WT	-
19	WT	-
20	unknown	-
21	V600E	-
22	WT	-
23	WT	-
24	unknown	-
25	unknown	-
26	unknown	-
27	unknown	+
28	unknown	-
29	unknown	-
30	V600E	-
31	unknown	-
32	WT	-
33	WT	-
34	WT	-
35	unknown	-
36	WT	-
37	V600E	+
38	WT	-
39	WT	-
40	WT	+
41	WT	-
42	WT	-
43	WT	-
45	V600E	+
46	WT	+
47	unknown	-
48	V600E	-
49	WT	+
50	WT	-
51	WT	+
52	WT	-
53	V600E	-
54	V600E	-
55	V600E	-