Role of RUNX3 in Bone Morphogenetic Protein Signaling in Colorectal Cancer

Cecilia Wei Lin Lee1,2,3, Kosei Ito4, and Yoshiaki Ito1,3

Abstract

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, are multifunctional cytokines regulating a broad spectrum of biological functions. Recent studies show the presence of BMP receptor 1a mutations in juvenile polyposis and frequent Smad4 mutations in colon cancer, suggesting that aberrations in BMP signaling play an important role in intestinal cancer pathogenesis. However, the exact molecular mechanisms remain poorly understood. The Runx domain transcription factor RUNX3 is an integral component of signaling pathways mediated by TGF-β and BMPs. RUNX3 is a gastric and colon tumor suppressor, functioning downstream of TGF-β. Recently, we showed the tumor-suppressive effects of RUNX3 by its ability to attenuate β-catenin/T-cell factors (TCFs) transactivation in intestinal tumorigenesis. Here, we explore the molecular basis of the tumor-suppressive function of the BMP pathway through RUNX3 in colorectal carcinogenesis. BMP exerted a growth-suppressive effect in HT-29, a human colorectal cancer cell line. c-Myc oncogene was found to be downregulated by BMP and/or RUNX3. We show that up-regulation of RUNX3 by BMP reduces c-Myc expression. Evidence is presented suggesting that RUNX3 down-regulates c-Myc expression by two parallel pathways—directly at the transcriptional level and through attenuation of β-catenin/TCFs, downstream of BMPs in colorectal cancer cells. Cancer Res; 70(10); 4243–52. ©2010 AACR.

Introduction

The RUNX family of transcription factors has attracted broad interest due to its involvement in many cancers (1). RUNX3 is a downstream target of the transforming growth factor-β (TGF-β) pathway, considered a tumor suppressor pathway, as components are frequently altered in cancers, especially those of the gastrointestinal tract (2). Physical interaction between RUNX3 and TGF-β pathway components has been shown (3). Inherent to the tumor suppressor activity of TGF-β is its ability to induce apoptosis. Transcriptional upregulation of Bim, mediated by RUNX3, is observed in TGF-β–induced apoptosis (4). RUNX3 also cooperates with FoxO3a/FKHR1 to induce apoptosis by activating Bim (5). Runx3−/− neonate mice exhibited hyperplasia of the stomach epithelial cells due to increased proliferation and diminished apoptosis (6). The epithelial cells were resistant to the growth-suppressive and apoptosis-inducing effects of TGF-β, suggesting that the tumor-suppressive activity of RUNX3 is associated with the TGF-β pathway.

Loss of RUNX3 has been reported in many cancers (7–9). RUNX3 is inactivated in gastric cancer by hemizygous deletion, promoter hypermethylation, histone modification, and protein mislocalization, suggesting a tumor-suppressive role of RUNX3 in this malignancy (6, 10, 11). The discovery of a single point mutation in RUNX3 in a patient sample was one of the most critical observations implicating RUNX3 as a gastric tumor suppressor (6). A single nucleotide C to T point mutation within the Runx domain of RUNX3 (R122C) completely abolished the tumor-suppressive activity of RUNX3 in nude mice and probably converted it into an oncogene. RUNX3 point mutations were also identified in two cases of bladder tumor, which abolished the DNA-binding ability of RUNX3 (12), strongly suggesting that RUNX3 is a tumor suppressor in bladder cancer. The human RUNX3 gene is located at chromosomal locus 1p36 (13), a region frequently deleted in cancers. In a large percentage of colorectal cancer cell lines and clinical specimens, RUNX3 is silenced by promoter hypermethylation (7, 14). We have reported that RUNX3, downstream of the tumor-suppressive TGF-β pathway, antagonizes the oncogenic Wnt pathway in intestinal carcinogenesis (15). RUNX3 and T-cell factor 4 (TCF4) bind directly to each other and form a ternary complex with β-catenin, negatively regulating Wnt signaling by inhibiting the transcriptional activity of β-catenin/TCF4 on promoters of Wnt target genes.

Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily and are multifunctional proteins with a wide range of biological activities, including proliferation and...
apoptosis. Two type I receptors, BMP receptor la (BMPRLa) and BMP receptor lb (BMPRLb), and one type II receptor, BMP receptor II (BMPRII), have been identified (16–18). BMP binding induces oligomerization of the receptor complex and type II receptor phosphorolyses type I receptor. The receptor complex phosphorylates the BMP-specific Smads, Smad1, Smad5, and Smad8, which complexes with Smad4 and translocates to the nucleus to activate gene expression that mediates the biological action of BMPs.

BMPs have been shown to function as tumor suppressors in cancer, including gastric and pancreatic cancer (19, 20). Recent findings suggest the involvement of BMPs in colon cancer. Frequent germline mutations in the SMAD4 gene were found in colon cancer patients (21). The most compelling evidence for the role of BMPs in colon cancer is the discovery of germline mutations in BMPRIA gene in patients with a rare inherited gastrointestinal cancer predisposition syndrome, familial juvenile polyposis (JP; ref. 22). Inhibiting BMP signaling in epithelial cells by transgenic overexpression of noggin, a BMP antagonist, resulted in the formation of ectopic crypts and polyps in the mouse intestine, mimicking the intestinal histopathology of JP (23). Another study showed that BMP suppression also causes JP-type gastric hamartoma development (24). Similarly, conditional inactivation of BMPRLa and BMPRII resulted in hyperplasia and development of hamartomatous polyps in the colon, recapitulating the human JP syndrome (25, 26). These findings further reinforce the role of BMP signaling in colonic malignancy.

Here, we investigated a potential role for RUNX3 as a tumor suppressor in colorectal cancer, downstream of the BMP pathway. In this study, we address the mechanism through which RUNX3 exerts its tumor-suppressive activity in response to BMP in colorectal cancer cells. Involvement of both BMP and Wnt pathways is shown, further strengthening the response to BMP in colorectal cancer cells. Involvement of RUNX3 exerts its tumor-suppressive activity in response to BMP in colorectal cancer cells.

**Materials and Methods**

**Cell culture and reagents.** Colorectal cancer cell lines, HT-29, HCT116, SW480, DLD1, WiDr, LS174T, Colo205, Colo320, BKO, LoVo, SW403, Colon201, CaCo2, SW837, LS513, LS1034, and SW620 were obtained from the American Type Culture Collection. Five colorectal cancer cell lines, OUMS23, CCK81, CoCM1, RCM1, and HCC56, were obtained from the Japanese Collection of Research Bioresources. Where indicated, cells were treated with 100 ng/ml of human recombinant BMP2 or BMP4, or 10 ng/ml of TGF-β (R&D Systems). As a control, cells were treated with the same volume of vehicle, 0.1% bovine serum albumin in 4 mmol/L HCl, used to reconstitute BMPs and TGF-β.

**Transfections.** RUNX3 mutants were constructed as described by Ito and colleagues (15). Transfection of plasmids into 293T cells was performed using Lipofectamine 2000 (Invitrogen). Transfection of plasmids and siRNAs into HT-29 was performed using FuGene HD (Roche) and DharmaFECT4 (Dharmacon), respectively, according to the manufacturer’s instructions. ON-TARGETplus SMARTpool RUNX3 and c-Myc siRNA (Dharmacon) were used to knock down the expression of RUNX3 and c-Myc, respectively. ON-TARGETplus siCONTROL nontargeting pool (Dharmacon) was used as a control. Cells were cotransfected with BLOCK-iT Fluorescent Oligo (Invitrogen). Forty-eight hours after transfection, FITC-positive cells were sorted by fluorescence-activated cell sorting using a FACS Vantage (BD Biosciences) and treated with BMP for 48 hours. RNA and protein were then extracted.

**Promoter studies.** The c-Myc promoter construct has been previously described (27). HT-29 cells were cotransfected with the c-Myc promoter and a Renilla luciferase construct, pRL-basic, for normalization of transfection efficiency. Forty-eight hours after transfection, cells were treated with BMP for 24 hours. Where BMP treatment was unnecessary, cells were lysed 48 hours after transfection. Luciferase activity was determined using the Dual-Luciferase Reporter Assay (Promega). Mutagenesis of the c-Myc promoter was performed using QuickChange Site-Directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing. To assay TCF-mediated transcription, a TOPFlash/FOPFlash reporter (Upstate) was used. All experiments were performed in triplicates and independently repeated.

**RNA extraction and reverse transcription.** Total RNA was extracted from cells using RNeasy Mini kit (Qiagen). To avoid genomic DNA contamination, DNease digestion was performed using RNAse-free DNase set (Qiagen). cDNA synthesis was performed with 1 µg of total RNA using Omniscript Reverse Transcription kit (Qiagen) and oligo (dT)15 primers (Roche).

**Real-time PCR analysis.** Real-time PCR was carried out using the ABI Prism 7500 Fast Sequence Detection System and ABI Taqman gene expression system (Applied Biosystems). Relative quantitation was calculated by ΔΔC_T method, normalized to either glyceraldehyde-3-phosphate dehydrogenase or β-actin, and analyzed using the Sequence Detection System 7500 Fast System v.1.4.0 software (Applied Biosystems). All analyses were performed in triplicates. For gene expression profiling, a gene is defined as undetectable when no C_T value can be obtained, indicating that mRNA is completely absent. We define a low-expressing gene when C_T value is above 30. We define a moderate expression when C_T value is between 25 and 30 and a high expression when C_T value is lower than 25.

**Western blot analysis.** Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer’s instructions. Lysates containing 30 µg of nuclear proteins or 50 µg of total cellular proteins were analyzed by Western blot using anti-phospho-Smad1/5 (Upstate), 5G4 anti-RUNX3 (10), anti-histone H3 (Upstate), and anti-β-actin (Sigma) antibodies. Immunoreactivity was visualized either by enhanced chemiluminescence (Amersham Biosciences) or by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

**Apoptosis detection.** Apoptosis was examined using an Annexin V-FITC Apoptosis Detection Kit II (BD Biosciences) as described (5). Cells were analyzed by flow cytometry using a FACS Vantage and FlowJo software (BD Biosciences).
Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed using the ChIP assay kit (Upstate), dephosphorylated β-catenin antibody (Alexis), or normal rabbit IgG. Primers used to amplify DNA fragments containing a TCF consensus site in the c-Myc promoter were 5′-GTGAATACGTTTGCGGGTTAC-3′ (forward) and 5′-AGAGACCTTGTGAAAAACCG-3′ (reverse; ref. 28).

Statistical analysis. Statistical evaluation was performed using the unpaired Student's t test. Data are presented as mean ± SD. A value of P < 0.05 was considered statistically significant. For proliferation over time, ANOVA analysis followed by Bonferroni’s multiple comparison test was used for statistical evaluation. All tests were applied using the GraphPad Prism software.

Results

Altered gene expression of RUNX3 in colorectal cancer cells. The gene expression profile of RUNX1, RUNX2, RUNX3, and PEBP2β was examined in a panel of 22 colorectal cancer cell lines (Table 1). RUNX1, RUNX2, and PEBP2β were expressed at moderate to high levels in all cell lines, except Colo205, CaCo2, and SW620, which expressed RUNX2 at a very low level. Interestingly, frequent loss of RUNX3 expression was observed. RUNX3 was expressed at decreased or undetectable levels in 9 of the 22 (40.9%) cell lines. Expression of RUNX3 was undetectable in RKO and was expressed at low levels in HT-29, DLD1, WiDr, LoVo, OUMS23, LS1034, and HCC56.

Human BMP receptors and Smad signaling components are expressed in majority of colorectal cancer cells. As a first step to studying BMP signaling in colorectal cancer, we characterized expression of BMP receptors and Smads in colorectal cancer cell lines (Table 1). All cell lines expressed Smad1 and Smad5. Smad8 was expressed at moderate to high levels in all cell lines except Colo201 and LS513. All cell lines expressed Smad4, except Colo205 and SW403. Smad6 was found at moderate to high levels in all cell lines except DLD1 and RKO. Most of the cell lines expressed moderate to high levels of BMPRIa and BMPRIb. BMPRIb was present in most cell lines, except LS174T, RKO, Colo201, SW403, CoCMI, and HCC56.

BMPs are moderately growth suppressive in HT-29 colorectal cancer cells. HT-29 cell line was chosen as a model to study BMP signaling in colorectal cancer as it expressed all components of the BMP pathway and the RUNX genes. In response to BMP, HT-29 cell line phosphorylated and translocated Smad1/5 to the nucleus in a time-dependent manner (Fig. 1A). This confirms that the BMP-SMAD signaling pathway is intact in HT-29 cells. Because BMPs are known to have a growth-suppressive effect on cancer cells, we examined the changes in cell

Table 1. Expression of BMP signaling pathway components and RUNX genes in colorectal cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Smad1</th>
<th>Smad5</th>
<th>Smad9</th>
<th>Smad4</th>
<th>Smad6</th>
<th>BMPRIa</th>
<th>BMPRIb</th>
<th>BMPRII</th>
<th>RUNX1</th>
<th>RUNX2</th>
<th>RUNX3</th>
<th>PEBP2β</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HCT116</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SW480</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DLD1</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WiDr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LS174T</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Colo205</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Colo320</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LoVo</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RKO</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Colo201</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SW403</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CaCo2</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OUMS23</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SW837</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CCK81</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CoCMI</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LS513</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LS1034</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SW620</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HCC56</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RCM1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

NOTE: ++, high expression; +, moderate expression; –, very low expression; —, undetectable.
growth in response to BMP in HT-29 cells. We first tested the sensitivity of HT-29 cells and found that both BMP2 and BMP4 inhibited HT-29 cell growth significantly at a concentration of 100 ng/mL (Fig. 1B). Lower concentrations of BMP showed a growth-inhibitory effect but could not attain statistical significance. Thus, a 100 ng/mL concentration of BMP was used for all further experiments.

A 37% and 34% growth reduction was observed on day 2 and day 4, respectively, when HT-29 cells were treated with BMP2 compared with vehicle-treated cells (Fig. 1C). Similarly, a 28% and 27% growth reduction was observed on day 2 and day 4, respectively, when HT-29 cells were treated with BMP4. Taken together, BMP confers a significant growth-suppressive effect. BMPs have been shown to attenuate cell growth by regulating apoptosis (29). In the absence of BMP, the percentage of apoptotic cells was 1.9% (Fig. 1D). When cells were treated with BMP2 and BMP4, the percentage of apoptotic cells was 3.0% and 2.2%, respectively. Because BMPs did not induce apoptosis in HT-29 cells significantly, growth-inhibitory effect of BMP is not associated with increased apoptosis.

**BMP treatment induced RUNX3 expression in colorectal cancer cells.** Changes in RUNX expression levels after BMP treatment were determined. Interestingly, RUNX3 gene expression was induced by 4- and 8-fold when HT-29 cells were treated with BMP2 and BMP4, respectively (Fig. 2A). There were no changes in RUNX1 and RUNX2 expression. An increase in RUNX3 protein expression was also observed (Fig. 2B). Both BMP and TGF-β have been reported to elicit a growth-suppressive effect in colorectal cancer cells (30, 31). In addition, TGF-β and BMP have been reported to induce RUNX2 (32). However, TGF-β failed to upregulate RUNX3 in HT-29 (data not shown). Therefore, these observations suggest that RUNX3 is the only member of the RUNX family specifically regulated by BMP.

**BMP and RUNX3 attenuate the transcriptional potential of β-catenin/TCF4 in Wnt signaling.** We investigated the effect of BMP on Wnt signaling using the TOPflash/FOPflash reporter system. Elevated TOPflash activity correlates with β-catenin/TCF transcriptional activity and Wnt signaling (33). BMP had a suppressive effect on TOPflash activity but had no effect on FOPflash activity (Fig. 2C). These data show that BMP inhibits the β-catenin/TCF–mediated transcriptional activation.

Next, we examined the transactivation of β-catenin/TCF4 in the presence of RUNX3 using the TOPflash/FOPflash reporter system. In HT-29, which expresses a low level of RUNX3, increasing amounts of exogenous RUNX3 progressively suppressed TOPflash activity (Fig. 2D). Therefore, RUNX3 also inhibits the β-catenin/TCF–mediated transcriptional activation in a dose-dependent manner.
BMP treatment repressed c-Myc mRNA expression by transcriptional mechanisms. Because c-Myc is a well-known β-catenin/TCF4 transcriptional target gene (34) and drives proliferation of intestinal epithelial cells, we questioned whether inhibition of proliferation by BMP could be attributed to changes in c-Myc expression. Interestingly, c-Myc gene expression was significantly downregulated in HT-29 by 55% and 60% when treated with BMP2 and BMP4, respectively (Fig. 3A). Similar results were observed when HCT116 cells were treated with BMP (Supplementary Fig. S1A). Next, we examined the effect of BMP on c-Myc promoter activity. BMP2 lowered c-Myc promoter activity moderately by 28%, whereas BMP4 only had a slight inhibitory effect of 14% (Fig. 3C).

An in silico analysis of the c-Myc promoter revealed the presence of three TCF-binding sites (34, 35) and two RUNX-binding sites (Fig. 3B). To determine if TCF-binding elements were required for c-Myc promoter activity repression by BMP, we mutated all three TCF-binding sites to generate a mutant c-Myc promoter construct that lacks intact TCF-binding elements. Mutation of TCF-binding sites effectively abolished the moderate repression of the c-Myc promoter by BMP (Fig. 3C).

To show DNA binding of β-catenin/TCFs onto the c-Myc promoter, a ChIP assay was carried out using an antibody against dephosphorylated β-catenin. β-Catenin exhibited significantly reduced binding to the c-Myc promoter when cells were treated with BMP (Fig. 3D). This suggests that BMP-mediated suppression of c-Myc involves occupation of β-catenin on the TCF-binding region of the c-Myc promoter.

From the results shown in Fig. 3C and D, it can be concluded that the TCF-binding site is crucial for the regulation of c-Myc expression by BMP, and further corroborates that c-Myc is a direct transcriptional target of β-catenin/TCF downstream of BMP signaling.

RUNX3 plays an essential role in BMP-mediated growth suppression. To assess the role of RUNX3 in BMP-mediated c-Myc suppression, the effect of RUNX3 on c-Myc promoter activity was examined. When both RUNX-binding elements in the c-Myc promoter were mutated, repression of the c-Myc promoter activity in response to BMP was completely lost (Fig. 3C). Increasing amounts of exogenous RUNX3 moderately and progressively repressed the c-Myc promoter activity in a dose-dependent manner (Fig. 4A). A dose-dependent effect of RUNX3 on c-Myc promoter repression was also shown in 293T cells (Supplementary Fig. S2B).

To map the region of RUNX3 responsible for this repression, six RUNX3 deletion mutants were constructed (Fig. 4B; ref. 15). Full-length RUNX3 strongly repressed the c-Myc promoter activity by >90% (Fig. 4C). Progressive truncations from its COOH terminus indicated that amino acid residues 1 to 373 are minimally required to observe a repressive effect, although the repression is reduced to 55% to 70%.
Interestingly, truncating the 1 to 182 residues from the NH₂-terminus of RUNX3 completely abolished repression of c-Myc promoter, suggesting that these residues are essential for RUNX3-mediated repression of the c-Myc promoter.

DNA methylation is an important mechanism in the activation of proto-oncogenes and plays a crucial role in cancer progression. Methylation-specific PCR was performed to evaluate the effect of RUNX3 on DNA methylation of the c-Myc promoter. Hypomethylation of c-Myc promoter was observed in both HT-29 and HCT116 (Supplementary Fig. S3). No differences were observed when cells were transfected with RUNX3 siRNA. Thus, downregulation of c-Myc is not the consequence of epigenetic control of gene expression by RUNX3.

Next, the extent of the contribution of RUNX3 to the growth-inhibitory effect of BMP was investigated. To determine if elevated levels of RUNX3 were responsible for inhibition of c-Myc gene expression, we tested whether suppression of endogenous RUNX3 could rescue the suppression of c-Myc by BMP. HT-29 cells were transfected with either control siRNA, siRNA against RUNX3, or c-Myc. RUNX3 expression was markedly inhibited by RUNX3 siRNA but not affected by control siRNA or c-Myc siRNA (Fig. 5A). Likewise, knockdown of RUNX3 protein expression was also observed (Fig. 5B). On the other hand, transfection of cells with c-Myc siRNA had no effect on RUNX3 expression levels, suggesting that c-Myc is downstream of RUNX3.

Interestingly, knockdown of RUNX3 abolished the growth-suppressive effect of BMP (Fig. 5C). In HT-29 cells where RUNX3 was knocked down, c-Myc expression levels remained unchanged in cells treated with BMP (Fig. 5D). In contrast, in cells transfected with control siRNA, a detectable repression of c-Myc expression was observed when cells were treated with BMP. This was also confirmed in HCT116 cells (Supplementary Fig. S1).

Taken together, the results clearly suggest that RUNX3 is essential for growth-inhibitory effects of BMP via suppression of c-Myc in colorectal epithelial cells.

**Discussion**

BMPs have been reported to be growth inhibitory in cancers, including breast, gastric, colon, and thyroid cancer...
However, the molecular mechanisms underlying its growth-suppressive effect are not well defined. In this study, the antiproliferative effect of BMPs was examined in colorectal cancer. We used HT-29, a colorectal adenocarcinoma cell line, as a model to examine BMP signaling in colorectal cancer as it expresses BMP-specific Smads and receptors and BMP signaling is intact. Both BMP2 and BMP4 inhibit HT-29 cell proliferation, consistent with a previous study that showed that growth of colon cancer cells, including HT-29, was modestly inhibited by BMP2 (30).

Because BMP has been reported to induce invasiveness of cancer cells (37, 38), we examined the effect of BMP on invasiveness of colorectal cancer cells. BMP2 and BMP4 did not have an effect on cell invasion in HT-29 cells (Supplementary Fig. S4). However, invasion of HCT116 cells was markedly enhanced by treatment with BMP. Taken together, these results strongly suggest that BMP plays an important role in metastatic ability of colorectal cancer cells by enhancing cell invasion in a cell line–dependent manner. To confirm the role of BMP in the metastatic process of colorectal cancer cells, additional in vivo studies are required.

Many important biological responses are coregulated by both Runx and TGF-β/BMP signaling (39). TGF-β induces Runx3 during Ig class switching (40) and RUNX2 in myoblast precursor cells (32). BMPs have also been shown to induce RUNX2 (32, 41). Additionally, RUNX1 was identified as a downstream target of the TGF-β/BMP pathway in the hematopoietic system (42). In this study, upregulation of RUNX3 was observed when HT-29 cells were treated with BMP. We show that RUNX3, but not RUNX1 and RUNX2, showed BMP-dependent expression. TGF-β had no effect on RUNX3 expression. These results suggest that induction of RUNX3 by BMP is unique and specific to RUNX3.

In this study, we show that BMP inhibits c-Myc transcriptional activity and expression in a RUNX3-dependent manner. MYC is located at 8q24, a region reported to be amplified in HT-29 cells, suggesting that c-Myc might be of utmost significance for the oncogenicity of HT-29 (43).

Figure 4. Repression of the c-Myc promoter by RUNX3. A, dose-dependent repression of the c-Myc promoter activity by exogenous RUNX3. HT-29 cells were cotransfected with c-Myc promoter and increasing amounts of RUNX3. All firefly luciferase activities were normalized to the Renilla luciferase activity of pRL-basic, which was used as an internal transfection control. B, schematic representation of wild-type RUNX3 and six RUNX3 deletion constructs (figure adapted from Ito and colleagues). C, repression of the c-Myc promoter activity by RUNX3 is dependent on the presence of an intact COOH-terminal domain. 293T cells were cotransfected with the empty vector (EV), full-length RUNX3 (1-415) or its deletion constructs shown in B, and the wild-type c-Myc promoter for 48 h.
Pretreatment with the transcriptional inhibitor actinomycin D and protein synthesis inhibitor cycloheximide abolished the downregulation of c-Myc by BMP (Supplementary Fig. S5). This suggests that both new mRNA and protein synthesis is essential for BMP-mediated repression of c-Myc. Because both RUNX3 mRNA and protein are upregulated downstream of BMP (Fig. 2), we hypothesize that loss of BMP-mediated c-Myc repression is due to absence of RUNX3 transcription and protein expression.

We propose a model whereby BMP inhibits RUNX3-dependent c-Myc expression by two parallel mechanisms. One mechanism is the direct binding of RUNX3 to RUNX-binding sites in the c-Myc promoter to inhibit c-Myc expression. Transcriptional activity of the c-Myc promoter was downregulated by both BMPs and RUNX3. We found that the major repression function was localized in the amino acid residues 1 to 182 of RUNX3, where the conserved DNA-binding Runt domain is located. Because loss of the Runt domain completely abolished the repressive effect of RUNX3 on the c-Myc promoter, this implies that the Runt domain of RUNX3 is required for it to exert its repressive effect.

Mutation of RUNX-binding sites in the c-Myc promoter abolished the responsiveness of the promoter to BMP, suggesting that RUNX-binding sites are critical for BMP signaling. Because knockdown of RUNX3 expression completely abolished the ability of BMP to repress c-Myc expression, expression of RUNX3 is essential for BMP growth-inhibitory effect. The effect of BMP in HT-29 was confirmed in HCT116 (Supplementary Fig. S1) and 293T (Supplementary Fig. S2), suggesting that the signaling effects are not idiosyncratic to HT-29. Taken together, these observations support a compelling argument that RUNX3 is essential for BMP-mediated suppression of c-Myc expression in colorectal cancer. TGF-β has been shown to directly repress c-Myc transcription induced by β-catenin and TCF4 (35). It is highly possible that BMP performs a similar function.

Another mechanism is an indirect effect of RUNX3 on c-Myc expression. Mutations of the TCF sites in the c-Myc promoter impaired the responsiveness of the c-Myc promoter to BMP. Repression of the c-Myc promoter depended on the presence of an intact TCF-binding site, suggesting that the TCF transcription factor is indispensable for BMP-mediated suppression of the c-Myc promoter activity. We show that both BMP and RUNX3 inhibit β-catenin/TCF4 transcriptional activity. Furthermore, BMP inhibits in vivo binding of β-catenin to c-Myc promoter. BMP inhibits the transcription

Figure 5. Suppression of RUNX3 expression rescues BMP-mediated c-Myc suppression. A, RUNX3, c-Myc, or control siRNA was transfected into HT-29. Changes in RUNX3 mRNA expression measured by real-time PCR. All values are relative to the RUNX3 levels in parental cells. B, changes in RUNX3 protein expression measured by Western blot. C, RUNX3, c-Myc, or control siRNA was transfected into HT-29. A cell count was performed to determine changes in proliferation. The number of siControl-transfected cells treated with vehicle was regarded as 100% and cell numbers are expressed as a percentage relative to the control. D, changes in c-Myc expression measured by real-time PCR. c-Myc levels are expressed relative to the untreated samples of siControl and siRUNX3 to determine the effect of BMP treatment on c-Myc expression. *, P < 0.05, significantly different from vehicle-treated group.
of c-Myc, a process mediated in part by β-catenin/TCF4. This subsequently decreases c-Myc expression, allowing BMP to exert its growth-suppressive effects. We propose that this is due, in part, to the mechanism suggested by Ito and colleagues (15), where RUNX3 and TCF4 bind directly to each other to form a ternary complex with β-catenin. We propose that BMP induces the formation of a RUNX3/β-catenin/TCF4 ternary complex, which in turn attenuates the DNA-binding activity of β-catenin/TCF4 to the c-Myc promoter. This temporarily releases cells from Wnt proliferative effect, thus allowing BMP to exert its growth-inhibitory effect. The physiological interaction between RUNX3 and β-catenin/TCF4 plays a role in coordinating signals from Wnt and BMP pathways, two opposing pathways in intestinal homeostasis, to permit tight regulation of proliferation. BMP2 has been shown to antagonize Wnt signaling in osteoblast progenitors by promoting an interaction between Smad1 and Dishevelled, thus restricting β-catenin activation (44). The model proposed in this study identifies an alternative level of interaction for BMP regulation and antagonism of Wnt signaling in the colon.

A mild but significant effect of BMP-induced repression of c-Myc was observed. The failure to obtain a more pronounced effect is probably because colorectal cancer cells do not respond well to the growth-inhibitory effect of BMP (45). This could be attributed to the fact that some transformed cells express high levels of BMP inhibitors (46). Alternatively, some cells also secrete endogenous BMP. Colon cancer cells, including HT-29, have been shown to secrete BMP4 (47). This is consistent with results shown in Fig. 1A, supporting the notion of a low basal level of BMP signaling in HT-29. Given endogenous BMP production in HT-29, it is not surprising that only a small increase in BMP-specific transcriptional activity is observed when cells were treated with exogenous BMP.

In this study, we observed differential effects of BMP2 and BMP4. Although they are highly homologous, divergent roles for BMP2 and BMP4 have been described (48). Thus, it is not surprising that the effect of BMP2 and BMP4 on regulation of c-Myc expression is dissimilar. Because there are no fundamental differences in the signaling pathways used by both BMP2 and BMP4 and they bind to the same receptors, this disparity could be due to different binding affinity of BMPs on these receptors. This could lead to differences in downstream signal transduction and variation in biological responses to BMPs.

In conclusion, our work has identified RUNX3 as a novel downstream target of the BMP pathway. We show that RUNX3 exerts its tumor suppressor effect downstream of BMP by inhibiting c-Myc. This study gives new insight into the mechanisms in which BMP suppresses cell growth and c-Myc expression in colorectal cancer. A disruption of BMP signaling leads to the deregulation of the intricate balance between promotion and inhibition of proliferation, which in turn is associated with increased tumorigenesis and colon cancer. It is intriguing to speculate that restoration of BMP pathway could contribute to new therapeutic strategies for colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Kazuo Sugamura for kindly providing the c-Myc promoter construct; Drs. Hiroyuki Kato, Motomi Osato, Dominic Voon, and Anthony Lim for providing advice; and Lynnette Chen for technical assistance in flow cytometric analysis.

Grant Support

Agency for Science, Technology and Research (A*STAR) and National Research Foundation through Cancer Science Institute of Singapore.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 10/16/2009; revised 03/10/2010; accepted 03/11/2010; published OnlineFirst 05/04/2010.

References


Role of RUNX3 in Bone Morphogenetic Protein Signaling in Colorectal Cancer

Cecilia Wei Lin Lee, Kosei Ito and Yoshiaki Ito

Cancer Res 2010;70:4243-4252. Published OnlineFirst May 4, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-3805

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/05/03/0008-5472.CAN-09-3805.DC1

Cited articles
This article cites 47 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/10/4243.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/70/10/4243.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.