Role of RUNX3 in Bone Morphogenetic Protein Signalling in Colorectal Cancer

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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 signalling play an important role in intestinal cancer pathogenesis. However, the exact molecular mechanisms remain poorly understood. The Runt domain transcription factor, RUNX3, is an integral component of signalling pathways mediated by TGF-β and BMPs. RUNX3 has been shown to be a gastric and colon tumour suppressor, functioning downstream of TGF-β. Recently we demonstrated the tumour suppressive effects of RUNX3 by its ability to attenuate β-catenin/TCFs transactivation in intestinal tumorigenesis. Here, we explore the molecular basis of the tumour 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 down-regulated 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.

INTRODUCTION

The RUNX family of transcription factors has attracted broad interest due to its involvement in many cancers. RUNX3 is a downstream target of the transforming growth factor-β (TGF-β) pathway, considered a tumour suppressor pathway as components are frequently altered in cancers, especially those of the gastrointestinal tract (1). Physical interaction between RUNX3 and TGF-β pathway components have been demonstrated (2). Inherent to the tumour suppressor activity of TGF-β is its ability to induce apoptosis. Transcriptional up-regulation of Bim, mediated by RUNX3, is observed in TGF-β induced apoptosis (3). RUNX3 also co-operates with FoxO3a/FKHRL1 to induce apoptosis by activating Bim (4). *Runx3*$^{-/-}$ neonate mice exhibited hyperplasia of the stomach epithelial cells, due to increased proliferation and diminished apoptosis (5). The epithelial cells were resistant to the growth-suppressive and apoptosis-inducing effects of TGF-β, suggesting that the tumour suppressive activity of *RUNX3* is associated with the TGF-β pathway.

Loss of RUNX3 has been reported in many cancers (6-8). RUNX3 is inactivated in gastric cancer by hemizygous deletion, promoter hypermethylation, histone modification and protein mislocalization, suggesting a tumour suppressive role of RUNX3 in this malignancy (5, 9, 10). 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 tumour suppressor (5). A single nucleotide C to T point mutation within the Runt domain of RUNX3 (R122C) completely abolished the tumour-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 tumour, which

abolished the DNA-binding ability of RUNX3 (11), strongly suggesting that RUNX3 is a tumour suppressor in bladder cancer. The human RUNX3 gene is located at chromosomal locus 1p36 (12), a region frequently deleted in cancers. In a large percentage of colorectal cancer cell lines and clinical specimens, RUNX3 is silenced by promoter hypermethylation (6, 13). We have reported that RUNX3, downstream of the tumour suppressive TGF-β pathway, antagonizes the oncogenic Wnt pathway in intestinal carcinogenesis (14). RUNX3 and TCF4 bind directly to each other and form a ternary complex with β-catenin, negatively regulating Wnt signalling 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 Ia (BMPRIa) and BMP receptor Ib (BMPRIb), and one type II receptor, BMP receptor II (BMPRII) have been identified (15-17). BMP binding induces oligomerization of the receptor complex and type II receptor phosphorylates 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 tumour suppressors in cancer, including gastric and pancreatic cancer (18, 19). Recent findings suggest the involvement of BMPs in colon cancer. Frequent germline mutations in the *SMAD4* gene were found in colon cancer patients (20). 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) (21). Inhibiting BMP signalling 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 (22). Another study showed that BMP suppression also causes JP-type gastric hamartoma development (23). Similarly, conditional inactivation of BMPRIa and BMPRII resulted in hyperplasia and development of harmatomatous polyps in the colon, recapitulating the human JP syndrome (24, 25). These findings further reinforce the role of BMP signalling in colonic malignancy.

Here, we investigated a potential role for RUNX3 as a tumour suppressor in colorectal cancer, downstream of the BMP pathway. In this paper, we address the mechanism through which RUNX3 exerts its tumour suppressive activity in response to BMP in colorectal cancer cells. Involvement of both BMP and Wnt pathways is demonstrated, further strengthening the recurrent theme of crosstalk between these two pathways in colorectal cancer.

MATERIALS AND METHODS

Cell culture and Reagents

Colorectal cancer cell lines, HT-29, HCT116, SW480, DLD1, WiDr, Ls174T, Colo205, Colo320, RKO, LoVo, SW403, Colo201, CaCo2, SW837, Ls513, Ls1034 and SW620 were obtained from American Type Culture Collection (ATCC). Five colorectal cancer cell lines, OUMS23, CCK81, CoCM1, RCM1 and HCC56 were obtained from Japanese Collection of Research Bioresources (JCRB). Where indicated, cells were treated with 100ng/ml of human recombinant BMP2 or BMP4, or 10ng/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 4mM HCl, used to reconstitute BMPs and TGF-β.

Transfections

RUNX3 mutants were constructed as described in Ito et al (14). Transfection of plasmids into 293T cells were performed using LipofectAMINE 2000 (Invitrogen). Transfection of plasmids and siRNAs into HT-29 was performed using FuGene HD (Roche) and Dharma*FECT*4 (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 non-targeting pool (Dharmacon) was used as a control. Cells were co-transfected with BLOCKiT^{TM}-Fluorecscent Oligo (Invitrogen). 48 hours post-transfection, FITC-positive cells were sorted by fluorescence-activated cell sorting (FACS) 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 (26). HT-29 cells were co-transfected with the *c-Myc* promoter and a renilla luciferase construct, pRL-basic, for normalization of transfection efficiency. 48 hours post-transfection, cells were treated with BMP for 24 hours. Where BMP treatment was unnecessary, cells were lysed 48 hours post-transfection. Luciferase activity was determined using the Dual-Luciferase Reporter Assay (Promega). Mutagenesis of the *c-Myc* promoter was performed using Quik-Change Site-Directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. To assay TCF-mediated transcription, a TOP/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, DNase digestion was performed using RNase-free DNase set (Qiagen). cDNA synthesis was performed with 1μg 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 $\Delta\Delta C_t$ method, normalized to either GAPDH or β-actin, and analysed using the Sequence Detection System 7500 Fast System v.1.4.0 software (Applied Biosystems). All analyses were done 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 25-30 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 30µg of nuclear proteins or 50µg of total cellular proteins were analyzed by Western Blot using anti-phospho Smad-1/5 (Upstate), 5G4 anti-RUNX3 (9), anti-histone H3 (Upstate) and anti-β-actin (Sigma) antibodies. Immunoreactivity was visualised either by ECL chemiluminescence (Amersham Biosciences) or by Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

Apoptosis detection

Apoptosis was examined using an AnnexinV- fluorescein isothiocyanate (FITC) apoptosis detection kit II (BD Biosciences) as described (4). Cells were analyzed by flow cytometry using a FACS Vantage (BD Biosciences) 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 forward, 5'-GTGAATACACGTTTGCGGGTTAC-3' and reverse, 5'-AGAGACCCTTGTGAAAAAAACCG-3' (27).

Statistical analysis

Statistical evaluation was performed using the student's unpaired *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 cell lines (40.9%). Expression of *RUNX3* was undetectable in RKO and was expressed at low levels in HT-29, DLD1, WiDr, Ls174T, LoVo, OUMS23, Ls1034 and HCC56.

Human BMP receptors and Smad signalling components are expressed in majority of colorectal cancer cells

As a first step to studying BMP signalling 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 *BMPRII*. *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 signalling in colorectal cancer as it expressed all components of the BMP pathway and the RUNX genes. In response to BMP, HT-29 cell line phoshorylated and translocated Smad1/5 to the nucleus in a time-dependent manner (Figures 1A). This confirms that the BMP-SMAD signalling pathway is intact in HT-29 cells.

Since BMPs are known to have a growth-suppressive effect on cancer cells, we examined the changes in cell 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 100ng/ml (Figure 1B). Lower concentrations of BMP showed a growth inhibitory effect but could not attain statistical significance. Thus, a 100ng/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 to vehicle-treated cells (Figure 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 (28). In the absence of BMP, the percentage of apoptotic cells was 1.9% (Figure 1D). When cells were treated with BMP2 and BMP4, the percentage of apoptotic cells was 3.0% and 2.2% respectively. Since 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 fold and 8 fold, when HT-29 cells were treated with BMP2 and BMP4 respectively (Figure 2A). There were no changes in RUNX1 and RUNX2 expression. An increase in RUNX3 protein expression was also observed (Figure 2B). Both BMP and TGF-β have been reported to elicit a growth suppressive effect in colorectal cancer cells (29, 30). In addition, TGF-β and BMP have been reported to induce RUNX2 (31). 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 signalling

We investigated the effect of BMP on Wnt signalling using the TOPflash/FOPflash reporter system. Elevated TOPflash activity correlates with β-catenin /TCF transcriptional activity and Wnt signalling (32). BMP had a suppressive effect on TOPflash activity but had no effect on FOPflash activity (Figure 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 (Figure 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

Since c-Myc is a well known β-catenin/TCF-4 transcriptional target gene (33) 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 down-regulated in HT-29 by 55% and 60% when treated with BMP2 and BMP4 respectively (Figure 3A). Similar results were observed when HCT116 cells were treated with BMP (Figure S1A). Next, we examined the effect of BMP on *c-Myc* promoter activity. BMP2 lowered *c-Myc* promoter transactivation moderately by 28%, whereas BMP4 only had a slight inhibitory effect of 14% (Figure 3C).

An in silico analysis of the *c-Myc* promoter revealed the presence of three TCF-binding sites (33, 34) and two RUNX-binding sites (Figure 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 which lacks intact TCF-binding elements. Mutation of TCF-binding sites effectively abolished the moderate repression of the *c-Myc* promoter by BMP (Figure 3C).

To demonstrate 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 (Figure 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 Figure 3C and 3D, 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 signalling.

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 (Figure 3C). Increasing amounts of exogenous RUNX3 moderately and progressively repressed the *c-Myc* promoter activity in a dose-dependent manner (Figure 4A). A dose-dependent effect of RUNX3 on *c-Myc* promoter repression was also demonstrated in 293T cells (Figure S2B).

To map the region of RUNX3 responsible for this repression, six RUNX3 deletion mutants were constructed (Figure 4B) (14). Full-length RUNX3 strongly repressed the *c-Myc* promoter activity by more than 90% (Figure 4C). Progressive truncations from its C-terminus indicated that amino acid residues 1-373 is minimally required to observe a repressive effect, although the repression is reduced to 55-70%. Interestingly, truncating the 1-182 residues from the N-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 (MSP) was performed to evaluate the impact of RUNX3 on DNA methylation of the *c-Myc* promoter. Hypomethylation of *c-Myc* promoter was observed in both HT-29 and HCT116 (Figure

S3). No differences were observed when cells were transfected with RUNX3 siRNA. Thus, down-regulation 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 was 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 (Figure 5A). Likewise, knockdown of RUNX3 protein expression was also observed (Figure 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, knock-down of RUNX3 abolished the growth suppressive effect of BMP (Figure 5C). In HT-29 cells where RUNX3 was knocked-down, c-Myc expression levels remained unchanged in cells treated with BMP (Figure 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 (Figure 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 (29, 35). However, the molecular mechanisms underlying its growth suppressive effect is 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 signalling in colorectal cancer as it expresses BMP-specific Smads and receptors and BMP signalling is intact. Both BMP2 and BMP4 inhibit HT-29 cell proliferation, consistent with a previous study which showed that growth of colon cancer cells, including HT-29, was modestly inhibited by BMP2 (29).

Since BMP has been reported to induce invasiveness of cancer cells (36, 37), 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 (Figure 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 signalling (38). TGF-β induces Runx3 during Ig class switching (39) and RUNX2 in myoblast precursor cells (31). BMPs have also been shown to induce RUNX2 (31, 40). Additionally, RUNX1 was identified as a downstream target of the TGF-β/BMP pathway in the haematopoietic system (41). In this study, up-regulation of RUNX3 was observed

when HT-29 cells were treated with BMP. We demonstrate 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 (42).

Pre-treatment with the transcriptional inhibitor actinomycin D and protein synthesis inhibitor cycloheximide abolished the down-regulation of c-Myc by BMP (Figure S5). This suggests that both new mRNA and protein synthesis is essential for BMP-mediated repression of c-Myc. Since both RUNX3 mRNA and protein is upregulated downstream of BMP (Figure 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 down-regulated by both BMPs and RUNX3. We found that the major repression function was localized in the amino acid residues 1-182 of RUNX3, where the conserved DNA-binding Runt domain is located. Since 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 signalling. Since knock-down 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 (Figures S1) and 293T (Figure S2), suggesting that the signalling 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 (34). 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 significantly 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 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 *et al* (14), 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 temporally releases cells from Wnt proliferative effect, thus allowing BMP to exert its growth-inhibitory effect. The physiological interaction between RUNX3 and β-catenin/TCF4 play a role in co-ordinating signals from Wnt and BMP pathways, two opposing pathways in intestinal homeostasis, to permit tight regulation of proliferation. BMP2 has been shown to antagonise Wnt signalling in osteoblast progenitors by promoting an interaction between Smad1 and Dishevelled, thus restricting β-catenin activation (43). The model proposed in this study identifies an alternative level of interaction for BMP regulation and antogansim of Wnt signalling 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 BMP's growth inhibitory effect (44). This could be attributed to the fact that some transformed cells express high levels of BMP inhibitors (45). Alternatively, some cells also secrete endogenous BMP. Colon cancer cells, including HT-29, have been shown to secrete BMP4 (46). This is consistent with results shown in Figure 1A supporting the notion of a low basal level of BMP signalling 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 (47). Thus, it is not surprising that the effect of BMP2 and BMP4 in c-Myc transactivation is dissimilar. Since there are no fundamental differences in the signalling 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 tumour 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 signalling 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.

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FIGURE LEGENDS

TABLE 1

Expression of BMP signalling pathway components and RUNX genes in colorectal cancer cells lines.

Legend: ++, high expression; +, moderate expression; -, very low expression; --, undetectable.

Cell Line	Smad 1	Smad 5	Smad 9	Smad 4	Smad 6	BMPRIa	BMPRIb	BMPRII	RUNX1	RUNX2	RUNX3	PEBP2β
HT29	+	+	+	+	++	+	+	++	+	+	-	+
HCT116	+	+	++	+	+	+	-	+	+	+	+	+
SW480	+	+	++	+	+	+	-	+	+	++	+	+
DLD1	+	+	++	+	-	+	++	+	+	++	-	+
WiDr	+	+	+	+	++	+	+	+	+	+	-	+
Ls174T	+	+	++	+	+	+	--	+	+	+	-	+
Colo205	+	+	+	--	+	+	-	+	+	-	+	+
Colo320	+	+	++	++	+	+	++	+	+	+	+	+
LoVo	+	+	++	+	+	+	++	+	+	+	-	+
RKO	+	+	++	+	-	+	--	+	+	+	--	+
Colo201	+	+	-	+	+	+	--	+	++	+	+	++
SW403	+	+	++	--	+	+	--	+	+	+	+	+
CaCo2	+	+	++	+	+	+	++	+	+	-	+	+
OUSM23	+	+	++	+	+	+	-	+	+	+	-	+
SW837	+	+	++	+	+	++	++	++	+	++	+	+
CCK81	+	+	++	++	+	+	+	+	+	+	+	+
CoCMI	+	+	+	+	+	+	--	+	++	+	+	+
Ls513	+	+	-	+	+	+	-	-	+	+	+	+
Ls1034	+	+	+	+	+	+	-	+	+	+	-	+
SW620	+	+	+	+	+	+	+	+	+	-	++	++
HCC56	+	+	++	+	+	+	--	+	+	+	-	+
RCM1	+	+	+	+	+	+	+	+	+	++	++	+

Legend

++	High
+	Moderate
-	Very low
--	not detected

Table 1

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FIGURE 1

BMP-SMAD signalling is intact in HT-29 and has a growth-suppressive effect.
(A) HT-29 cells were treated with BMP for 30, 60 and 120 minutes. Nuclear extracts were prepared and a Western blot was carried out to determine changes in phosphorylated Smad1/5. BMP2 and BMP4 induce the phosphorylation and nuclear accumulation of Smad1/5 in HT-29 cells.
(B) HT-29 cells were treated with different concentrations of BMP2 or BMP4, namely 10, 50, 100 and 200ng/ml, for 48 hours. The number of cells in the control medium was regarded as 100% and total cell number after BMP treatment was expressed as a percentage relative to the control sample. *$p<0.05$, significantly different from vehicle-treated control group.
(C) Cell growth determined by counting cell numbers in vehicle-treated (♦), BMP2-treated (▲) and BMP4-treated cells (■). Data represents the average of experiments carried out in triplicates. (Control vs BMP2 : $p = 0.0002$; Control vs BMP4 : $p = 0.0025$)
(D) HT-29 cells were treated with BMP for 48 hours. Apoptosis was determined by staining cells with AnnexinV-FITC and propidium iodide and analysed by flow cytometry. Lower left quadrant: unstained cells; upper left quadrant: necrotic cells; lower right quadrant: early apoptotic cells; upper right quadrant: late apoptotic and necrotic cells.

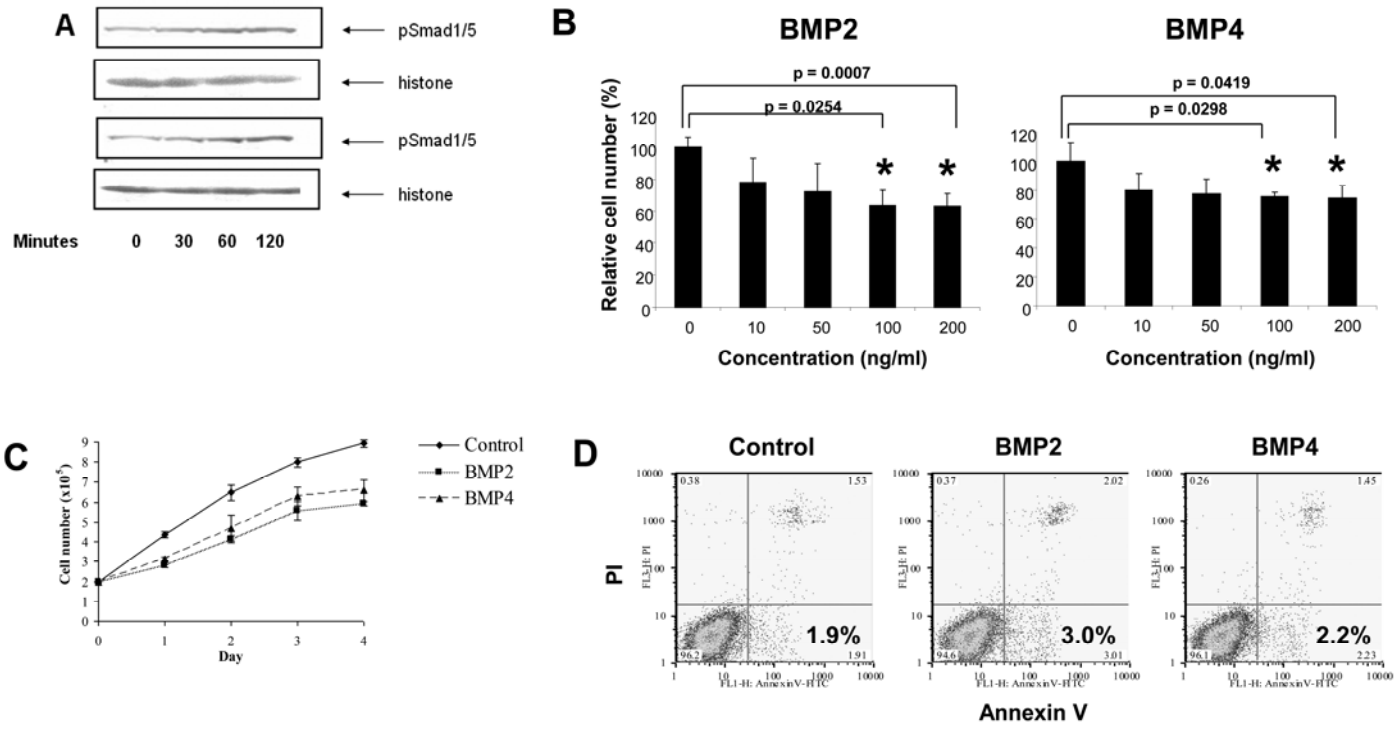

Figure 1

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FIGURE 2

BMP2/BMP4 increases RUNX3 expression, which then represses TOPflash activity in HT-29 cells.
(A) Changes in *RUNX1*, *RUNX2* and *RUNX3* mRNA expression, after BMP treatment for 48 hours, measured by real-time PCR
(B) Changes in RUNX3 protein expression after BMP treatment for 48 hours, analyzed by Western Blot.
(C) BMP2/BMP4 represses TOPflash activity
(D) Reduction of TOPflash activity by exogenous RUNX3 in a dose-dependent manner. HT-29 cells were co-transfected with TOPflash or FOPflash, and increasing amounts of RUNX3. All firefly luciferase activities were normalised to the renilla luciferase activity of pRL-basic, which was used as an internal transfection control.

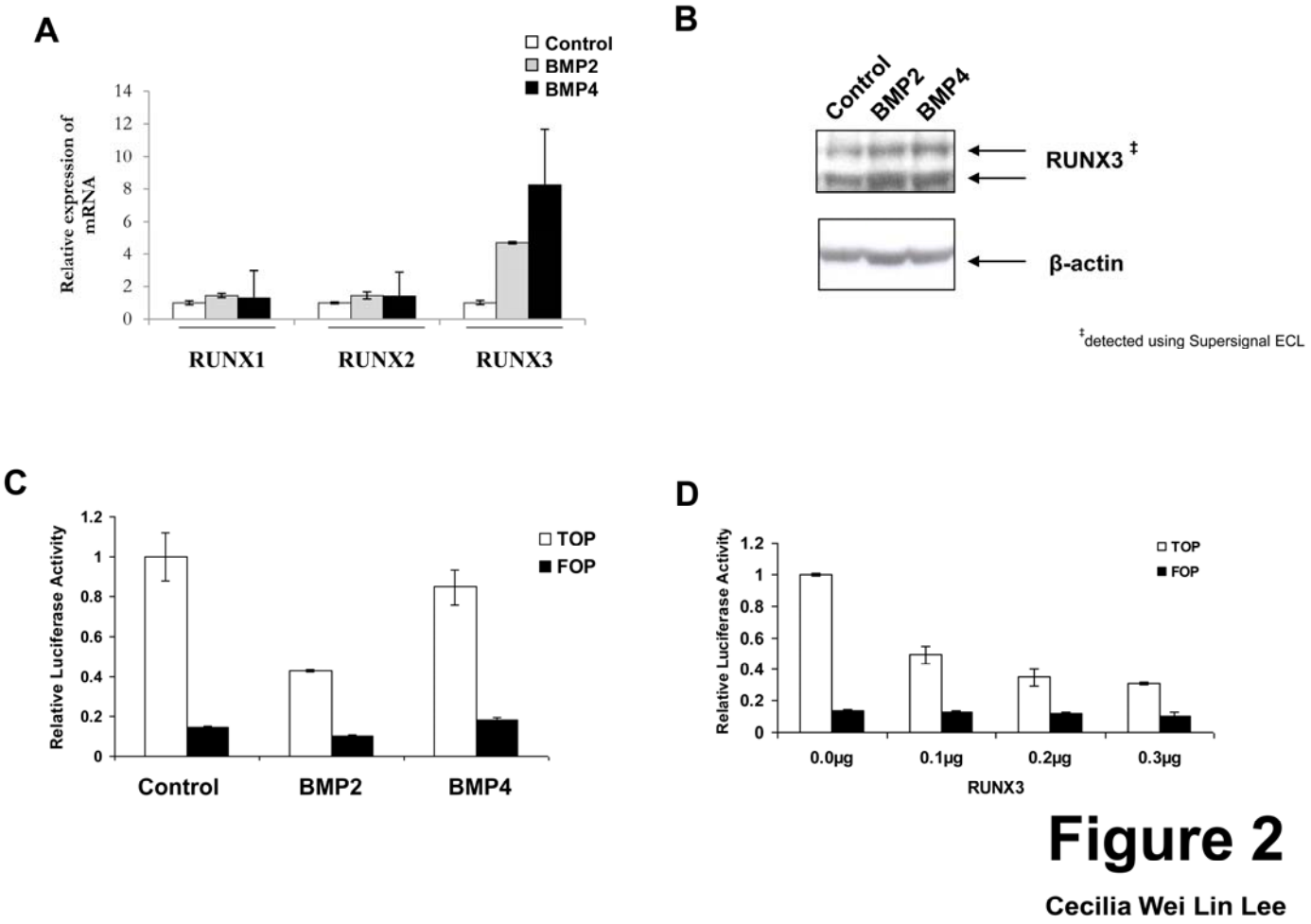

Figure 2

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FIGURE 3

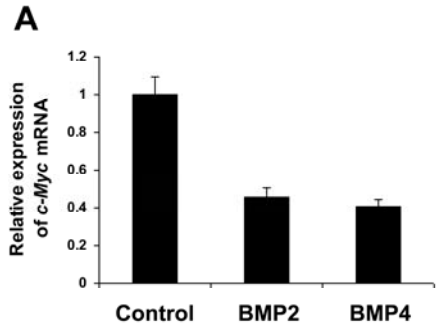
BMP2/BMP4 represses *c-Myc* expression and promoter transactivation in a TCF-dependent manner.

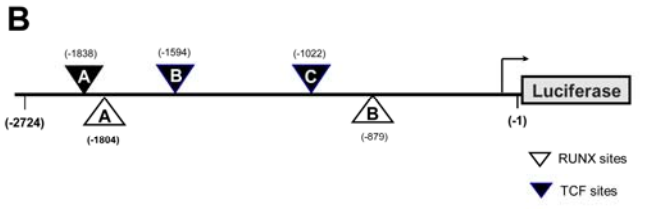
(A) Changes in *c-Myc* mRNA expression levels, after BMP treatment for 48 hours, measured by real-time PCR

(B) Schematic representation of the 2.5kB *c-Myc* promoter showing two RUNX-binding elements and three TCF-binding elements. Two mutant *c-Myc* promoters, containing either mutant RUNX-binding elements or mutant TCF-binding elements, were generated.

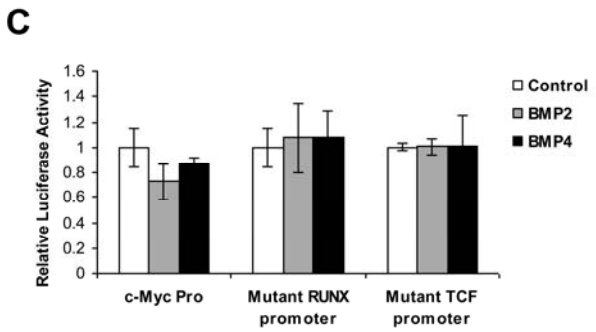
(C) Repression of the *c-Myc* promoter activity by BMP. Mutations of either the TCF-binding elements or RUNX-binding elements abolished repression of *c-Myc* promoter activity by BMP. HT-29 cells were transfected with the wild-type *c-Myc* promoter or promoter with either mutant TCF-binding or mutant RUNX-binding sites for 48 hours and treated with 100ng/ml of BMP for 24 hours. All firefly luciferase activities were normalised to the renilla luciferase activity of pRL-basic, which was used as an internal transfection control.

(D) ChIP assays demonstrate interaction of β-catenin with the *c-Myc* promoter region containing TCF-binding sites. ChIP was done using anti-dephosphorylated-β-catenin antibody or IgG. PCR was performed using a specific primer for the promoter region of *c-Myc* containing TCF-binding elements. As a control, one-fiftieth of the starting chromatin (Input) was used.





Putative binding sites		A	B	C
RUNX	Wild-type	ACCGCA	TGCGGT	-
	Mutant	CTAACA	CTAACA	-
TCF	Wild-type	TCTTTGAT	CCTTTGATT	GATCAAAGCG
	Mutant	CCTTTGGC	CCTTTGGCC	GGCCAAAGGG



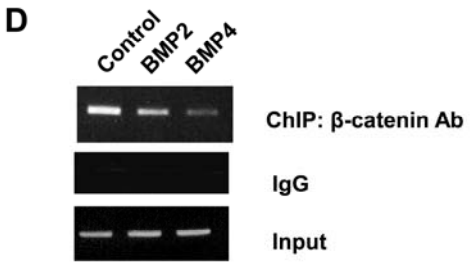


Figure 3

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FIGURE 4

Involvement of RUNX3 in BMP-dependent suppression of c-Myc transactivation.
(A) Dose-dependent repression of the *c-Myc* promoter activity by exogenous RUNX3. HT-29 cells were co-transfected with *c-Myc* promoter and increasing amounts of RUNX3. All firefly luciferase activities were normalised to the renilla luciferase activity of pRL-basic, which was used as an internal transfection control.
(B) Mapping of the RUNX3 region which interacts with the *c-Myc* promoter. Schematic representation of wild-type RUNX3 and six RUNX3 deletion constructs. (Figure adapted from Ito *et al*).
(C) Repression of the *c-Myc* promoter activity by RUNX3 is dependent on presence of an intact C-terminal domain. 293T cells were co-transfected with the empty vector (EV), full-length RUNX3 (1-415) or its deletion constructs and the wild-type *c-Myc* promoter for 48 hours. All firefly luciferase activities were normalised to the renilla luciferase activity of pRL-basic, which was used as an internal transfection control.

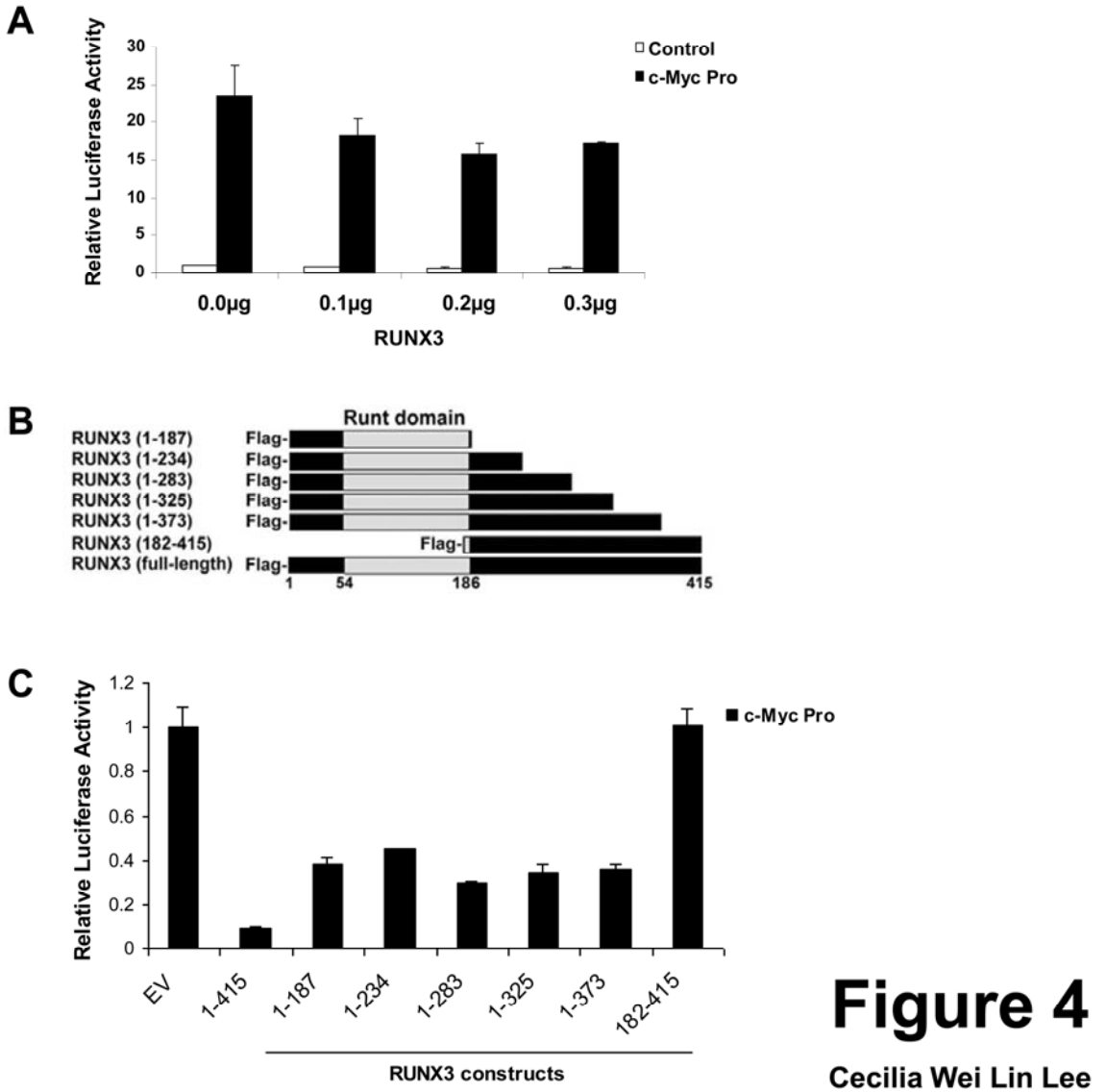

Figure 4

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FIGURE 5

Suppression of RUNX3 expression rescues BMP-mediated c-Myc suppression.
(A) RUNX3, c-Myc or control siRNA were 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 were 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.

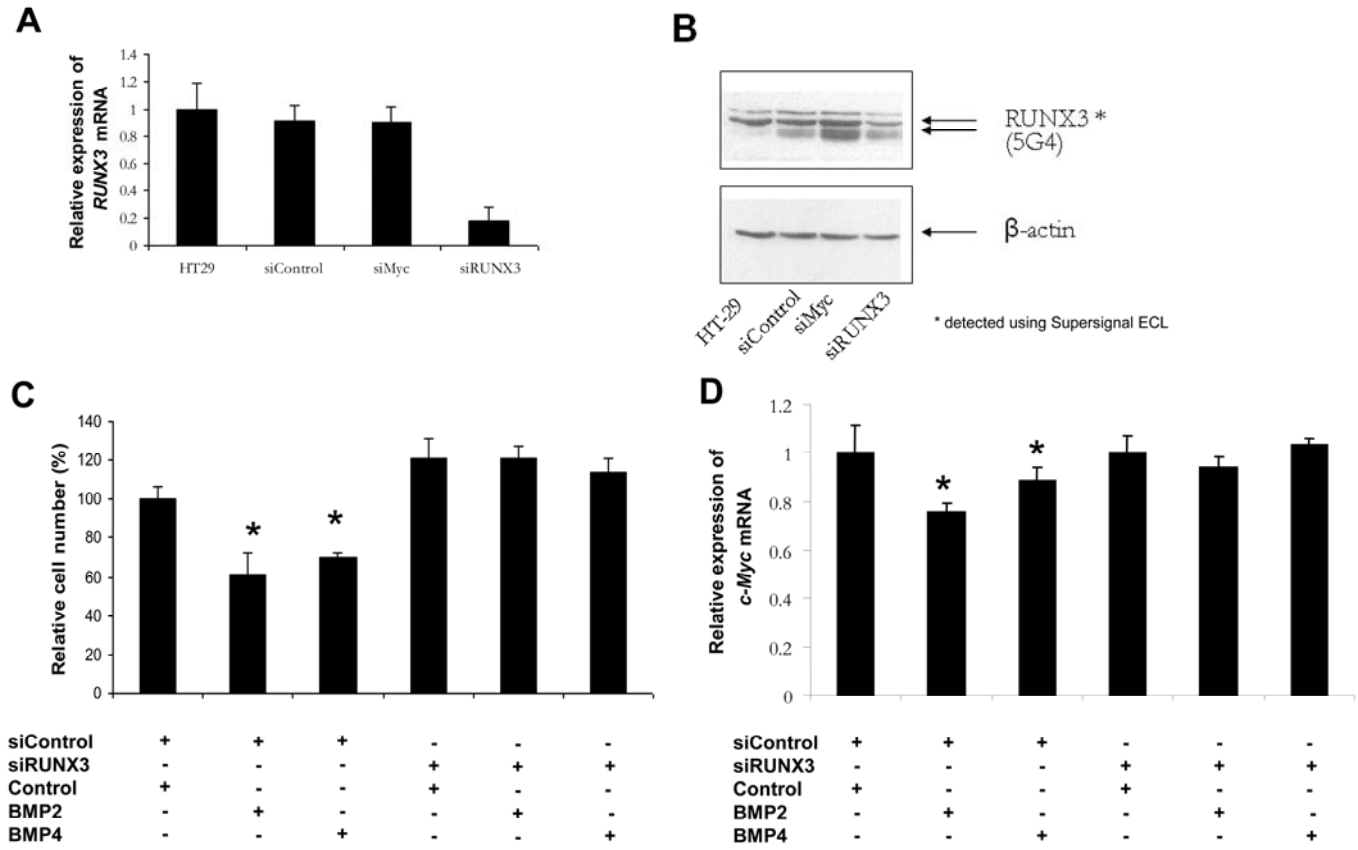

Figure 5

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Supplemental Data

Article

Role of RUNX3 in Bone Morphogenetic Protein Signalling in Colorectal Cancer

Cecilia Wei Lin Lee, Kosei Ito and Yoshiaki Ito

Supplemental Experimental Procedures

Methylation-specific PCR (MSP)

Methylation-specific PCR (MSP) was performed in CpG-rich regions of c-Myc to evaluate the impact of RUNX3 on DNA methylation of the *c-Myc* promoter. HT-29 and HCT116 cells were transfected with control siRNA or siRNA against RUNX3 for 48 hours. Extraction of genomic DNA from HT-29 and HCT116 cells was then performed using the QIAGEN genomic DNA kits for preparation of genomic DNA from cultured cells (Qiagen) according to the manufacturer's instructions. Bisulphite conversion of the DNA was carried out using the EZ DNA-Methylation-Gold Kit (Zymo Research) according to the manufacturer's instructions. The chemically modified DNA was then used as a template for PCR. The primer sets used for detection of methylated DNA were forward,
5'-tagaattggattggggtaaa -3' and reverse, 5'-cgaccgaaaatcaacgcgaat -3'. The primer sets used for detection of unmethylated DNA were forward, 5'-tagaattggattggggtaaa -3' and reverse, 5'-ccaaccaaaaatcaacatgaat -3', as reported previously (1).

Matrigel invasion assay

Invasion assays were performed using the BD BioCoat Matrigel Invasion Chamber (BD Biosciences) according to the manufacturer's instructions. Briefly, serum-free DMEM containing 100ng/ml of BMP2 or BMP4 was added to the bottom chamber to serve as a chemoattractant. After 24 hours of incubation, non-migrating cells were removed from the upper chamber using a cotton swab. Cells which migrated through the Matrigel were fixed, stained and counted. Mean values for four randomly selected fields were obtained for each sample. All experiments were repeated three times.

Actinomycin D and cycloheximide analysis

HT-29 cells were pre-treated with 1μg/ml of Actinomycin D (Sigma) and 10μg/ml of Cycloheximide (Sigma) for 4 hours, followed by treatment with BMP2 or BMP4 for 48 hours. As a control, cells were treated with the same volume of vehicle, 0.1% bovine serum albumin in 4mM HCl, used to reconstitute BMPs. RNA from the control and BMP-treated plates were harvested and real-time PCR was performed.

Promoter studies

Transfection of plasmids into 293T and HCT116 cells were performed using LipofectAMINE 2000 (Invitrogen). All transfections were carried out according to the manufacturer's instructions. 48 hours post-transfection, cells were treated with BMP for 24 hours. Where BMP treatment was not required, cells were lysed 48 hours post-transfection. Luciferase activity was determined using the Dual-Luciferase Reporter Assay (Promega). All experiments were performed in triplicates and independently repeated.

Supplemental References

1. Weng YR, Sun DF, Fang JY, Gu WQ, Zhu HY. Folate levels in mucosal tissue but not methylenetetrahydrofolate reductase polymorphisms are associated with gastric carcinogenesis. World J Gastroenterol 2006;12(47):7591-7.

Figure Legends

Figure S1
Involvement of RUNX3 in BMP-dependent suppression of c-Myc transactivation in HCT116 colorectal cancer cell line.
(A) BMP2/BMP4 increases RUNX3 expression and represses *c-Myc* expression in HCT116 colorectal cancer cell line. Changes in *RUNX3* and *c-Myc* mRNA expression, after BMP treatment for 48 hours, measured by real-time PCR.
(B) Repression of the *c-Myc* promoter activity by BMP in HCT116 cells. Mutations of either the TCF-binding elements or RUNX-binding elements abolished repression of *c-Myc* promoter by BMP. HCT116 cells were transfected with the indicated promoter constructs. 48 hours post-transfection, cells were treated with 100ng/ml of BMP2 or BMP4. Relative luciferase activity was measured after 24 hours. Only wild-type *c-Myc* promoter activity was inhibited by BMP2 and BMP4. Mutations of either the RUNX- or TCF-binding elements abolished the repression of the human *c-Myc* promoter activity by BMP2 and BMP4. All firefly luciferase activities were normalised to the *Renilla* luciferase activity of pRL-basic, which was used as an internal transfection control.
(C) RUNX3, c-Myc or control siRNA were transfected into HCT116. Changes in RUNX3 mRNA expression were measured by real-time PCR. All expression values are relative to the RUNX3 levels in the parental HCT116 cells. Western blot analysis was performed to determine changes in RUNX3 protein expression. SNU16 cell line was used as a positive control for RUNX3 expression.
(D) RUNX3 or control siRNA were co-transfected with BLOCK-iT Fluorescent Oligo into HCT116. RNA was prepared from FITC-positive cells. Real-time PCR was performed to determine changes in c-Myc expression. 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.

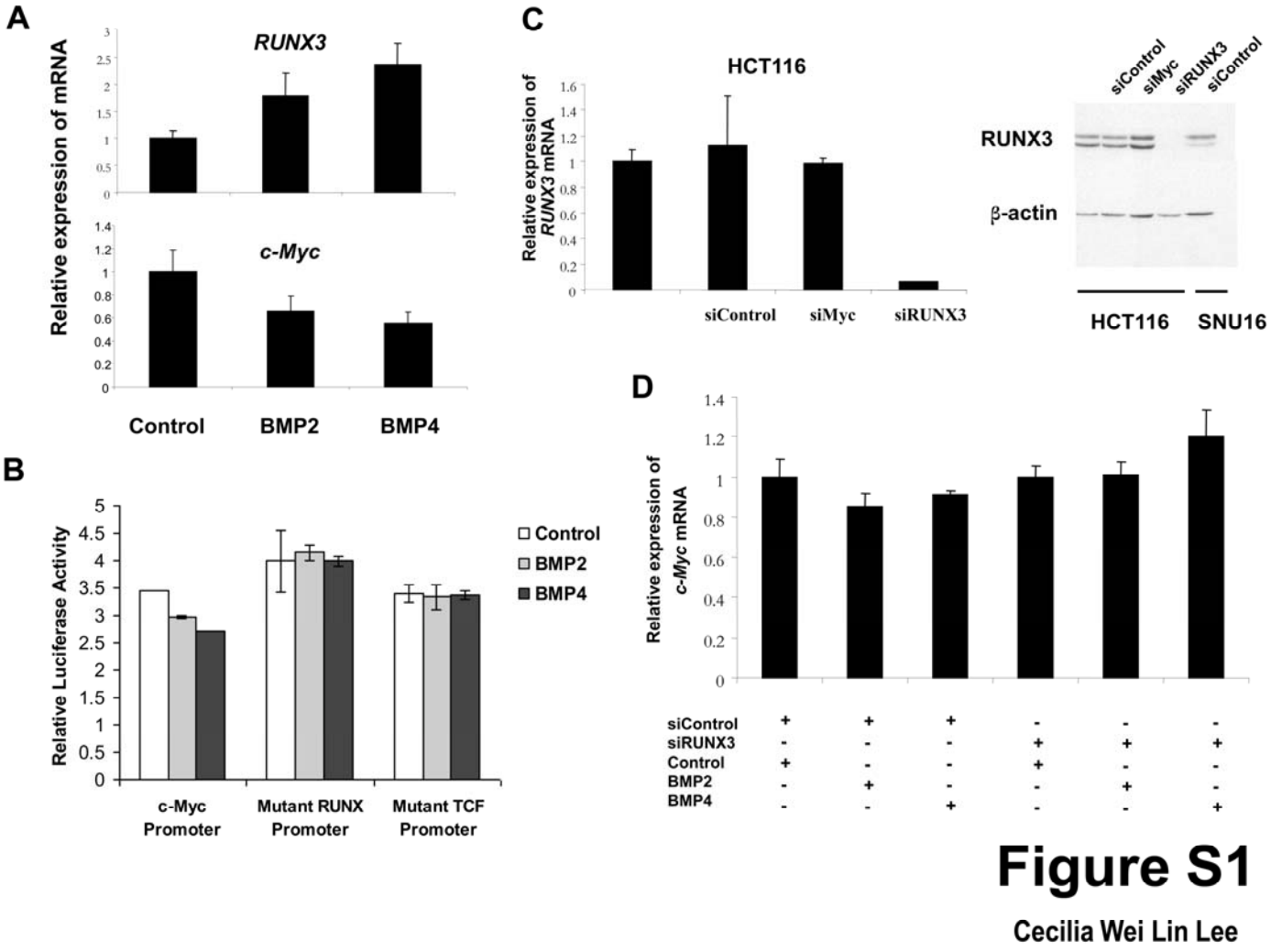

Figure S1

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Figure S2
Repression of Luciferase reporter assays using the human wild-type *c-Myc* promoter in 293T cells.
(A) Repression of the *c-Myc* promoter activity by transient co-transfections of CA-ALK3 and Smads 1 and 5. 293T cells were co-transfected with the *c-Myc* promoter, Smad1 and Smad5 expression constructs and the constitutively-active BMP Receptor Ia (CA-ALK3). As a negative control, cells were transfected with the dominant-negative BMP Receptor Ia (DN-ALK3).
(B) Dose-dependent repression of the *c-Myc* promoter activity by transient transfection of RUNX3 in 293T cells. 293T cells were co-transfected with *c-Myc* promoter and increasing amounts of RUNX3. All firefly luciferase activities were normalized to the *Renilla* luciferase activity of pRL-TK, which was used as an internal transfection control.

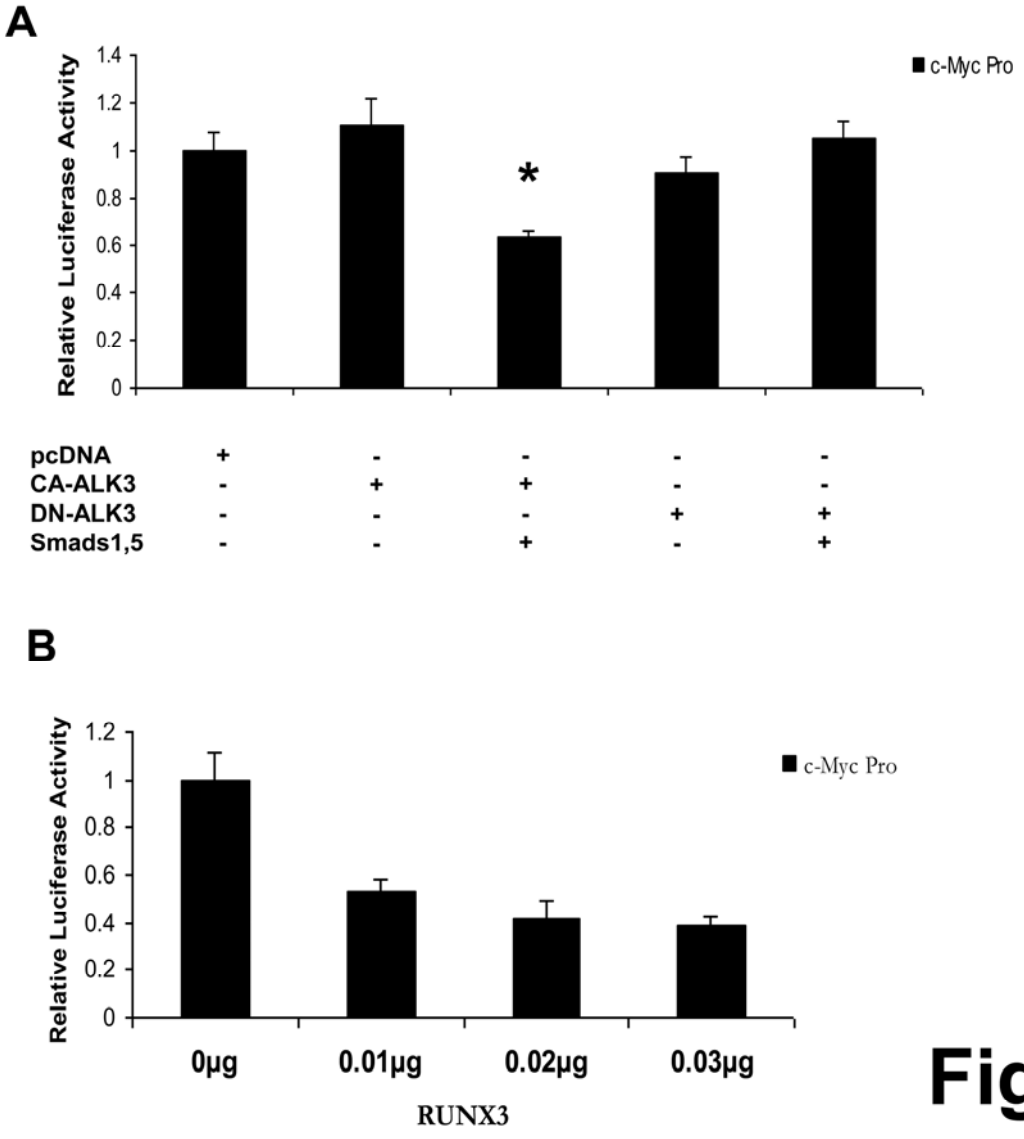

Figure S2

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Figure S3
Methylation specific PCR (MSP) for c-Myc gene promoter in HT-29 and HCT116 cells.
RUNX3 or control siRNA were transfected into HT-29 and HCT116 cells for 48 hours. MSP was performed on DNA from cells with primers designed to specifically detect methylated and unmethylated promoter regions. The data shown is representative of three replicate MSP experiments. U: unmethylation-specific PCR; M: methylation-specific PCR.

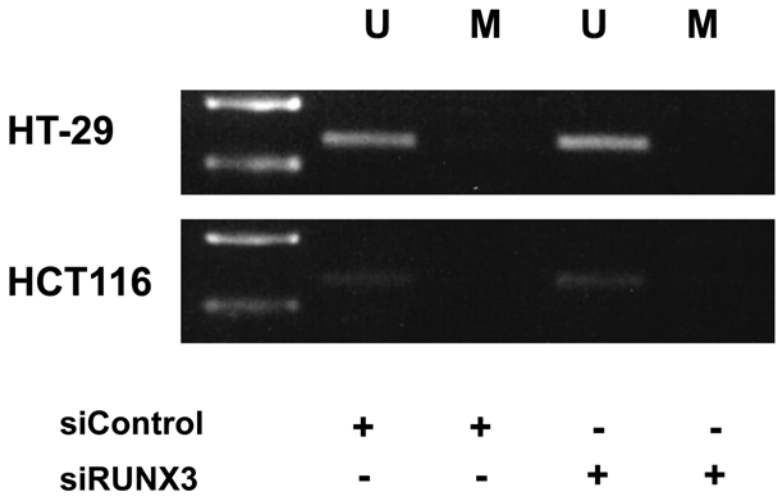

Figure S3

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Figure S4
Effect of BMP on the invasiveness of colorectal cancer cells.
(A) HT-29 and HCT116 cells were seeded in the upper chamber, which was coated with Matrigel. Serum-free medium containing 100ng/ml of BMP2 or BMP4 was added to the lower chamber. After 48 hours of incubation, migrated cells were fixed and stained with crystal violet. A representative field of the membrane with migrated cells. The 8μm membrane pores are visible in the background
(B) Effect of BMP on invasiveness of HT-29 and HCT116 cells. Migrated cells were counted from four random fields of view. Data is expressed as means of three independent experiments.

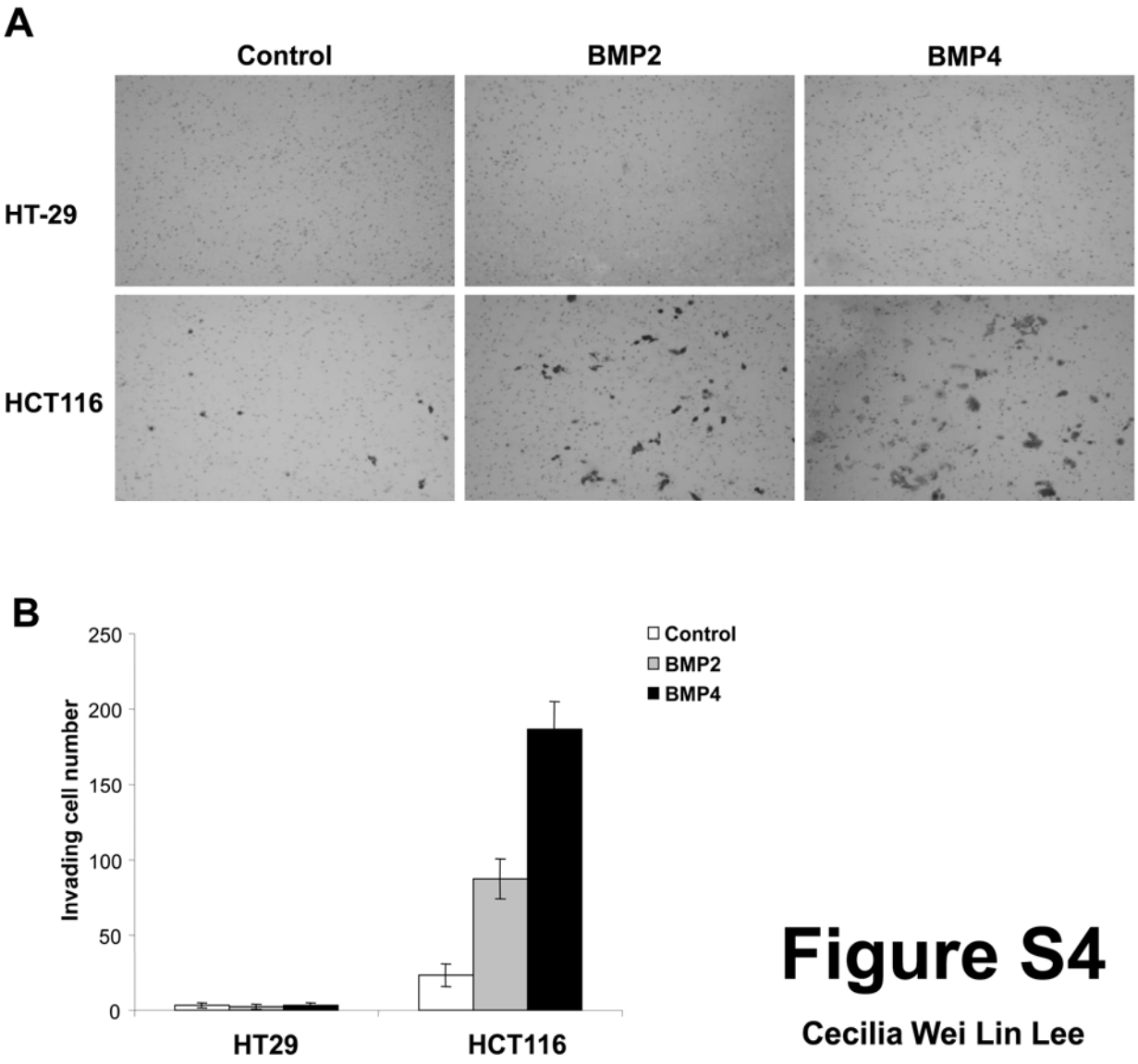

Figure S5
Effects of Actinomycin D and cycloheximide on BMP-dependent c-Myc suppression
Colorectal cancer cells were pre-treated with actinomycin D (1ug/ml) and cycloheximide (10ug/ml) for 4 hours, followed by BMP2 or BMP4 treatment for 48 hours. Changes in *c-Myc* expression levels were measured by real-time PCR. $^{*}p<0.05$, significantly different from vehicle-treated group.

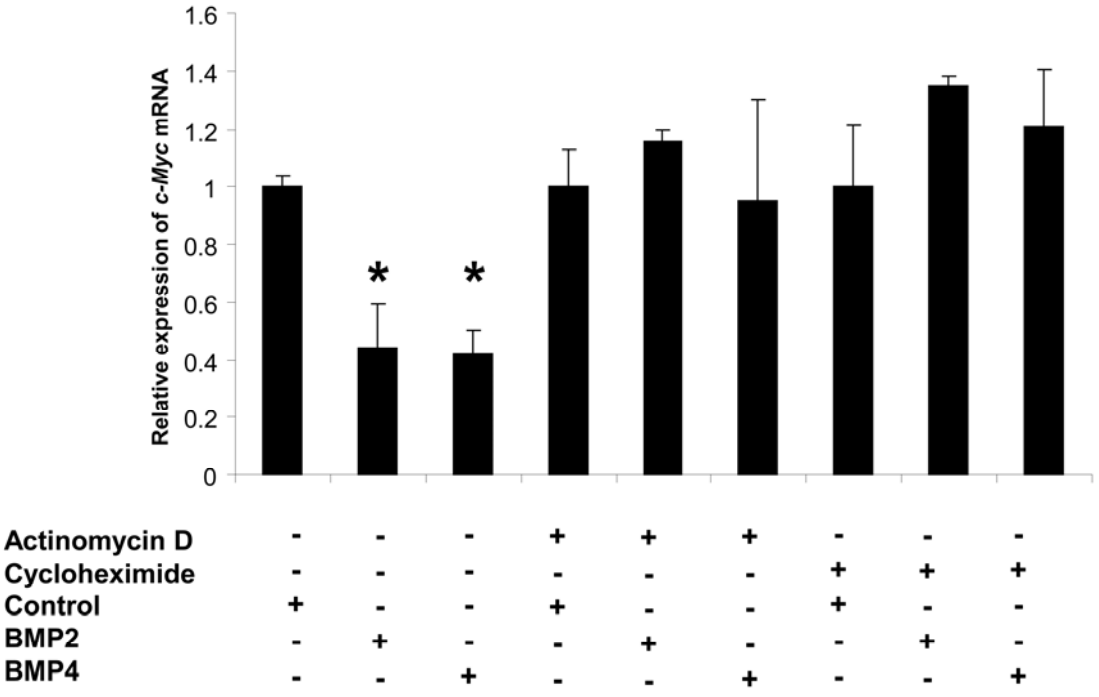

Figure S5

Cecilia Lee Wei Lin