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Statins augment the chemosensitivity of colorectal cancer cells inducing epigenetic reprogramming and reducing colorectal cancer cell ‘stemness’ via the bone morphogenetic protein pathway

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ABSTRACT

Background Promoter hypermethylation is an important and potentially reversible mechanism of tumour suppressor gene silencing in cancer. Compounds that demethylate tumour 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). The authors have previously shown that statins act by activating tumour suppressive bone morphogenetic protein (BMP) signalling 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 Methylation-specific PCR, bisulphite sequencing, immunoblotting, reverse transcription PCR, quantitative PCR, fluorescence-activated cell sorting analysis, an in vitro DNA methyltransferase (DNMT) assay, and cell viability studies were performed 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 tumours. Treatment with lovastatin downregulates DNMT activity, leading to BMP2 promoter demethylation and to upregulation of expression of BMP2 as well as other genes methylated in CRC. Statins alter gene expression, indicating a shift from a stem-like state to a more differentiated state, thereby sensitising cells to the effects of 5-fluorouracil. In a xenograft mouse model, simvastatin treatment induces BMP2 expression, leading to differentiation and reduced proliferation of CRC cells.

Conclusions Statins act as DNMT inhibitors, demethylating the BMP2 promoter, activating BMP signalling, inducing differentiation of CRC cells, and reducing ‘stemness’. This study indicates that statins may be able to be used as differentiating agents in combined or adjuvant therapy in CRC with the CpG island methylator phenotype.

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 tumour 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),3 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 catalyse the addition of methyl groups to cytosine residues in DNA. The activity of DNMTs is elevated in CRC cells, and the inhibition of DNMT activity can strongly inhibit the formation of tumours in vivo. DNMT inhibitors have been intensively studied as promising new drugs for cancer therapy. 5-Azacytidine and decitabine have already entered clinical practice, but currently known DNMT inhibitors cause significant toxicity, as they become incorporated into RNA and interfere with protein translation. 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. Bone morphogenetic proteins (BMPs) have also been used for this purpose by intratumoural injection of recombinant protein in animal models. This methodology is not attractive for clinical application in humans, but small molecular compounds that activate the BMP pathway could be an attractive alternative strategy.

The BMP pathway plays an important role in intestinal epithelial homeostasis and CRC. BMP signalling promotes intestinal differentiation and inhibits stem cell activation. Germline mutations in BMPRIA and SMAD4 are the cause of familial juvenile polyposis syndrome, 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. This makes the study of compounds that specifically modify the BMP pathway even more relevant.

3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors, better known as statins, not only reduce serum cholesterol and decrease the incidence of cardiovascular and cerebrovascular events, but also reduce the risk of developing CRC. In vitro studies show that statins inhibit cellular proliferation and induce apoptosis in CRC cells and in animal models. A screen of 30,000 compounds for their ability to enhance BMP2 expression and activate the BMP pathway 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 SssI methyltransferase (New England Biolabs, Beverly, Massachusetts, USA) was used as a positive control for the methylated reaction.

**Bisulphite sequencing (BS)**

For BS, cell line-derived DNA was treated with sodium bisulphite and amplified by PCR. The primers of BMP2 for BS were Forward (5'-GATTTGTGTTTTAGGTTAGGAGAG-3') and reverse (5'-CCAAATACACATACAAACCAACC-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 the 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). For each sample, five to ten separate clones were sequenced on an ABI 377 or 3100 automated sequencer (Applied Biosystems, Foster City, California, USA) using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and the original primers of BMP2 for BS.

**In vitro DNMT assay**

HCT116 cells were treated with different concentrations of lovastatin or 5 μM 5-azacytidine (Sigma, St Louis, Missouri, USA) for 48 or 72 h. Cells were washed in ice-cold phosphate-buffered saline (PBS) and scraped into 200 μl ice-cold cell extract buffer (10 mM Hepes/KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulphonyl fluoride (PMSF). The cells were kept on ice for 10 min, vortex-mixed for 10 s, and centrifuged at 4°C at 14,000 rpm for 30 s. The supernatant was discarded, and the pellet was resuspended in 30 μl nuclear extraction buffer (20 mM Hepes/KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF), placed on ice for 20 min, and centrifuged at 4°C at 14,000 rpm for 2 min. The supernatant was saved as the nuclear
extract and used to measure total DNMT activity using the EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit (ITK Diagnostics BV (Uithoorn, The Netherlands)) according to the manufacturer’s instructions.

**Xenograft mouse model**

Eight female NMRI nu/nu mice were injected subcutaneously in the flank with $1 \times 10^5$ HCT116 cells in Matrigel (BD Bioscience, 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 3 weeks, initiated when the tumour volume reached 100–200 mm$^3$. After the mice had been killed, the tumours were harvested and either frozen in liquid nitrogen and later homogenised in Trizol (Invitrogen) or embedded into paraffin blocks. We chose simvastatin because it was the second most potent statin in our in vitro experiments, and because it is licensed for use in humans in the Netherlands whereas lovastatin is not.

**Chemosensitivity assay**

Cells were plated in 96-well plates and treated with low-dose lovastatin (Sigma) (0.2 μM) or vehicle control in Dulbecco’s modified Eagle’s medium (DMEM) with 0.5% fetal calf serum (FCS). This low concentration of FCS allowed 5 days of exposure without the cells reaching confluence. Most studies use doses between 10 and 30 μM in vitro. After 5 days of statin exposure, cells were incubated in fresh DMEM with 10% FCS for 24 h. Subsequently, cells were treated with 5-FU (Sigma) (1–50 μM) for 48 h in DMEM with 0.5% FCS. After treatment, MTT solution was added (5-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; final concentration 0.5 mg/ml, stock solution 5 mg/ml MTT in PBS), for 3 h. The medium was discarded and cells were lysed in acidic propan-2-ol. Absorbance was measured at 550–560 nm.

**Fluorescence-activated cell sorting (FACS) analysis**

Cells were treated with 0.2 μM lovastatin or vehicle control for 72 h, and then harvested, washed and stained in PBS buffer (PBS containing 1% bovine serum albumin) on ice with anti-CD166-PE (Becton Dickinson, Breda, The Netherlands). Cells were washed again, and CD166 cell surface levels were analysed by flow cytometry using a FACS Calibur (BD Biosciences) and FlowJo Software (Treestar, Ashland, Oregon, USA).

**Statistical analysis**

Statistical analysis was performed using the two-tailed Student t test, and p<0.05 was considered significant. Data are shown as mean±SEM.

**RESULTS**

**BMP2 expression is often impaired due to promoter hypermethylation in CRC**

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 three cell lines are known to exhibit the CIMP phenotype, with hypermethylation of the promoters of several tumour suppressor genes. The main genetic characteristics of the cell lines used in this study are presented in online supplementary table 1.

We performed MSP for the BMP2 promoter region starting 214 bp in front of exon 1 (figure 1B). This region contains a CpG island and has been shown to be methylated in gastric cancers. 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 (figure 1C). We verified the results of MSP by direct BS (figure 1D). 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 CpGps 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 BS on SW48 and RKO cell lines probably because of a mixture of methylated and unmethylated signals. Therefore we performed BS 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 it is 72%.

To determine whether BMP2 is methylated in a subgroup of patients with CRC, we performed MSP on bisulphate-modified DNA from 55 CRC tumours. Thirteen out of 55 tumours (25%) showed a methylated signal on MSP, confirming the relevance of our in vitro findings for patients (figure 1F). Further characterisation of these tumours by BRAF V600E mutation analysis was informative in 40 tumours and revealed nine BRAF V600E mutations. Three of the 15 cancers with BMP2 methylation had BRAF mutations, one was uninformative for BRAF status and nine were wild-type (online supplementary table 2).

Lovastatin treatment leads to demethylation of the BMP2 promoter region, upregulation of BMP2 expression, and 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 2 μM lovastatin for 48 h and 72 h 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 BS of multiple independent clones from HCT116 cells and HCT116 cells treated for 72 h with lovastatin. The BS results show that BMP2 promoter methylation decreases from 97% to 40% after treatment with 2 μM lovastatin for 72 h (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 2 μM lovastatin for 3, 5 and 7 days. As shown in figure 2C, lovastatin treatment, as well as treatment with a strong demethylating agent, 5-deoxyazacytidine-C, upregulates the mRNA level of BMP2 in a time-dependent manner.

We performed MSP analysis of three other genes methylated in HCT116 cells before and after treatment with 2 μM lovastatin for 72 h. 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 three genes belong to a panel of genes hypermethylated in HCT116 cells and in CRCs.

Lovastatin inhibits DNMT activity in vitro

To further investigate the mechanism by which lovastatin leads to promoter demethylation, we performed an in vitro DNMT assay. The DNMT assay shows that treatment with different concentrations of lovastatin leads to a dose-dependent
downregulation of DNMT activity in HCT116 cells. Remark-
ably, even low concentrations of lovastatin (0.25 and 0.5 µM),
approximating those found in the serum of patients taking
standard doses of statins, downregulate DNMT activity,
implying a speci
fic effect on DNMT and not a consequence of
a general toxicity (figure 2E). The effect seems to be through
inhibition of DNMT function, since protein and mRNA levels of
DNMTs are not in
fluenced by lovastatin treatment (online
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
tumour suppressors such as BMP2.

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 investi-
gated whether lovastatin induces differentiation in CRC cells.
We performed immunoblotting on HCT116 cells treated with
lovastatin for markers of absorptive cell differentiation and
quantitative RT-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). Lovas-
statin 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 signalling. 31 We see downregulation of the Notch
pathway target,
HES1, and upregulation of KLF4, a goblet cell-
specific differentiation factor in the colon33 regulated by Notch
signaling33 in HCT116 cells treated with lovastatin. An impor-
tant Wnt target and oncogene c-Myc and gene inducing
stemness is also downregulated by lovastatin treatment (figure 3A), as well as the expression of two markers of cancer stem cells, CD166 and EpCAM (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 the HCT116 cell line and was therefore not used in this study.

Lovastatin induces differentiation in a xenograft model

Our previous work showed that simvastatin inhibited the growth of HCT116 xenografts in mice, but the effect of oral administration of statins on the differentiation of cells within the xenografts had not yet been studied. We performed immunohistochemical 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 (figure 4A). Simvastatin treatment also results in downregulation of c-Myc and HES1 mRNA in xenografts (figure 4C).
Activation of BMP signalling leads to the differentiation of CRC cells

To test the hypothesis that statin-induced differentiation in CRC cells is due to the activation of BMP signalling, we transfected HCT116 cells with BMPR2 and activated BMP signalling sixfold (figure 5A). This results in upregulation of Villin and Mucin2 expression, downregulation of Notch pathway activity, and downregulation of expression of cancer stem cells markers, CD166 and EpCAM, as seen with statin treatment (figure 5B, C). These data suggest that activation of the BMP pathway induces differentiation and reduces 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 signalling.

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 with untreated cells or ethanol-treated control cells (figure 6B). After the removal of lovastatin, cells were treated with different concentrations of 5-FU for 48 h. Lovastatin treatment significantly increases the sensitivity of cancer cells to 5-FU (figure 6C). Even at low concentrations of 5-FU that do not inhibit the viability of HCT116 cells when used alone, lovastatin pretreatment sensitises CRC cells and...
Figure 4  (A) Immunohistochemistry for Ki-67, villin, BMP2 and periodic acid—Schiff (PAS) staining of goblet cells of HCT116 xenografts from mice treated with simvastatin and from controls. Original magnification: 20×. (B) The percentage of Ki-67-positive cells relative to all cells per 20× field (for every xenograft three images were scored). Error bars represent SEM (n=8). ***p<0.001. (C) Quantitative reverse transcription PCR analysis of c-Myc and HES1 in HCT116 xenografts from mice treated with simvastatin and from controls. *p<0.05. Error bars represent SEM (n=8). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
leads to significant inhibition of cell viability. Importantly, concentrations of 5-FU of 1–10 μM are clinically relevant, as they approximate serum (6–12 μM)39 and tissue (2–5 μM)40 levels in patients.

**DISCUSSION**

DNA hypermethylation of the promoter region of tumour suppressor genes occurs often in cancer and results in the transcriptional silencing and loss of function of critical tumour 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 have often been used for methylation studies42 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 analysed BRAF V600E mutation status in the primary cancer specimens in which we analysed BMP2 methylation. BRAF V600E mutations are often used as a marker of CIMP status, but may underestimate the prevalence of CIMP by ~50%.45 Of the cancers we tested, 25% show concomitant BMP2 methylation and BRAF mutation. Although in cell lines BMP2 promoter methylation is seen exclusively in those with the CIMP phenotype, analysis of BRAF only as a marker of CIMP is insufficient to confirm or refute this in tumour specimens.

We report that statins exhibit demethylating properties. Inhibition of DNMTs can be seen even at low statin concentrations (0.25 μM), which are comparable to the serum levels of ~0.1 μM measured in patients treated with standard doses for...
hypercholesterolaemia 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 with most known DNMT inhibitors, statins inhibit DNMTs and induce DNA demethylation at non-toxic doses. Our experiments in xenografts investigated whether the demethylation 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 and leads to differentiation and to down-regulation of proliferation of colon cancer cells in a xenograft mouse model.

Lovastatin treatment induces DNA demethylation and the re-activation of BMP2 gene expression, which is silenced by hypermethylation in CRC cells. Importantly, we find demethylation of two other methylated 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 that are not influenced by statin treatment—for example, histone modification—mediate methylation more prominently in this gene.

From these studies, it is unclear how statins inhibit DNMTs. We show that they have little or no influence on expression levels of DNMTs, and further studies are needed to determine the mechanism by which they inhibit DNMTs. It is also unclear from these studies what the influence of statins is on global methylation levels. Although CIMP CRCs exhibit widespread promoter hypermethylation, global levels of DNA methylation are often found to be lower in CRC. 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 and FPR2-γ agonists, and with multiple interventions aimed at differentiating CRC stem cells and thus improving their chemosensitivity, including the use of BMPs. Delivery of BMP to tumours is highly challenging and at present has only been successfully performed by intratumoural 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, and we have shown that this also holds true for CRC cells. Although in bone cells the mechanism appears to be direct activation of the BMP2 promoter, the promoter is often silenced in CRC, as we show here, but this can be reversed by statin therapy.

The CIMP is found not only in CRCs but also in colonic polyps and even in the normal colorectal mucosa in patients with hyperplastic polyposis. It has been observed that cigarette smoking is strongly associated with CIMP CRC, with a dose–response relationship with respect to the amount smoked. 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 patients with CRC. 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, sensitising cells to 5-FU chemotherapy. Our study suggests a potential role for statins as chemopreventive or therapeutic agents in a subgroup of patients with CRC: those with, or prone to develop, CRC with the CIMP.

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