CHAPTER SEVEN

STATINS INCREASE CELL MEMBRANE LEVELS OF BMP RECEPTORS AND CAUSE A SHIFT FROM NON-CANONICAL TO CANONICAL BMP SIGNALING IN COLON CANCER

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ABSTRACT

Background: Statin use is associated with a reduced risk of colorectal cancer (CRC). This effect of Statins is mediated through the Bone Morphogenetic Protein (BMP) tumor suppressor pathway. BMP receptor (BMPR) expression is commonly altered in CRC. BMPR levels are influenced by endocytosis, occurring either via Caveosomes or Clathrin-coated-pits. The manner of endocytosis determines whether BMP-signaling is canonical (SMAD-dependent) or non-canonical (MAP-Kinase-dependent). Statins have been shown to alter membrane-receptor endocytosis and target Caveolin in endothelial cells. We therefore hypothesized that Statins may act by altering BMPR endocytosis, expression and localization in CRC, increasing canonical BMP-signaling.

Methods: CRC cell lines were treated with Lovastatin and analyzed. Membrane BMPR expression was assessed by flowcytometry and confocal microscopy; total cellular BMPR protein levels by immunoblotting. Caveolin and Clathrin expression was assessed by immunoblotting. Canonical and non-canonical BMP-signaling was investigated using the BRE-luc reporter and immunoblotting for pp42/44MAPK. Apoptosis was measured using flowcytometry for Annexin-5.

Results: Statin treatment reduces non-canonical BMP-signaling by reducing Caveolin-1 expression. This leads to increased membrane BMPR localization and a shift towards Clathrin-dependent BMPR endocytosis and canonical BMP signaling, leading to apoptosis induction.

Conclusion: Modulation of cancer cell signaling at the receptor endocytosis level represents a promising new therapeutic approach.

INTRODUCTION

Formation of colorectal carcinoma (CRC) is a process that takes approximately 10 years [1]. The development from normal epithelial tissue to carcinoma is preceded by the formation of a premalignant adenomatous polyp, which malignantly transforms over time. This adenoma to carcinoma sequence is well described [2] and is driven by the sequential accumulation of DNA mutations that allow cells to escape the multiple layers of molecular control that instruct normal cell behavior within the intestinal epithelium. Downregulation of tumor suppressor signaling pathways, like the Bone Morphogenetic Protein (BMP) pathway is one of these events [3].

BMPs are members of the Transforming Growth Factor-β family. They act as morphogens playing important roles both in development and in maintenance of adult tissues by influencing cell proliferation, differentiation, chemotaxis, and apoptosis [4, 5]. The activity of BMP signaling is tightly regulated at several molecular levels. Defects in one of the BMP pathway components can lead to developmental disorders or cancer [6]. BMP ligands signal through two different types of transmembrane serine/threonine kinase receptors. Together with extracellular modulators, co-receptors and cytosolic receptor-associated proteins, they determine signaling specificity [7].

Several studies have shown that Statins (3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors) can induce canonical BMP signaling [8] and epidemiological studies and meta-analyses have shown an association between Statin usage and a reduced incidence of colorectal cancer [9]. We have previously shown that Statins induce apoptosis of CRC cells both *in vitro* and *in vivo* in mice and that this occurs via the induction of BMP2 and activation of canonical BMP signaling. We have also shown that this activation of BMP signaling by Statins induces epigenetic changes and reduces the 'stemness' of CRC cells, sensitizing cells to standard chemotherapy [10]. However, we have also found that many CRCs have very low expression of BMPR2 [11] and lack other type 2 receptors [12]. How Statins can activate BMP signaling in cells with very low levels of type 2 receptors is unclear.

BMP ligands bind to BMP receptors which are comprised of type 1 and type 2 transmembrane receptors. Type 1 receptors include the BMP receptor 1a (BMPR1a/ALK3), BMP receptor 1b (BMPR1b/ALK6) and the Activin receptor 1a (ActR1a/ALK2). Type 2 receptors include BMP receptor 2 (BMPR2) and Activin receptor 2a (ActR2a) and 2b (ActR2b) [13]. In this study we have focused on the BMP specific receptors 1a, 1b and 2. To activate specific signaling, both type 1 and type 2 receptors are required. Ligands bind to receptors in one of two ways, either binding first to BMPR1 which then recruits BMPR2 forming a BMP-induced signaling-complex (BISC), or ligands can bind preformed complexes (PFC) of type 1 and 2 receptors. Whether ligands bind BISC or PFC further determines how the ligand/receptor complex is internalized and determines which of two signal transduction routes ligand ultimately activates. Ligand binding to PFC leads to internalization of the ligand/re-

ceptor complex via Clathrin coated pits and initiation of canonical BMP signal transduction; after ligand binding, BMPR2 phosphorylates BMPR1 to activate its kinase function [14]. The activated BMPR1 subsequently phosphorylates the BMP specific intracellular proteins SMAD1, SMAD5 and SMAD8 (further mentioned as SMAD1,5,8). After phosphorylation these receptor specific SMADs form a complex with SMAD4. This complex translocates to the nucleus regulating expression of canonical BMP target genes [15]. Alternatively, when ligand binding induces receptor complex formation (BISC) the complex is internalized via Caveosomes [16] and activated BMPR1 phosphorylates upstream members of the Mitogen Activated Protein Kinase (MAPK) signal transduction pathways thus activating non-canonical BMP signaling [17].

TGF-β signaling can be upregulated by increases in the cell surface expression of TGF-β receptors. This occurs to cells upon glucose treatment without an overall change in total cellular protein levels of the receptors, an effect responsible for cell hypertrophy in response to glucose [15]. Altered membrane expression of BMP receptors could similarly be responsible for the observed increased BMP signaling and could conceivably even influence whether ligand binding leads to canonical or non-canonical BMP signaling. The balance between canonical and non-canonical signaling might in turn determine whether BMP signaling is tumor suppressive (proapoptotic) or tumor promoting (pro-proliferative).

In this study we investigated the effect of Statin treatment on the expression and localization of the BMP receptors, changes in Caveolin and Clathrin expression and the balance between canonical and non-canonical BMP signaling in colorectal cancer cells. Our results show that Statin treatment increases cell surface expression of the BMPRs, induces a shift toward the Clathrin dependent endocytosis pathway and thus to canonical BMP signaling in colorectal cancer cells. These Statin-induced Clathrin-dependent changes lead to activation of apoptosis in cancer cells.

MATERIALS AND METHODS

Cell Culture

HCT116, RKO and SW837 colon cancer cell lines were obtained from ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL, Breda, the Netherlands) containing 4,5mg/L glucose supplemented with 10% fetal calf serum (Gibco-BRL, Breda, the Netherlands), penicillin (50 U/mL) and streptomycin (50 µg/mL) (Life technologies, Invitrogen, Breda, the Netherlands). Cells were grown in monolayers in a humidified atmosphere containing 5% CO_2. Lovastatin was obtained from Sigma Aldrich. All 3 cell lines express SMAD4. HCT116 cells are microsatellite instable (MSI) as are RKO[18]. SW837 colon cancer cells are microsatellite stable (MSS).

Immunofluorescence and flow cytometry

Anti-BMPR1a (BAF820), Anti-BMPR1b (BAF505) and Anti-BMPR2 (BAF811) were obtained from R&D Systems (Abingdon, UK). Anti-phospho-SMAD 1/5/8 was obtained from Cell Signaling Technology (Leiden, the Netherlands). Steptavidin-488 and 594 and anti-rabbit-AlexaFluor 488 were obtained from Invitrogen (Carlsbad, CA). For immunofluorescence, cells were allowed to adhere to poly-l-lysine (Sigma Aldrich, Zwijndrecht, Netherlands) coated cover slips, treated with Lovastatin for 24 hours, fixed in 4% paraformaldehyde, stained in permeabilisation buffer (PBS containing 0.05% Triton X-100) and embedded in SlowFade Gold (Invitrogen). Images were obtained on a Leica TCS SP2 confocal system equipped with 488 nm argon and 543 HeNe lasers (Leica, Mannheim, Germany) and processed using ImageJ software.

For flow cytometry of surface receptors, cells were treated with Lovastatin for 24, 48 or 72 hours. Cells were trypsinized on ice, washed in PBS and subsequently stained for the receptors in PBS containing 0.5% BSA and washed again. All samples were analyzed using a FACS-Calibur (BD Bioscience) and FlowJo Software (Treestar, Ashland, OR, USA). SW837 cells which have been shown to express all components of the BMP pathway[19], were used as a positive control.

Immunoblotting

Cells at 60% to 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 200 μL of cell lysis buffer (Cell Signaling, Leiden, the Netherlands) containing a protease inhibitor cocktail (MP Biochemicals, Illkirch, France). Protein concentration was measured using the RC DC protein assay kit (Biorad, Hercules, CA) according to the manufacturer's instructions. Samples were diluted in 3 sample buffer (125 mmol/L Tris/HCl, pH 6.8; 4% sodium dodecyl sulphate (SDS); 2% β-mercaptoethanol; 20% glycerol, 1 mg bromophenol blue), sonicated and then heated at 95° for 5 minutes. 50μg of protein from each sample was loaded onto SDS-PAGE and blotted onto PVDF membrane (Millipore, Bedford, MA). The blots were blocked by air drying 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). Primary antibodies to BMPR1a, BMPR1b, BMPR2 and SMAD4 were from Santa Cruz (Heidelberg, Germany). Goat polyclonal antibodies to pSMAD1,5,8, pCaveolin-1 and Clathrin Heavy chain were from Cell Signaling Technology (Leiden, the Netherlands). Blots were then washed 3 × 10 minutes in TBST and incubated for 1 hour at room temperature in 1:1000 horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-goat, or goat anti-mouse secondary antibody (Dako, Glostrup, Denmark) in block buffer. After a final 3- × 10-minute wash in TBST, blots were incubated Lumi-light (Roche, Woerden, the Netherlands), and chemiluminescence was detected using a VersaDoc imaging system (Bio-Rad).

RNA interference

Cells were transfected with either negative control small interfering RNA (siRNA) or with siRNA against Clathrin or Caveolin-1 (Dharmacon, ID 1213 resp. 857; Dharmacon Inc., Chicago (IL), USA) using Dharmafect transfection reagent according to the manufacturer's instructions (Dharmacon, Etten-Leur, The Netherlands).

Luciferase Reporter Assay

After treatment with siRNA for Clathrin or Caveolin-1 for 24 hours, cells were transiently transfected with the BRE-Luc vector reporter in combination with a cytomegalovirus promoter-driven Renilla luciferase vector (Promega, Madison (WI), USA) using Lipofectamine Plus (Invitrogen, Breda, The Netherlands) according to manufacturer's instructions. After the second transfection, cells were treated for 24 hours with either a low dose Lovastatin (2μM) or vehicle control. After treatment, 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 Renilla luciferase standard to correct for transfection efficiency or dilution effects.

Apoptosis Assay

Cells were harvested and stained for Annexin V and with Propidium Iodide using the FITC-Annexin V Apoptosis Detection Kit 1 (556547; BD Pharmingen, Breda, the Netherlands) according to the manufacturer's protocol. Cells were analyzed using a FACS-Calibur (BD Bioscience) and FlowJo Software (Treestar, Ashland, OR, USA).

Statistical Analysis

Statistical analysis was performed using 2-tailed Student's t test, and $p < .05$ was considered statistically significant. Data are shown as mean ± SEM. All experiments were done with a minimum of 3 independent experiments. Asterisks indicate a significant difference between two groups. ($^{*}P < 0{,}05$; $^{**}P < 0{,}005$;*** $P < 0{,}0001$)

RESULTS

Statin treatment increases cell surface expression of BMP Receptors

The concentration of 2μM Lovastatin was chosen as being achievable with high dose oral Statins in humans *in vivo* [17]. Given the fact that Statin treatment increases BMP signaling, [20] even in cells expressing low basal total cellular levels of BMPR, we hypothesized that Statins affect the expression of BMP receptors. We first examined the effects of treatment

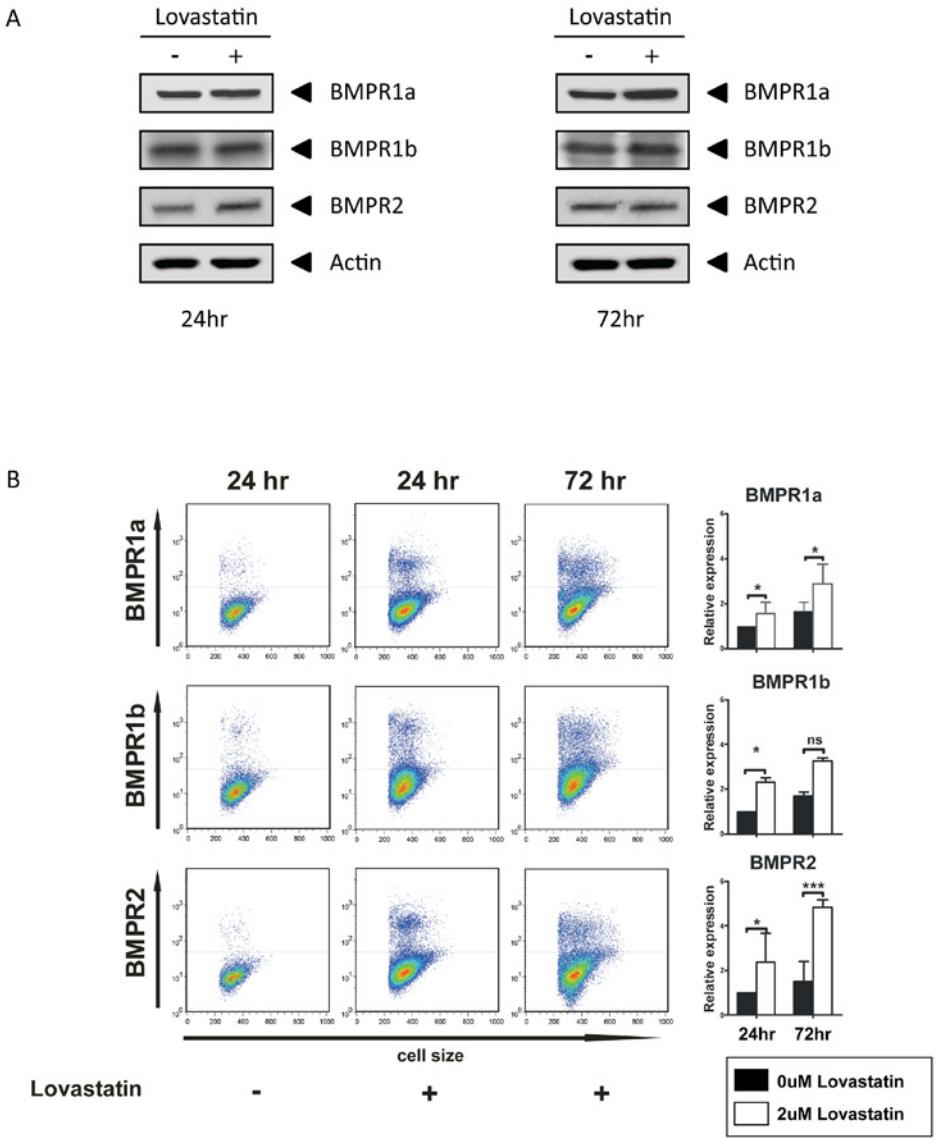

Figure 1.

A: Representative immunoblots for BMPR1a, BMPR1b and BMPR2 of HCT116 cells treated with 0μM or 2μM of Lovastatin for 24hr and 72hr. The protein expression was analyzed using the corresponding specific antibody. Actin served as a loading control. Total cellular BMP receptor expression did not change significantly with Statin treatment, although there was a tendency to increased levels of BMPR1a after 72 hours. B: FACS plots for membranous BMPR1a, BMPR1b and BMPR2 of HCT116 cells treated with 0μM or 2μM of Lovastatin for 24hr and 72hr. The receptor expression was analyzed by flowcytometry using antibodies directed against the extracellular domain of the individual receptors. Quantification the FACS analysis (right panel) showed a significant increase in membranous expression of all three BMP receptors with Lovastatin treatment.

on total cellular protein levels of BMPR1A, BMPR1B and BMPR2. HCT116 colon cancer cells were treated with 2μM Lovastatin or vehicle control for 24, or 72 hours. Lovastatin treatment leads to minimal increases in the total protein expression of BMPR1a, but no significant changes in the expression of BMPR1b or BMPR2 (Figure 1A).

Since the changes in total protein expression are minor and insufficient to explain the increase in BMP signaling seen with Statin treatment, we went on to investigate whether Statin treatment might change surface expression levels of BMPRs in HCT116 cells. Assessment of cell surface levels of BMP receptors was performed by flow cytometric analysis. We observed that Statin treatment indeed increased cell surface expression of all BMPRs, with the strongest effect observed for BMPR2 (Fig. 1B). SW837 cells with known high expression of all components of the BMP pathway were used as a positive control (supplementary figure 1) [21]. We repeated these experiments in the colon cancer cell line RKO. Here Statin treatment also increased BMPR1A expression at the cell surface two-fold but did not significantly increase cell surface BMPR2 expression. This is likely to be due to the fact that basal cell surface BMPR2 expression is already high in these cells in contrast to HCT116 (supplementary figure 2).

Given the much smaller effects on total BMPR protein expression, the observed increases in membrane BMPR expression are likely to be due to altered subcellular distribution of the receptors. To confirm this the relative expression of the BMPRs at the cell surface compared to intracellular BMPR protein was assessed by fluorescent staining of the BMP receptors and creating z-stacks using confocal microscopy (Fig. 2A). The ratio of membrane associated versus cytosolic BMP receptor expression increased significantly for all BMP receptors (Fig. 2B) upon Statin treatment. These data support the hypothesis that Statin treatment of colorectal cancer cells primarily induces changes in BMPR cellular distribution rather than total protein levels.

Statins influence endocytic pathways

Receptor density at the cell surface is largely controlled by endocytosis through Clathrin and Caveolin-dependent pathways [22]. BMP receptors undergo constant endocytosis and either enter the cell as preformed complexes (PFC) via Clathrin-coated vesicles or as BMP-induced signaling complexes (BISC) via Caveolin-dependent internalization [23]. Changes in Clathrin and/or Caveolin levels might explain the altered BMPR distribution observed after Statin treatment. Therefore, we evaluated protein levels of Caveolin-1 and Clathrin by immunoblotting. Statin treatment results in a decrease in Caveolin-1 and to a lesser extent an increase in Clathrin expression (Figure 3A), suggesting that Statins lead to a shift in balance between these two mechanisms of BMPR endocytosis with predominantly a decrease in receptor endocytosis via Caveosomes resulting in increased membrane BMPR levels.

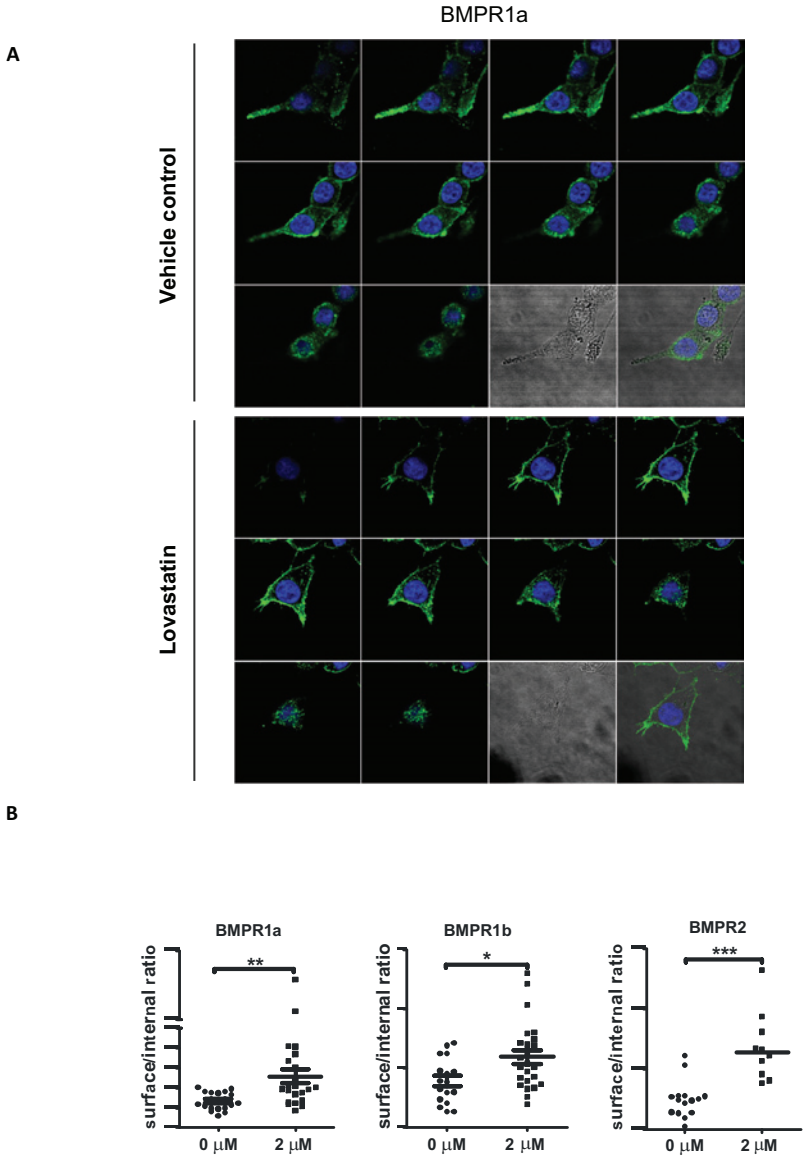

Figure 2.

A: Confocal microscopy of HCT116 cells treated with 0μM (-) or 2μM (+) Lovastatin and stained with the BMPR1a antibody. Confocal pictures show representative pictures of BMPR1a expression and cellular localization in HCT116 cells. Statin treatment increases cell surface expression of BMPR1a. The confocal pictures for BMPR1b and BMPR2 showed similar patterns and were analyzed in exactly the same manner. the last two pictures show a phase contrast and a z-stack merged picture of the cell. B: Quantification of the cell surface expression of BMPR1a, BMPR1b and BMPR2 relative to their cytoplasmic expression in HCT116 cells treated with 0 or 2μM Lovastatin. Images were obtained on a Leica TCS SP2 confocal system and analyzed with ImageJ software. The ratio of membrane associated versus cytosolic BMP receptor expression increased significantly after Statin treatment for all BMP receptors.

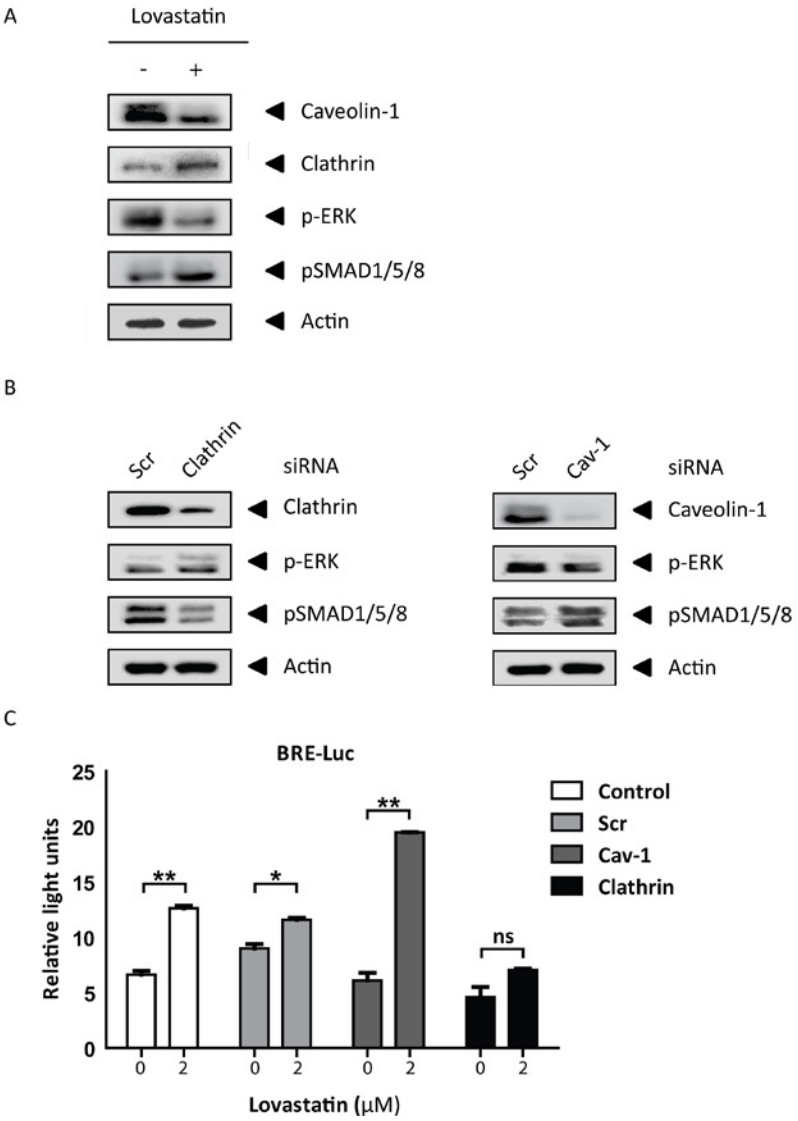

Figure 3.

A: Immunoblots for Caveolin-1, Clathrin, pp42/44 MAPK and pSMAD1,5,8 of HCT116 cells treated with 2 μM Lovastatin for 24hr or vehicle control. The protein expression was analyzed using the corresponding specific antibody. Actin served as a loading control. Statin treatment decreases the level of Caveolin-1 and increases the level of Clathrin. This is associated with a decrease in pp42/44 MAPK as a downstream measurement for Caveolin-1 dependent non-canonical BMP signaling, and an increase in the phosphorylation of the SMAD1,5,8-complex which in turn is a measurement for Clathrin dependent canonical BMP signaling. B: Immunoblots for Clathrin, Caveolin-1, pp42/44 MAPK and pSMAD1,5,8 of HCT116 cells transiently transfected with siRNA against Clathrin or Caveolin-1. The protein expression was analyzed using the corresponding specific antibody. Actin served as a loading control. Clathrin siRNA successfully knocked down Clathrin and results in a slight increase in the level of pp42/44 MAPK and a strong decrease in SMAD1,5,8 phosphoryla-

tion. Caveolin-1 siRNA successfully knocked down Caveolin-1 resulting in a decrease in pp42/44 MAPK and an increase in pSMAD1,5,8. Thus siRNA mediated knockdown of Caveolin-1 mimics the effects observed after treatment with Lovastatin while siRNA mediated knockdown of Clathrin leads to opposite effect of Lovastatin. C: HCT116 cells were transiently transfected with either the BRE-Luc BMP reporter alone, or together with scrambled siRNA, siRNA against Caveolin-1 or siRNA against Clathrin. Dual Luciferase assays were performed 24hr after transfection. Data were normalized to Renilla luciferase activity. Caveolin-1 knockdown alone has little effect on BRE-luc activity but together with Statin treatment there is an enhanced increase in BRE-luc activity compared to Statin treatment alone. siRNA against Clathrin slightly reduces BRE-Luc activity and almost completely abolishes the effect of Statins on BMP signaling. Statins no longer increase BRE-Luc activity after Clathrin knockdown.

Statins alter BMP signal transduction through effects on endocytic pathways

In the same experiment we assessed the influence of Statin treatment on readouts of both canonical BMP signal transduction, pSMAD1,5,8 levels, and non-canonical BMP signal transduction, pp42/44 MAPK levels. Statin treatment leads to increases in pSMAD1,5,8 and decreased levels of pp42/44 MAPK (Figure 3A).

To further assess whether the observed changes in Caveolin-1 and Clathrin expression could underlie the effects of Statins on BMP signaling we assessed whether knockdown of Caveolin-1 or Clathrin mimicked the effects of Statins on BMP signaling. To do this we transfected cells with siRNA against either Caveolin-1 or Clathrin and assessed the same readouts of BMP signal transduction, pSMAD1,5,8, pp42/44 MAPK, by immunoblotting. Caveolin-1 and Clathrin protein expression was efficiently knocked down by their respective siRNAs. Caveolin-1 knockdown leads to increases in pSMAD1,5,8 and a decrease in pp42/44 MAPK mimicking the effect seen with Statins. Clathrin knockdown leads to the opposite with reduced pSMAD1,5,8 and increased pp42/44 MAPK (Figure 3B).

In further experiments we assessed the effect of both Caveolin-1 and Clathrin knockdown with siRNA and simultaneous Statin treatment on BRE-luc activation as a readout of canonical BMP pathway signal transduction. Caveolin knockdown enhances the effect of Statin treatment. However, Caveolin knockdown alone did not lead to increased BRE-luc activity while Statin treatment does, perhaps reflecting the fact that Statins simultaneously enhance Clathrin levels. While Clathrin knockdown alone leads to a non-significant reduction in BRE-Luc activity, it completely abolishes the effect of Statins on BRE-Luc activity. In other words, Statins are no longer able to induce canonical BMP-signaling after Clathrin knockdown (Figure 3C). This confirms the importance of Clathrin-mediated endocytosis for the ability of Statins to induce canonical BMP signaling. Together these data suggest that Statins alter BMP signaling through a combination of a reduction in Caveolin-dependent BMPR endocytosis and an increase in Clathrin-dependent BMPR endocytosis.

As further confirmation of the effect of Statins on canonical BMP signaling we performed fluorescent immunocytochemistry for pSMAD1,5,8 in HCT116 cells treated with Statins, BMP2 or Statins and the BMP inhibitor Noggin. Statins induce activation and nuclear

translocation of pSMAD1,5,8, similarly to BMP2 (Figure 4). This effect of Statins is abolished when cells are co-treated with Noggin.

Statin induced apoptosis is Clathrin dependent

We have previously shown that treatment of colon cancer cells with low-dose Statin leads to apoptosis through induction of the BMP signaling pathway [24]. Here we have so far presented evidence that the effects of Statins on BMP signaling can be explained by their effects on endocytic pathways. However, we have not as yet confirmed whether these effects on endocytic pathways are sufficient to explain the biological effects of Statins in inducing apoptosis. To investigate whether Statin-induced apoptosis is dependent on endocytosis pathways, cells were transiently transfected with control siRNA or siRNA against either Clathrin or Caveolin-1. Lovastatin treatment with control siRNA leads to a significant induction of apoptosis as indicated by Annexin V (FITC) expression measured by flow cytometry (Figure 5). Treatment with Caveolin-1 siRNA alone leads to an increase in apoptosis and when combined with Statin treatment even higher levels of apoptosis are seen. Treatment with Clathrin siRNA alone also leads to a significant induction in apoptosis but

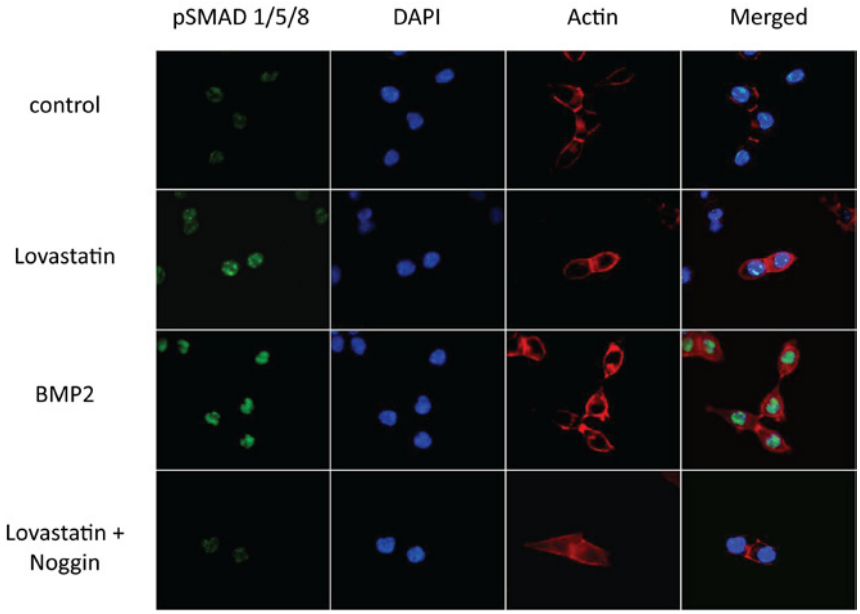

Figure 4. Fluorescent immunocytochemistry to show the nuclear localization of pSMAD1,5,8 as a measurement of canonical BMP signaling. HCT116 cells were either left untreated or treated with 2 μM Lovastatin, BMP2 or combination of Lovastatin and Noggin for 24hr, and the nuclear localization of phospho-SMAD1,5,8 (pSMAD1,5,8) was visualized (green). Dapi was used to stain the nuclei (blue) and Actin to stain the cytoskeleton (red). The "merged" panel shows an overlay of all three stainings. Lovastatin treatment leads to increased nuclear pSMAD1,5,8. This increase is abolished when cells are co-treated with Noggin. BMP2 treatment was used as positive control for activation of canonical BMP signaling.

it abolishes the increase in apoptosis seen with Statin treatment. These data suggest that the influence of Statins on endocytic pathways explain the observed induction of CRC cell apoptosis upon Statin treatment.

Figure 5

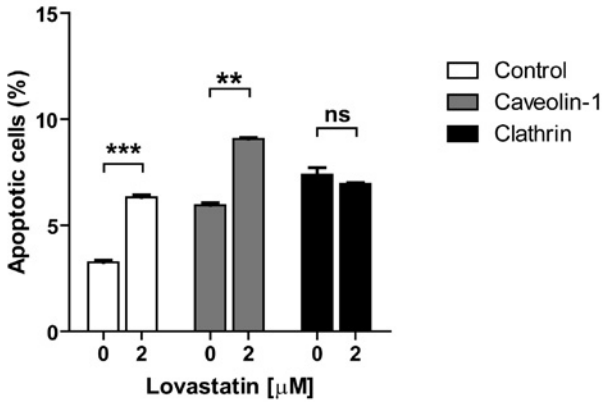

Figure 5. Apoptosis induced by Statin treatment is Clathrin dependent. Annexin V binding at the cell surface was studied by flow cytometry. HCT116 colon cancer cells were transfected with control siRNA, siRNA against Caveolin-1 or against Clathrin. Subsequently cells were treated with 2μM Lovastatin for 24 hours. All cells were harvested and labeled with Annexin V-FITC conjugated (BD Pharmingen). Immediately before cell acquisition cells were stained with Propidium Iodide allowing differentiation between necrotic and apoptotic cells. Reported values represent three independent experiments. (*$P < 0,05$; **$P < 0,005$; ***$P < 0,0001$)

DISCUSSION

Induction of apoptosis in colorectal cancer cells by Statins (e.g. Lovastatin, Simvastatin) has been described many times and we have shown previously that this effect is dependent on the induction of canonical BMP signaling [7]. We have chosen to use Lovastatin for our experiments as being the most potent inducer of apoptosis *in vitro*. How Statins influence BMP signaling is still not fully understood. In this paper we provide evidence that this effect is at least in part mediated through a change in receptor cycling leading to an increase in BMP receptor levels at the cell surface and a shift towards canonical BMP signaling.

BMPs, as a part of the transforming growth factor beta (TGF-β) superfamily, play an important role in epithelial cell differentiation and normal intestinal homeostasis. In previous work we have shown that loss of canonical BMP signaling correlates with the progression of adenoma to carcinoma, a well-established sequence of events [8]. For TGF-β signaling it is known that its role in carcinogenesis is dual [24]. Initially it functions as a tumor suppressor by potent growth inhibition in epithelial tissues. However at later stages of tumorigenesis it plays an important protumorigenic role through stimulating invasion and metastasis.

Whether this is also the case for BMP signaling is not known. Here we show that Statin treatment leads to a shift from pro-proliferative non-canonical signaling to proapoptotic canonical BMP signaling, which may explain the beneficial effects of Statins in colorectal cancer.

There are several potential explanations as to how Statins lead to this shift in the balance between canonical and non-canonical signaling. Statins reduce Caveolin-1 levels and an increase in Clathrin levels. Caveolin-1 is essential for Caveosome mediated internalization of receptor complexes leading to the activation of non-canonical BMP signaling, while Clathrin-mediated receptor-complex internalization leads to canonical signaling. Several groups have recently published data providing evidence that SMAD phosphorylation can be independent of receptor endocytosis [25-27]. However BMP signal transduction is still strongly influenced by receptor endocytosis. That altered receptor endocytosis is the main mechanism by which Statins influence BMP signaling is confirmed by the fact that inhibition of the Clathrin endocytosis pathway by siRNA abolishes the effect of Statins on BMP signaling and that inhibition of Caveosome-mediated endocytosis by siRNA against Caveolin-1 mimics the effect of Statin treatment (Figure 3B), as also described by others [28-30]. It has been shown that Caveosome-mediated endocytosis is dependent on the ratio between Caveolin-1 and the caveolar coat protein Cavin[31]. We have not investigated whether Statin treatment influences Cavin expression or its possible influence on Caveosome-mediated endocytosis. Our data showing that transient knockdown of Caveolin-1 using siRNA shows

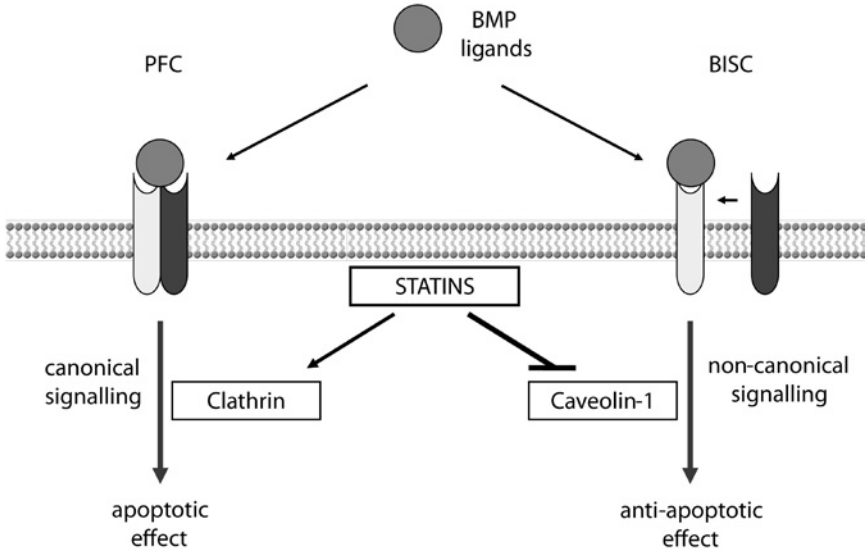

Figure 6. Schematic representation of the proposed mechanism of action of Statins at the level of receptor endocytosis.

similar effects on BMP signaling as Statin treatment, suggest to us that Statins act through effects on Caveolin-1, independently of Cavin.

The effect of Statins seems strongest on the expression of BMPR2, whose decreased expression is also associated with colorectal carcinogenesis. Although very slight changes in expression of total protein of BMPR1a are seen, far larger increases in cell surface expression of all the receptors are seen suggesting that this change in sub-cellular distribution is the predominant effect. In normal intestinal epithelial cells multiple BMP receptor complexes are present on the cell surface, most prominently heterocomplexes containing both BMPR1 and BMPR2. Type 1 receptors can also form homodimers unlike BMPR2, which cannot bind BMP ligand by themselves. Canonical BMP signaling has to involve BMPR2 in order to phosphorylate SMAD1, 5 and 8. Non-canonical signaling, on the other hand, is activated directly by type 1 receptors. The activation of BMP signaling in response to Statin appears to result from an increase of BMPRs at the cell surface, mainly the type 2 receptor. In HCT116 cells total BMPR2 expression is low and almost absent on the cell membrane. This would favor the formation of type 1 receptor dimers leading to non-canonical BMP signaling. The increase in BMPR2 upon Statin treatment would be expected to increase the amount of heterocomplexes, shifting the balance back to canonical BMP signaling.

Statins may also influence BMP signaling through entirely separate mechanisms. In a screen of 30,000 compounds 2 Statins were found to most highly activate a BMP2 reporter construct. We have shown *in vitro* that Statins can demethylate the BMP-2 promoter, which is often silenced by CpG island methylation in CRC. The internalization of receptor/ligand complexes is required for signaling in multiple pathways, predominantly through Clathrin-mediated endocytosis. Why alterations in receptor endocytosis due to Statin treatment seems to have more important effects on the BMP pathway than on other pathways is beyond the scope of this paper and should be further investigated. It may be due to the fact that BMP receptors are unique within the TGF-beta family in existing as pre-formed complexes and in having two competing mechanisms of endocytosis. The importance of Caveosomes for BMP signaling and the disruption of Caveosomes by Statins coupled with the effects of Statins at other points in the BMP pathway may explain the important effects of Statins specifically on this pathway in colorectal cancer.

In conclusion Statins have anti-tumor effects in colorectal cancer by inducing changes in BMP signaling through reducing Caveolin-1 levels. Caveosome-mediated BMP receptor endocytosis is reduced leading to increased membrane BMP receptor localization and a shift towards Clathrin-mediated endocytosis. These two effects combine to increase proapoptotic canonical BMP signaling and decrease pro-proliferative non-canonical BMP signaling. The modulation of oncogenic pathways through the manipulation of receptor endocytosis is an intriguing new potential therapeutic option in cancer treatment.

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