Autocrine BMP-4 Signaling Is a Therapeutic Target in Colorectal Cancer

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Abstract

Poor prognoses for colorectal cancer patients with metastatic lesions have driven demand for the development of novel targeted therapies. Here, we demonstrate that expression of bone morphogenetic protein 4 (BMP-4) is universally upregulated in human colorectal cancer cells and tissues, resulting in activated BMP signaling. Inhibition of endogenous BMP signaling by the BMP type I receptor inhibitor LDN-193189 elevated expression of the phosphatase DUSP5 in colorectal cancer cells, inducing apoptosis via dephosphorylation of Erk MAPK. Administering LDN-193189 to mice diminished tumor formation of colorectal cancer cells. Our findings suggest inhibition of autocrine BMP-4 as a candidate treatment strategy for colorectal cancer. Cancer Res; 77(15); 4026–38. ©2017 AACR.

Introduction

Colorectal cancer is the third most common cancer and the fourth most common cause of cancer-related death worldwide (1). Although surgical resection can cure early-stage colorectal cancer, a combination of surgery and chemotherapeutic agents is recommended in advanced stages of colorectal cancer (1). In addition to conventional cytotoxic agents, new agents targeting VEGF signaling and EGFR signaling have been introduced during the last decade (2). Although colorectal cancer prognoses have steadily improved, the 5-year survival rate remains low, especially in patients with metastatic lesions (1). Thus, the development of new molecular targets for treatment of advanced colorectal cancer is critical for improving patient outcomes.

Bone morphogenetic proteins (BMP) are members of the TGF-β family and are multifunctional cytokines (3–5). BMPs recognize two distinct receptors, termed type I and type II receptors, with serine/threonine and tyrosine kinase activities. Type I BMP receptors include activin receptor-like kinase (ALK)-1, -2, -3, and -6, and type II receptors include BMP type II receptor (BMPR-II), activin type II receptor (ActR-II), and activin type IIB receptor (ActR-IB). BMPs are classified into several subgroups, including the BMP-2/4 group, BMP-5/6/7/8 group, BMP-9/10 group, and growth and differentiation factor (GDF)-5/6/7 group, according to structural similarities and their ability to bind certain type I receptors. Upon binding to type I and type II receptors, BMPs form heterotetrameric complexes; the protein kinase of the type II receptor activates the protein kinase of the type I receptor, which in turn phosphorylates the BMP-specific receptor-regulated Smads (R-Smads), Smad1 and Smad5. Phosphorylated R-Smads induce a heteromeric assembly with common-partner Smad (Co-Smad; Smad4) and translocate into the nucleus, regulating the transcription of target genes. BMPs can also activate non-Smad signaling pathways, including the MAPK pathway (3).

Divergent roles of BMPs have been reported during cancer progression (4–6). BMPs inhibit proliferation of gastric cancer, breast cancer, and prostate cancer cells, induce differentiation of glioma-initiating cells, and inhibit glioblastoma tumor formation (5), indicating a tumor-suppressive role of BMPs. In contrast, BMPs have been reported to enhance the motility and invasiveness of various types of cancer cells, such as breast cancer, prostate cancer, and malignant melanoma cells, suggesting that BMPs also function as tumor-promoting factors (4).

In this study, the role of BMP-4 produced by colorectal cancer cells in cancer progression was investigated. We reveal for the first time that inhibition of BMP-4 induces the apoptosis of colorectal cancer cells through the attenuation of MAPK activity in culture and that the small-molecule BMP inhibitor LDN-193189 diminishes colorectal cancer formation in vivo.

Materials and Methods

Cell culture and reagents

Human colon adenocarcinoma cells HT29 and DLD-1 (Japanese Cancer Research Resource Bank) were cultured in RPMI containing 10% FBS, penicillin, and streptomycin. Human colon adenocarcinoma SW480 cells (ATCC) were cultured in DMEM containing 10% FBS, penicillin, and streptomycin. Routine Mycoplasma testing was performed by PCR regularly on these cells. The cells were bought in 2002 and have been stocked as cryopreserved aliquots in liquid N2. The cells were used within 8 passages after thawing and reauthenticated by short tandem repeat profiling in 2017. LDN-193189 was obtained from Wako or RIKEN.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Quantitative real-time RT-PCR analyses and chromatin immunoprecipitation–qRT-PCR analyses

Quantitative real-time RT-PCR (qRT-PCR) analysis and chromatin immunoprecipitation (ChiP)–qRT-PCR analysis were performed as previously described (7, 8). Primer sequences are described in Supplementary Table S1. Anti-TCF4 antibody (sc-8631) was purchased from Santa Cruz Biotechnology.

Immunoblotting

Immunoblotting was performed as previously described (7). Antibodies are described in Supplementary Materials and Methods. ImageJ (NIH) was used to quantify blot band intensities.

Apoptosis assay

Terminal deoxynucleotidyl transferase-mediated digoxigenin nick end labeling (TUNEL) assay was performed as previously described (7). Fluorescence was examined using a Leica DMi6000 B.

Lentiviral production and infection

We used a lentiviral vector system to induce specific gene introduction and knockdown as previously described (9, 10). The target sequences for shRNA are described in the Supplementary Materials and Methods.

RNA-sequence analyses

RNA-sequence (RNA-seq) analysis was performed as described previously (11). Raw and processed data are available at GEO (GSE96914). Gene Ontology analysis was performed using CLC Genomics Workbench (Qiagen Bioinformatics).

Subcutaneous xenograft model

BALB/c nu/nu female mice (4–5 weeks) were obtained from Sankyo Labo Service Corporation. A total of 5 × 10⁶ cells in 100 μL of culture medium were subcutaneously inoculated. Tumor volume was estimated as previously described (12). All animal experiments were performed under the policies of the Animal Ethics Committee of The University of Tokyo (approval number: 12312). The bioavailability of administered LDN-193189 was examined as described previously (13).

IHC

Formalin-fixed, paraffin-embedded human clinical samples were obtained from patients at The University of Tokyo Hospital with informed consent. The protocol was approved by the Research Ethics Committee at The University of Tokyo, Graduate School of Medicine (approval number: 10475). IHC was performed as previously described (14). Antibodies are described in the Supplementary Materials and Methods.

Results

Colorectal cancer produces BMP-4 through aberrant activation of the Wnt/β-catenin pathway

To investigate the expression of BMP mRNAs in colorectal cancer, data from several public databases were re-analyzed. The NCI-60 cell line panel indicated that, among various BMPs, expression of BMP4 was commonly elevated in colon cancer cells (Fig. 1A). NCBI GEO database GSE14256 revealed that expression of BMP4, but not of other BMPs, was significantly higher in colon cancer tissues than in normal colon tissues (Fig. 1B). Next, the correlation between BMP4 expression and colorectal cancer patient prognosis was examined. GSE14333 showed that elevated expression of BMP4 was associated with poor prognosis in patients with stage II colorectal cancer (Fig. 1C). Furthermore, multivariate analysis demonstrated that BMP4 expression was an independent prognostic factor in stage II colorectal cancer (Supplementary Table S2). Expression of BMP-4 and activation of Smad-dependent BMP signaling was then examined using human colorectal cancer tissues and cells. IHC analysis revealed that expression levels of BMP-4 and phosphorylated Smad1/5 were upregulated in colorectal cancer tissues compared with those in corresponding normal tissues (Fig. 1D). ELISAs demonstrated that these colorectal cancer cells produced BMP-4, whereas, with the exception of SUIT-2 pancreatic cancer cells, other cancer cells examined did not (Fig. 1E). These findings suggest that colorectal cancer cells produce BMP-4, which may act in an autocrine manner, and that BMP-4 expression may be related to colorectal cancer progression.

Next, we sought to clarify the mechanism by which BMP4 mRNA was elevated in colorectal cancer. Mutations in the APC gene occur in the early phase of colorectal cancer progression, which in turn increases the stability of β-catenin (15). Because BMP4 expression is reported to be regulated by the Wnt/β-catenin pathway (16), the involvement of Wnt/β-catenin in the induction of BMP4 expression in colorectal cancer cells was assessed. β-Catenin protein levels were elevated in colorectal cancer cells but not in non-colorectal cancer cells, such as pancreatic cancer cells (SUIT-2) and breast cancer cells (MDA-231-D; Fig. 2A). Knockdown of the CTNNB1 gene (encoding β-catenin) in colorectal cancer cells by siRNAs suppressed BMP4 mRNA expression and BMP-4 protein production, as well as expression of a direct downstream target of the Wnt/β-catenin pathway, AXIN2 (Fig. 2B and C). Similar to colorectal cancer cells, introduction of siCTNNB1 to SUIT-2 cells also decreased BMP4 mRNA, indicating that regulation of BMP4 by the Wnt/β-catenin pathway was not restricted to colorectal cancer cells (Fig. 2B). Although colorectal cancer cells were stimulated with Wnt-3a, increase of AXIN2 was not observed (Fig. 2D), suggesting that signaling activity of Wnt/β-catenin might have already been saturated in colorectal cancer cells. Likewise, stimulation of pancreatic cancer cells with Wnt-3a increased the expression of both AXIN2 and BMP4. Pretreatment of BxPC-3 cells with cycloheximide did not suppress BMP4 expression (Supplementary Fig. S1). ChiP–qRT-PCR analysis revealed a binding of TCF4 to the previously reported enhancer region of BMP4 gene, which was enhanced by Wnt-3a in SUIT-2 cells and attenuated by introduction of siCTNNB1 in HT29 cells (Fig. 2E; ref. 16). These findings suggest that BMP-4 is directly regulated by the Wnt/β-catenin pathway.

Colorectal cancer cells undergo apoptosis following the inhibition of autocrine BMP-4 signaling

To examine the role of BMP-4 on colorectal cancer tumor growth, shRNA-targeting BMP4 was introduced into HT29 and SW480 cells. As a result, expression of inhibitor of DNA binding 1 (ID1), a direct target gene of BMPs, and production of BMP-4 protein in culture supernatants were significantly diminished.

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Figure 1.

BMP-4 is produced in colorectal cancer cells and colorectal cancer tissues. 

A, Comprehensive gene-expression analysis from NCI-60 cell line panels showing gene expression profiles of BMP mRNA in leukemia (n = 6), breast cancer (n = 5), central nervous system (CNS) tumor (n = 6), colon cancer (n = 7), renal cancer (n = 8), lung cancer (n = 9), ovarian cancer (n = 7), prostate cancer (n = 2), and melanoma (n = 9) cells. Color indicates distance from log2 6.

B, Gene expression analysis from the NCBI GEO database (GSE14258). Box plot reveals expression of BMP mRNA in normal colon epithelium (n = 54) and colon cancer (n = 186) tissues.

C, Kaplan-Meier plot of disease-free survival of patients with stage II colorectal cancer (CRC; n = 94) stratified by median BMP4 mRNA expression based on data from NCBI GEO database (GSE14333).

D, IHC of colorectal cancer tissues and corresponding normal colon tissues from two colorectal cancer patients (Patients #1 and #2), stained with hematoxylin and eosin (H&E), anti-BMP-4 antibody, and antiphospho-Smad1/5 antibody (pSmad1/5); scale bars, 50 μm.

E, Concentrations of BMP-4 proteins in cancer cell culture supernatants (48 hours) determined by ELISA (n = 2). Figure data are shown as box whisker plots (B) or as means ± SD (E). *, P < 0.05; **, P < 0.001, as determined by Student t test (B) or by log-rank test (C).
Figure 2.
Elevated expression of BMP4 in colorectal cancer cells is due to aberrant activation of the Wnt/β-catenin pathway. A, Top, immunoblotting of cell lysates with indicated antibodies. Bottom, relative expression of β-catenin protein in indicated cells. B, qRT-PCR analysis of CTNNB1, AXIN2, and BMP4 expression in cancer cells (n = 2). Indicated cells were transfected with control siRNA (siNTC) or siRNA targeting CTNNB1 (siCTNNB1#1 and #2), cultured for 72 hours, and analyzed by qRT-PCR. C, Concentrations of BMP-4 proteins in cells in B. Cell culture supernatants (48 hours) were examined by ELISA (n = 4). D, qRT-PCR analysis of AXIN2 and BMP4 expression in indicated cancer cells after Wnt-3a stimulation (200 ng/mL) at the indicated time points (n = 2). E, ChIP-qRT-PCR analysis of TCF4-bound DNA using primers designed at the enhancer region of BMP4. SUIT-2 cells and HT29 cells were fixed and harvested 1.5 hours after Wnt-3a (200 ng/mL) stimulation or 48 hours after introduction of siCTNNB1, respectively. Sine oculis binding protein homolog (SOBP) was used as negative control. Figure data are shown as means ± SD (B-E).
BMP-4 as a New Therapeutic Target in Colorectal Cancer

...of cancer cells with BMP-4 attenuated DUSP5 mRNA expression, confirming that DUSP5 is regulated by BMP signaling (Fig. 5F). Because DUSP5 acts as an inducible nuclear MAPK phosphatase and specific dual phosphatase for extracellular signal-regulated kinase (Erk) MAPK, the effects of LDN-193189 and noggin on MMP signaling in colorectal cancer cells were examined. Although different effects on the phosphorylation of p38 MAPK and INK were observed in a cell type-dependent manner, phosphorylation of Erk MAPK was commonly attenuated in the colorectal cancer cells examined (Fig. 5G; Supplementary Fig. S3A and S3B). Moreover, although siBMP4#2 exhibited a partial effect probably because of inefficient knockdown of BMP-4, siBMP4#1 caused a similar result on DUSP5 expression to LDN-193189 (Supplementary Fig. S3C), suggesting that regulation of Erk MAPK by BMP signaling is mediated through DUSP5 in colorectal cancer cells.

To determine whether the prosurvival effect of BMP-4 is mediated by DUSP5, DUSP5 was knocked down in colorectal cancer cells with siRNAs (Fig. 6A). Knockdown of DUSP5 attenuated LDN-193189-induced apoptosis (Fig. 6B and C). Furthermore, the enhancement of PARP cleavage and the attenuation of Erk phosphorylation were diminished following silencing of DUSP5 in colorectal cancer cells (Fig. 6D). Because expression levels of other DUSP members in colorectal cancer cells were upregulated by the transfection of siDUSP5 (data not shown), siDUSP5 appeared to induce some off-target effects on other phosphatases, which might have influence on phosphorylation of Erk.

To clarify the proapoptotic effect of DUSP5, DUSP5 was introduced into colorectal cancer cells using a lentiviral vector (Supplementary Fig. S4A). Under serum-free conditions, overexpression of DUSP5 resulted in cell-number reduction and elevated apoptosis of colorectal cancer cells (Supplementary Fig. S4B and S4C). PARP cleavage was also enhanced and Erk phosphorylation was attenuated by overexpression of DUSP5 (Supplementary Fig. S4D).

Finally, we examined whether attenuation of Erk MAPK results in apoptosis of colorectal cancer cells. Treatment of U0126, a MEK inhibitor, abolished phosphorylation of Erk and enhanced cleavage of PARP and apoptosis (Supplementary Fig. S5A and S5B). These results suggest that LDN-193189–induced apoptosis of colorectal cancer cells is regulated by DUSP5-mediated dephosphorylation of Erk MAPK.

LDN-193189 inhibits tumor formation in vivo

On the basis of the above findings, the efficacy of LDN-193189 as a new therapeutic agent for colorectal cancer was evaluated. Colony formation of colorectal cancer cells in soft agar was...
Figure 4.
LDN-193189 induces apoptosis of colorectal cancer cells. A, Immunoblot analysis of colorectal cancer cells treated with BMP inhibitors. Colorectal cancer cells were cultured with 0.2–0.3 μmol/L LDN-193189 (LDN) or 50 ng/mL noggin for 2 hours. Immunoblotting of cell lysates was conducted with indicated antibodies. B, qRT-PCR analysis of ID1 expression in colorectal cancer cells in A (n = 2). C, Effects of LDN-193189 on proliferation of colorectal cancer cells. Cells were seeded in 6-well plates. On the following day, cells were deprived of serum and cultured with DMSO or 0.2–0.3 μmol/L LDN-193189 for 3 days. Cell numbers are indicated (n = 2). D, TUNEL staining of cells in C. Left, red, TUNEL; blue, SYTOX green. Right, the percentage of TUNEL-positive cells among SYTOX green-positive cells. Data represent the mean of six microscopic fields. E, Left, Immunoblotting of lysates from cells in C with indicated antibodies. Right, cleavage of PARP protein in indicated cells. Data represent a fold increase compared with untreated control (n = 2). Data are shown as means ± SD (C–E). *, P < 0.05; **, P < 0.01; ***, P < 0.001, as determined by Student t test (C–E).
Data are shown as means ± SD (E–G). *, P < 0.05; **, P < 0.01; ***, P < 0.001, as determined by Student t test (G).

Figure 5.
LDN-193189 inactivates MAPK through induction of DUSP5 in colorectal cancer cells. A, Identification of genes regulated by LDN-193189 in colorectal cancer cells using RNA-seq analysis. Cells were seeded in 6-well plates. On the following day, cells were deprived of serum and cultured with DMK0 or 0.2–0.3 μmol/L LDN-193189 for 3 days. All genes whose RPKM values were >3 were included in analysis. Left Venn diagrams, number of genes upregulated >1.5-fold by LDN-193189. Right Venn diagrams, number of genes downregulated >1.5-fold by LDN-193189. B, Gene ontology analysis of genes upregulated by LDN-193189 in indicated cells. C, Genes upregulated by LDN-193189 (>1.5-fold) that belong to the gene ontology “inactivation of MAPK activity.” Red genes belong to DUSP family. D, Expression of DUSP family genes in colorectal cancer cells. Data were extracted from RNA-seq analysis. E, qRT-PCR analysis of DUSP5 expression in colorectal cancer cells (n = 2). Cells were seeded in 6-well plates. On the following day, cells were deprived of serum and cultured with DMK0 or 0.2–0.3 μmol/L LDN-193189 (LDN) for 5 days. F, qRT-PCR analysis of DUSP5 expression in cancer cells (n = 2). Cells were seeded in 6-well plates. On the following day, cells were deprived of serum and cultured with BMP-4 (30 ng/mL) for 3 days (MDA-231-D cells) or 24 hours (A549 cells). G, Left, Immunoblotting of lysates from cells in E with indicated antibodies. Right, expression of pErk MAPK protein in indicated cells. Data represent as a fold decrease compared with untreated control (n = 2). Data are shown as means ± SD (E–G). *, P < 0.05; **, P < 0.01; ***, P < 0.001, as determined by Student t test (G).
Figure 6.
Silencing of DUSP5 in colorectal cancer cells abolishes proapoptotic effect of LDN-193189. A, qRT-PCR analysis of DUSP5 expression in colorectal cancer cells (n = 2). Colorectal cancer cells were transfected with control siRNA (siNTC) or siRNA-targeting DUSP5 (siDUSP5 #1 and #2). On the following day, cells were deprived of serum and cultured with DMSO or 0.2–0.3 μmol/L LDN-193189 for 3 days, followed by qRT-PCR analysis. B, Number of cells in A (n = 2). C, TUNEL staining of cells in A. Left, red, TUNEL; blue, SYTOX green. Right, the percentage of TUNEL-positive cells among SYTOX green-positive cells. Data represent the mean of six microscopic fields. D, Top, immunoblotting of lysates from cells in A with indicated antibodies. Bottom, cleavage of PARP and expression of pErk MAPK protein in indicated cells. Data represent a fold change compared with negative control. Data are shown as means ± SD (A–C). *, P < 0.05; **, P < 0.01; ***, P < 0.001; n.s., nonsignificant, as determined by Student t test (B and C).
Figure 7.
(Continued on the following page.)
significantly suppressed by LDN-193189 (Fig. 7A). To confirm the bioavailability of administered LDN-193189, an ex vivo bioassay was performed using DLD-1 cells. qRT-PCR analysis revealed that serum from LDN-193189–treated mice successfully suppressed ID1 expression (Fig. 7B). Body-weight loss was not observed in LDN-193189–treated mice (data not shown), suggesting that LDN-193189 acted as a potent BMP inhibitor in vivo without severe toxicity. Finally, the effect of LDN-193189 on the tumor formation of colorectal cancer cells was investigated in vivo. Notably, LDN-193189 inhibited tumorigenesis in mice bearing colorectal cancer cells (Fig. 7C). Furthermore, LDN-193189 attenuated the phosphorylation of Smad1/5 and Erk MAPK in vivo (Fig. 7D). These results indicate that the inhibition of endogenous BMP signaling by LDN-193189 may represent a potential strategy for treatment of colorectal cancer.

Discussion

In this study, we demonstrated the protumorigenic role of BMP-4 in colorectal cancer (Fig. 7E). aberrant activation of the Wnt/β-catenin pathway increases BMP4 expression in colorectal cancer cells. Autocrine BMP-4 signaling protects cells from apoptosis through suppression of DUSP5-mediated dephosphorylation of Erk MAPK. When autocrine BMP-4 signaling is inhibited by LDN-193189, Erk MAPK is dephosphorylated via induction of DUSP5, resulting in colorectal cancer cell apoptosis. These results suggest that autocrine BMP-4 represents a potential target for colorectal cancer treatment.

We demonstrated that endogenous BMP signaling was activated by autocrine BMP-4 in colorectal cancer cells and tissues. Elevated BMP4 expression is unique to colorectal cancer, as other BMPs are not elevated in colorectal cancer cells, and BMP4 appears to be elevated only in colorectal cancer. Elevated expression of BMP ligands in colorectal cancer was shown previously and was reported to correlate with poor prognosis (4, 5, 19); however, activation of endogenous signaling by autocrine BMPs in colorectal cancer remains controversial. Kodach and colleagues (20) reported that Smad1/5 phosphorylation was not observed in most colorectal cancer cases due to mutations in Smad4 or BMPR-II. In contrast, Beck and colleagues (21) showed that Smad1 phosphorylation was detected in colorectal cancer tissues and observed in colorectal cancer cells with BMPR2 mutations. In this study, BMP-4 expression and Smad1/5 phosphorylation were observed in colorectal cancer tissues and correlated with each other. Furthermore, Smad1/5 phosphorylation was detected in DLD-1 cells, which are reported to carry mutations in BMPR-II (21), suggesting that BMP signaling is transduced in these cells through ActR-II and ActR-IB. On the basis of these findings, we concluded that BMP signaling was activated in colorectal cancer cells and tissues.

The role of BMP signaling during colorectal cancer progression has not been fully elucidated. Some studies have revealed that BMPs promote invasiveness and tumor formation of colorectal cancer cells (4, 5), whereas other reports have demonstrated growth-suppressive roles of BMPs in colorectal cancer (22, 23). Smad4 and p53 are reported to be key molecules affecting the functional roles of BMPs in colorectal cancer progression. Voorneveld and colleagues reported that loss of Smad4 switches BMPs from antitumorigenic to protumorigenic, whereas p53 mutations suppress the enhancement of chemosensitivity by BMP signaling in colorectal cancer cells with wild-type Smad4 (24, 25). Because p53 was mutated in all colorectal cancer cells used in the current study (26), the association between p53 status and the protumorigenic role of BMP signaling in colorectal cancer could not be assessed in our study. However, we demonstrated that inhibition of endogenous BMP signaling induced apoptosis of not only Smad4-null cells (HT29 and SW480) but also cells with wild-type Smad4 (DLD-1). Although factors other than Smad4 and p53 may modulate the role of BMP signaling in colorectal cancer progression, our findings suggest that autocrine BMP-4 exerts a prosurvival effect on colorectal cancer cells regardless of Smad4 status.

Erk enhances the proliferation, survival, and metastasis of cancer cells and acts as an oncogenic signaling pathway (27). Although BMPs have been known to phosphorylate Erk MAPK and enhance its activity, the underlying molecular mechanism is not fully understood (28–30). Using RNA-seq, we identified DUSP5 as a BMP target gene and determined that DUSP5 was important for the Erk-mediated prosurvival effect of BMP-4 on colorectal cancer cells. DUSP5 is a member of the four inducible nuclear MAPK phosphatases and dephosphorylates Erk1/2 (31, 32). DUSP5 has been implicated as a tumor suppressor in various types of cancer, including skin, gastric, prostate, colon, and lung cancer (33–36). In this study, we also showed that enhancement of Erk MAPK phosphorylation by BMP-4 is mediated by a reduction in DUSP5. Although we did not examine whether the regulation of DUSP5 family genes by BMPs occurs via a Smad-dependent pathway, our findings may provide insight into the mechanism of regulation of MAPK signaling by BMP signaling.

Because BMPs provide a potential target for cancer treatment, various BMP inhibitors have been developed. Calpe and colleagues (37) showed that neutralizing antibodies for BMP-4 increase the chemosensitivity of HT29 cells. Dorosornorphin was identified as a potent BMP inhibitor, and its analogue, DMH-1,
disturbs lung cancer growth and breast cancer metastasis (38, 39). LDN-193189 was reported to inhibit growth of breast and prostate cancers in vivo and prolong survival of mice bearing ovarian cancer cells (40–42). In colorectal cancer, Voorneveld and colleagues demonstrated that LDN-193189 reduced the viability and enhanced the chemosensitivity of Smad4-silenced colorectal cancer cells in vivo (25). On the basis of these reports, we attempted to determine whether LDN-193189 inhibits colorectal cancer tumor formation in vivo. Tumor formation in mice bearing colorectal cancer cells was significantly diminished by LDN-193189, suggesting that this therapeutic strategy may potentially be of use in colorectal cancer treatment. However, the risk of intestinal carcinogenesis must be noted when LDN-193189 is used in vivo. Because inhibition of BMP signaling in mice by transgenic expression of noggin under control of the villin promoter or by conditional knockout of BMPR1A leads to intestinal polyposis (43, 44), inhibition of BMP signaling in colon epithelial cells may increase the risk of intestinal carcinogenesis. Whissell and colleagues (16) demonstrated that orally administered LDN-193189 increased intestinal tumor formation in conditional Apc knockout mice; however, we did not detect intestinal tumor formation in LDN-193189-treated mice (data not shown). One possible explanation for this discrepancy is a difference in the route of LDN-193189 administration. Intermittent dosing of inhibitors may be required to avoid the risk of tumorigenesis. Another possible explanation is the use of different strains of mice in the experiments. The balance between BMP signaling and Wnt/β-catenin pathways is important for maintenance of homeostasis of intestinal epithelial regeneration (44). Because Apc knockout mice exhibit activation of the Wnt/β-catenin pathway in the intestine, Apc knockout mice are more sensitive to BMP inhibitors than wild-type mice.

In this study, we also demonstrated that LDN-193189 attenuated phosphorylation of not only Smad1/5 but also Erk MAPK in colorectal cancer tissues in mice. Because the Ras–Raf–MEK–Erk signaling cascade is activated by mutations in the signaling components of cancer cells, this signaling pathway is considered an important target for cancer treatment (27). Indeed, Ras mutations are detected in 50% of colorectal cancer cases (45). Anti-EGFR antibodies targeting this signaling, such as cetuximab and panitumumab, are effective against certain types of colorectal cancer; however, their usefulness is limited to colorectal cancer cases without KRAS mutations (1). We showed that LDN-193189 was also effective against SW480 cells harboring KRAS mutations in vivo (46). Together, these results suggest that BMP inhibitors, especially small-molecule kinase inhibitors such as LDN-193189, may represent attractive new therapeutic strategies for colorectal cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Yokoyama, K. Miyazono, S. Ehata Development of methodology: Y. Yokoyama, S. Ehata Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Yokoyama, Y. Tamura, S. Ehata Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Yokoyama, Y. Tamura, S. Ehata Writing, review, and/or revision of the manuscript: Y. Yokoyama, Y. Hashizume, K. Miyazono, S. Ehata Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Yokoyama, T. Watanabe, S. Ehata Study supervision: T. Watanabe, K. Miyazono, S. Ehata Other [preparation of chemical tool and evaluation of the properties; designed the administration method (solvent, administration route, and dosage) based on the above data]: Y. Hashizume

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