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Targeting BMP9-Promoted Human Osteosarcoma Growth by Inactivation of Notch Signaling

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Abstract: Osteosarcoma (OS) is the most common primary malignancy of bone and is usually associated with poor prognosis due to its high incidence of metastasis and chemoresistance. Molecular pathogenesis of OS is poorly understood. We previously showed that OS cells are refractory to BMP9-induced osteogenesis and respond favorably to proliferation and tumor growth. Here we investigate if Notch signaling mediates the BMP9-promoted cell proliferation and tumor growth of human osteosarcoma (OS). We find that the expression of Notch1, Notch2, Notch3, DLL1, JAG1 and JAG2 is readily detected in most of the tested OS cell lines. BMP9-promoted OS cell proliferation, migration, and cell cycle S/G2 progression are effectively inhibited by a dominant-negative mutant of Notch1 (dnNotch1) or the γ-secretase inhibitor Compound E (ComE). Furthermore, BMP9-promoted tumor growth and osteolytic lesions *in vivo* are significantly inhibited by dnNotch1. BMP9 up-regulates the expression of Notch1, Notch3, DLL1, and JAG1 in OS cells. Accordingly, BMP9 stimulation induces a nuclear accumulation of NICD, which is blocked by ComE. Our results demonstrate that BMP9-promoted OS proliferation and tumor growth is at least in part mediated by Notch signaling, suggesting that osteogenic BMPs may function as upstream regulators of Notch signaling in OS tumorigenesis. Thus, pharmacologic intervention of Notch signaling may be explored as a new therapeutic strategy for human OS tumors.

Keywords: BMP9, bone tumorigenesis, notch signaling, osteogenesis, osteosarcoma, soft tissue tumors.

INTRODUCTION

Osteosarcoma (OS) is the most common primary malignancy of bone [1, 2]. OS usually involves the metaphysis of long bones where high bone turnover occurs during longitudinal growth spurts [2]. These pulmonary lesions are responsible for the high mortality associated with OS [1, 2]. Only about 15-20% of patients have radiographically detectable pulmonary metastases, while approximately 80% of the patients either will develop or already have radiographically undetectable micrometastases [1-3]. Treatment of OS includes surgical resection of both primary and/or pulmonary lesions combined with pre- and post-operative chemotherapy [1-3]. Once a metastasis has been detected, the disease is likely to relapse. Although the pathogenesis underlying OS is poorly understood, the tumors often develop in settings of high bone turnover, such as the adolescent growth spurt [1, 2, 4]. Numerous genetic and cytogenetic abnormalities have been associated with OS, including mutations of tumor suppressors and oncogenes, as well as chromosomal amplifications, deletions, rearrangements, and translocations [1, 2, 4-7]. However, no consensus genetic changes have been identified.

Increasing evidence indicates that OS may result from disruptions that prevent osteogenic terminal differentiation, thereby allowing tumor-initiating cells to retain their highly proliferative precursor cell phenotypes [1, 6-8]. Consistent with this possibility was our recent findings in which potent osteogenic factors, such as BMP9, fail to induce osteogenic differentiation of human OS cells and rather promote OS tumor growth *in vivo* [1, 8]. BMPs are important members of TGFβ superfamily and play an important role in the development and osteogenic differentiation [9, 10]. We previously identified BMP9 as one of the most potent BMPs among the 14 types of BMPs in inducing osteogenic differentiation of MSCs [9, 11-14]. Through expression profiling analysis, we identified several early target genes

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that may mediate BMP9 signaling in MSCs. One such downstream target is Hey1 [15], a well-characterized target of Notch signaling.

Notch signaling regulates cell fate decision, stem cell renewal and proliferation [16, 17]. Notch pathway includes Notch receptors (Notch 1-4), ligands (DLLs and JAGs), negative and positive regulators, and transcription factors [16]. Upon ligand binding, Notch proteins are proteolytically cleaved in two steps by ADAM10 and γ-secretase, resulting in the Notch intracellular domain (NICD) which is translocated to the nucleus, interacting with transcription factor CSL/RBPJk and the mastermind-like protein, and regulating CSL target genes, such as the HES family and Hes-related repressor genes [16, 17]. Both promoting and inhibitory roles of Notch signaling have been reported in several types of human cancers [17], including human OS [18-23]. Thus, it remains to be fully elucidated if Notch signaling plays an important role in the development of human OS.

In this study, we investigate if Notch signaling mediates BMP9-promoted OS cell proliferation and tumor growth. We find that the expression of Notch receptors and ligands is readily detected in most of the tested 11 OS cell lines. BMP9-promoted OS cell proliferation, migration, and cell cycle S/G2 progression are effectively blocked by a dominant-negative mutant of Notch1 (dnNotch1) or the γ-secretase inhibitor Compound E (ComE). Moreover, BMP9-promoted OS tumor growth and osteolytic lesions are significantly inhibited by the forced expression of dnNotch1 mutant. Mechanistically, BMP9 is shown to up-regulate the expression of Notch receptors and ligands, and to induce the nuclear accumulation of NICD in OS cells. These results suggest that BMP9-promoted OS proliferation and tumor growth may be mediated by Notch signaling. Thus, targeting Notch pathway may provide an important alternative to OS therapies.

MATERIALS AND METHODS

Cell Culture and Chemicals

Human OS lines MG63, 143B, U2OS, SaO2, MNG/HOS, and TE85 were recently purchased from ATCC (Manassas, VA, USA). The highly metastatic subline MG63.2 was previously described [24]. Human OS lines UCOS4, UCOS7, UCOS10, and UCOS11 were isolated from primary OS tumors as previously described [8]. Cells were maintained in complete DMEM containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 units of penicillin and 100μg of streptomycin at 37°C in 5% CO_2 [8, 25-29]. The γ-secretase inhibitor Compound E (ComE) was purchased from Enzo Life Sciences (NY, USA) and dissolved in DMSO. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Recombinant Adenoviruses Expressing BMP9, dnNotch1, RFP and GFP

Recombinant adenoviruses were generated using AdEasy technology [11, 12, 30-32]. The coding regions of human BMP9 and the extracellular domain with the transmembrane region (aa #1-aa #1705) of mouse Notch1 (Suppl. Fig. **1A**) were PCR amplified and cloned into an adenoviral shuttle vector, and subsequently used to generate recombinant adenoviruses in 293 packaging cells. The resulting adenoviruses were designated as AdBMP9 which also expresses GFP, and AdR-dnNotch1 which expresses RFP. Analogous adenovirus expressing only RFP (AdRFP) or GFP (AdGFP) was used as controls [15, 25, 30, 32-34].

RNA Isolation and Semi-quantitative RT-PCR (sqPCR)

Total RNA was isolated by using TRIZOL Reagents (Invitrogen) and used to generate cDNA templates by RT reaction with hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA). The cDNA products were used as PCR templates. The sqPCR were carried out as described [35-38]. PCR primers (Suppl. Table **1**) were designed by using the Primer3 program and used to amplify the genes of interest (approximately 150-180bp). A touchdown cycling program was used as follows: 94°C for 2 min for 1 cycle; 92°C for 20 s, 68°C for 30 s, and 72°C for 12 cycles decreasing 1°C per cycle; and then at 92°C for 20 s, 57°C for 30 s, and 72°C for 20 s for 20-25 cycles, depending on the abundance of target genes. PCR products were resolved on 1.5% agarose gels. All samples were normalized by the expression level of GAPDH.

Crystal Violet Staining

Subconfluent cells were seeded in 12-well plates, infected with the indicated adenoviruses and/or treated with drug. Cells were subjected to crystal violet staining after 48h infection. Images were taken from the plates. For quantitative measurement, the stained cells were dissolved in 10% acetic acid at room temperature for 20min with agitation. Absorbance was measured at 570~590nm [39-41].

FACS Analysis

Subconfluent cells were harvested, fixed with 70% ethanol, washed with PBS and stained with Hoechst 33342. Cell cycles were analyzed using the BD™ LSR II Flow Cytometer and the FlowJo software.

Gap Closure Cell Migration Assay

A modified gap closure migration assay was performed as described [42, 43]. Subconfluent cells were treated with drug and/or infected with AdRFP, AdGFP, AdBMP9 and/or AdR-dnNotch1 for 24h. Cells were trypsinized, counted and replated in 1% FBS complete medium in 12-well plates containing a sterile pyrex cylinder (6.0mm outside diameter) to create a gap on the monolayer cells. At 6-8h after plating, the cylinder was removed and recorded for bright field images of the gaps at 0, 18h and 36h. The remaining open areas were quantitatively measured by using ImageJ software. The assays were done in triplicate. Representative images are shown.

Immunofluorescence Staining

Subconfluent cells were treated with drug and/or adenoviral infection and fixed with 4% paraformaldehyde. The fixed cells were then treated with 0.1% Triton-100 and blocked with 10% bovine serum albumin. Cells were

incubated with Notch1 antibody (against the NICD domain) (Santa Cruz Biotechnology) in 4°C overnight and stained with Cy3-anti-mouse IgG secondary antibody (Jackson ImmunoResearch). Cell nuclei were counter-stained with DAPI, followed by fluorescence microscopic imaging.

Tibial Subperiosteal Tumor Formation

143B cells tagged with firefly luciferase (i.e., 143B-Luc) were infected with AdRFP, AdGFP, AdBMP9 and AdR-dnNotch1 as described [8, 37-40, 44]. Cells were harvested and resuspended in sterile PBS at 2×10^7 cells/ml. Cells (1×10^6, or 50μl of resuspended mix) were injected into the subperiosteum of proximal tibia of athymic nu/nu mice (4-6wk old, female, 5 mice/group) (Harlan Laboratories, Indianapolis, IN). Volumes of the leg and tumor were calculated by using the following equation: volume = (L + W)(L)(W)(0.2618)[24, 45]. Width (W) was an average of the distance at the proximal tibia at the level of the knee joint in the anterior–posterior and medial–lateral planes. Length (L) was the distance from the most distal extent of the calf musculature or distal tumor margin to the knee joint or proximal tumor margin.

Xenogen Bioluminescence Imaging

For bioluminescence imaging, mice were anesthetized with isoflurane attached to a nosecone mask within Xenogen IVIS 200 imaging system [8, 37-40, 44]. Mice were injected (i.p.) with D-Luciferin sodium salt (Gold BioTechnology) at 100 mg/kg in 0.1ml PBS. The pseudoimages were obtained by superimposing the emitted light over the gray-scale photographs of the mice. Quantitative analysis was done with Xenogen's Living Image software.

Micro-computed Tomography Analysis

All retrieved specimens were fixed and imaged using the μCT component of a GE triumph (GE healthcare, Piscataway, NJ, USA) trimodality preclinical imaging system. All image data analysis was performed using Amira 5.3 (Visage Imaging, Inc., San Diego, CA), and 3D volumetric data were obtained as described [36, 41, 46-49].

Histological Evaluation and Immunohistochemical Staining

Retrieved tissues were fixed, decalcified in 10% buffered formalin, and embedded in paraffin. Serial sections were stained with H & E. For immunohistochemical staining, sections were deparaffinized, rehydrated, subjected to antigen retrieval, and probed with anti-PNCA antibody (Santa Cruz Biotechnology) [8, 12, 15, 25, 31, 34, 36, 46, 47], followed by incubation with biotin-secondary antibodies and streptavidin-horseradish peroxidase. PCNA protein was visualized by 3,3'-diaminobenzidine staining. Control IgG and minus-primary antibody staining were used as negative controls.

Statistical Analysis

All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean ± SD. Statistical significances were determined by one-way analysis of variance and the student's *t* test. A value of $p<0.05$ was considered statistically significant.

RESULTS

Notch Receptors and Ligands Are Expressed in Human OS Cells

We first examined the endogenous expression of Notch receptors and ligands in six commonly-used OS cell lines (143B, MG63, U2OS, SaOS2, TE85, MNNG/HOS) and a MG63-derived highly metastatic subline MG63.2 [24]. All seven OS lines expressed detectable levels of DLL1, but relatively undetectable levels of DLL4, while MG63.2 exhibited a highest level of DLL3 expression (Fig. **1A**). Both JAG1 and JAG2 were expressed in all seven OS lines (Fig. **1A**). Among the four Notch receptors, all but Notch4 were expressed in the seven OS lines (Fig. **1A**).

We also analyzed the endogenous expression of Notch receptors and ligands in four OS lines, which were previously characterized [8]. As shown in Fig. **1B**, three of the four lines expressed high levels of DLL ligands, JAG1, and three Notch receptors. UCOS11 line expressed low or undetectable levels of DLL ligands. JAG2 expression was high in UCOS4 line, while Notch4 expression was undetectable in all four OS lines (Fig. **1B**). Furthermore, we examined the expression of Notch1 receptor in OS xenograft tumor samples derived from 143B periosteal injection in nude mice. The immunohistochemical staining indicates that the xenograft OS tumors expressed a high level of Notch1 receptor (Fig. **1C**, panels ***a*** *vs.* ***b***). These results strongly suggest that most of Notch receptors and/or ligands may be readily expressed in human OS cells.

BMP9 Promotes OS Cell Proliferation and Migration, Which Can Be Effectively Inhibited by the Blockade of Notch Pathway *in vitro*

We previously demonstrated that BMP9 can promote OS tumor growth in an orthotopic tumor model [1, 8]. Here, we sought to test if BMP9 promotes OS growth through the activation of Notch signaling. To achieve this goal, we constructed a recombinant adenovirus expressing dnNotch1 (AdR-dnNotch1) (Suppl. Fig. **1A**). We confirmed that the dnNotch1 was highly expressed in AdR-dnNotch1 infected cells (Suppl. Fig. **1B**), and the adenoviral vector infected OS cells with high efficiency (Suppl. Fig. **1C**). When 143B and MG63 were transduced with GFP, BMP9, and/or dnNotch1, BMP9 was shown to enhance OS cell proliferation, which could be inhibited by dnNotch1 ($p<0.001$) (Fig. **2A**, panels a *vs*. b). It is noteworthy that the forced expression of dnNotch1 alone slightly inhibited OS cell proliferation, compared with that of the GFP control (Fig. **2A**). Several Notch pathway inhibitors, most of which are small molecule inhibitors of γ-secretase, have been recently characterized and widely used. We tested one of such inhibitors ComE and found that BMP9-stimulated cell proliferation was significantly inhibited by ComE at a modest concentration (1μM) (Fig. **2B**, panels **a** & **b**). Thus, using both dnNotch1 and Notch signaling inhibitor ComE, we demonstrate that BMP9-stimulated OS cell proliferation can be effectively inhibited by blocking the Notch signaling pathway.

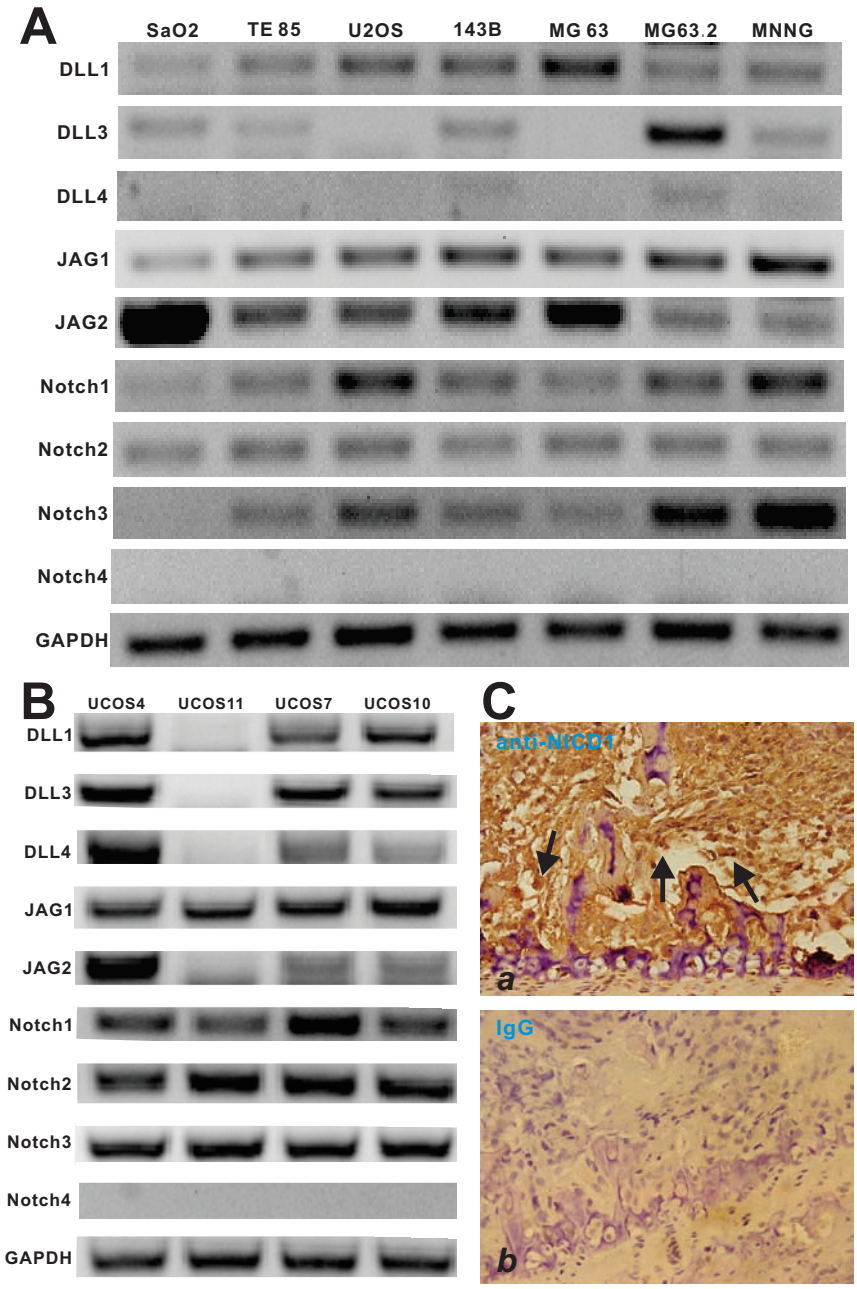

Fig. (1). Endogenous expression of Notch receptors and ligands in human OS cells. (**A**) Endogenous expression of Notch ligands and receptors in human OS cell lines. Cells were maintained in 1% FBS complete medium for 24h. Total RNA was isolated and subjected to semi-quantitative RT-PCR (sqPCR) analysis. All samples were normalized for GAPDH expression. Reactions were repeated in at least three independent experiments, and representative results are shown. (**B**) Endogenous expression of Notch ligands and receptors in 4 human OS lines derived from OS patients. The established OS lines were cultured in subconfluenct condition and subjected to sqPCR analysis as described in (A). (**C**) Endogenous expression of the NICD domain of Notch1 in OS xenograft tumors. The paraffin-embedded OS xenograft tumor samples derived from 143B periosteal injection in nude mice were subjected to deparaffinization, rehydration, and immuno-histochemical staining with an antibody against the NICD region of Notch1. (a) Control IgG was used as a negative control. (b) Arrows indicate the positively stained OS cells.

We also analyzed the effect of Notch signaling blockade on BMP9-promoted cell migration. Using gap closure migration assay, we found that BMP9 promoted gap closures in both OS lines, which was blocked by a forced expression of dnNotch1 ($p<0.001$) (Fig. **2C**, panels a & b). Exogenous expression of dnNotch1 alone in 143B cells was shown to slow down gap closures (Fig. **2C**). Accordingly, ComE was also shown to blunt the BMP9-promoted gap closures in OS lines (Fig. **2D**, panels a & b). These results indicate that BMP9 can promote OS cell migration, which may be mediated by Notch signaling.

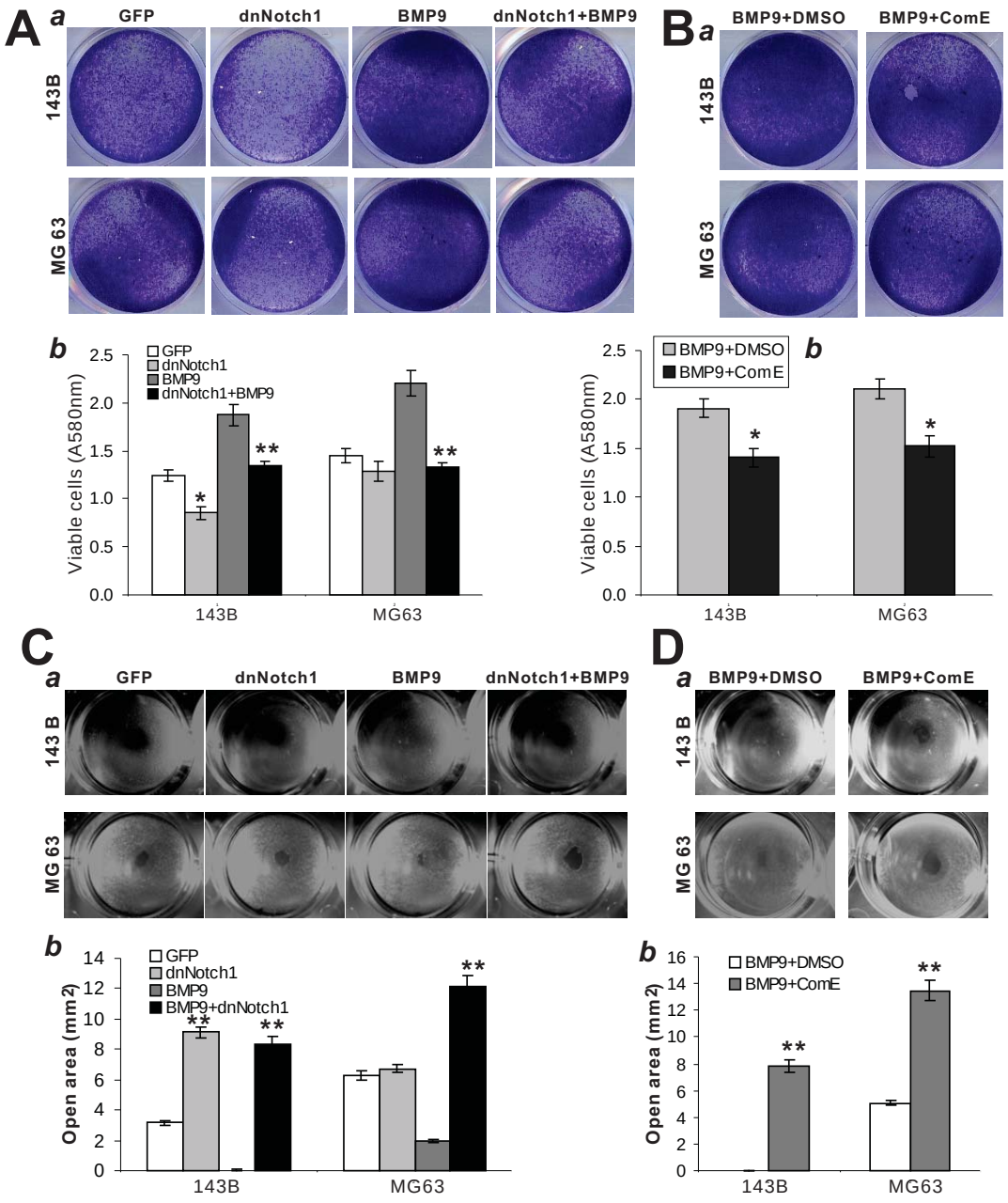

Fig. (2). BMP9-stimulated OS proliferation and migration are inhibited by the blockade of Notch signaling *in vitro*. (**A** and **B**) Cell proliferation assessed by crystal violet staining. 143B or MG63 cells were infected with AdGFP, AdR-dnNotch1, AdBMP9, and AdBMP9+AdR-dnNotch1 (**A**) and seeded in 12-well cell culture plates at a low density. Alternatively, 143B or MG63 OS cells were seeded at a low density and treated with DMSO or ComE (1μM) (**B**). Viable cells were stained with crystal violet at day 5 (**a**). For quantitative analysis, the stained cells were dissolved with 10% acetic acid, and A580nm was measured (**b**). (**C** and **D**) Gap closure migration assay. Sterile cylinders were first placed at the center of each well of 12-well culture plate. Freshly harvested OS cells, which were infected with adenoviral vectors (**C**), were seeded outside the cylinders at subconfluency. For the cells treated with ComE, the OS cells were infected with BMP9 adenovirus first, seeded with the pre-placed cylinders, and treated with ComE (1μM) (**D**). Cylinders were removed at 6-8h post plating. At 72h, cells were fixed and imaged under a microscope (**a**). ImageJ software was used to quantitatively determine the unclosed areas (**b**). All assay conditions were done in triplicate. The representative images are shown. "*" $p<0.05$; "**" $p<0.001$ (*vs.* respective controls).

BMP9 Promotes S/G2 Progression in OS Cells, Which Can Be Inhibited by Blocking Notch Signaling

To further test if Notch signaling mediates BMP9-regulated cell proliferation, we analyzed the cell cycle profiles in the presence of BMP9 and/or blockade of Notch signaling. When 143B cells were transduced with BMP9, the S/G2 phase increased from 53.5% to 61.5%, which was reduced to 56.8% by the forced expression of dnNotch1 (Fig.

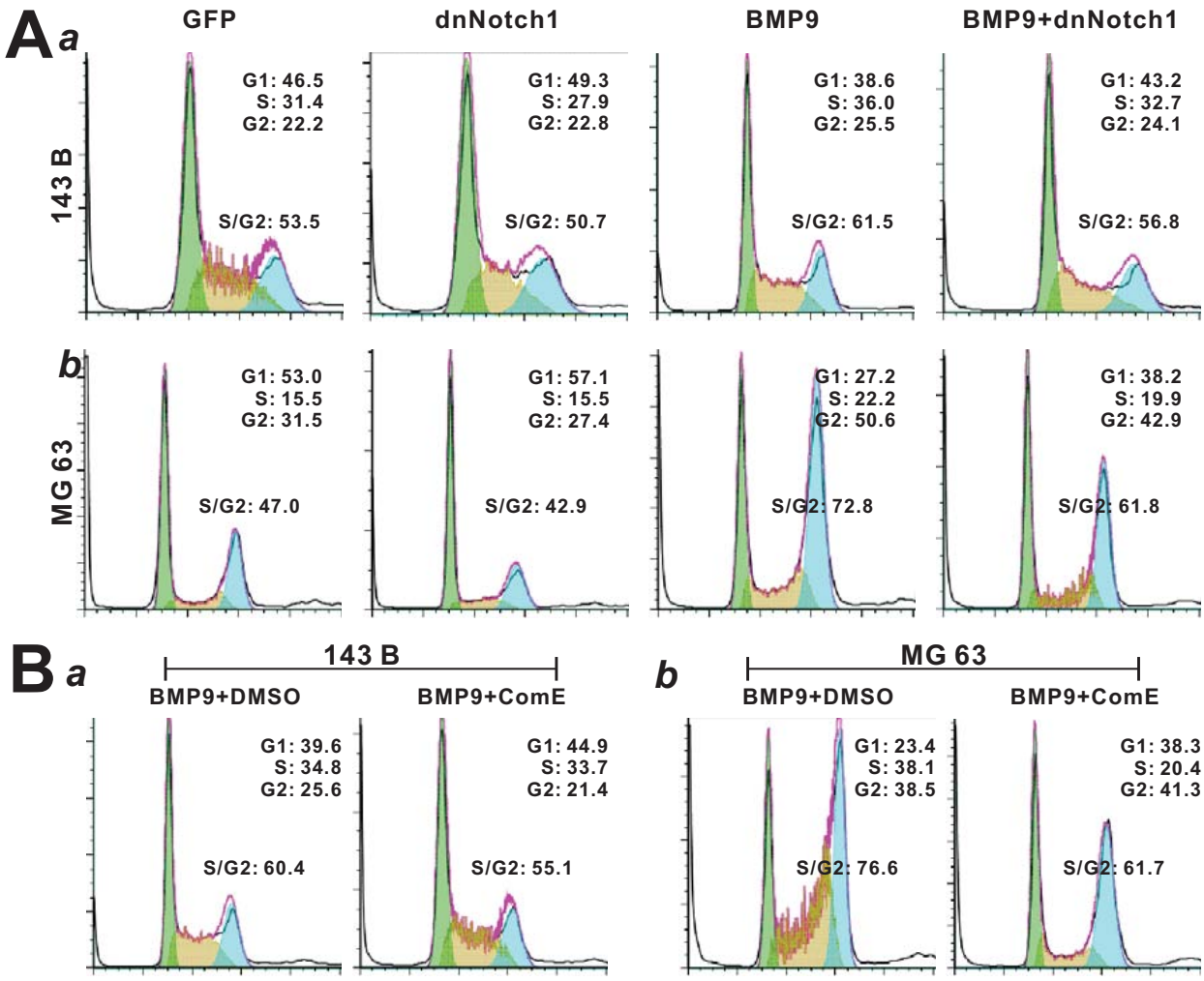

Fig. (3). BMP9 promotes the S/G2 progression of OS cells, which can be blocked by inhibiting Notch signaling. (**A**) Dominant-negative Notch1 (dnNotch1) can reduce BMP9-promoted S/G2 phase of OS cells. OS cells were infected with the indicated adenoviral vectors and maintained in 1%FBS medium for 48h. Cells were harvested, stained with Hoechst 33342 for FACS analysis. (**B**) BMP9-promoted S/G2 progression of OS cells can be inhibited by ComE. OS cells were seeded in T-25 flasks and infected with AdBMP9, followed by a treatment with either DMSO or ComE (1μM). At 48h, cells were collected and stained with Hoechst 33342 for FACS analysis. Each assay condition was done in triplicate. Representative results are shown.

3A, panel a). Similar results were obtained in MG63 cells except with a greater effect. BMP9 stimulation increased the S/G2 phases of MG63 to 72.8% from 47.0%, which was reduced to 61.8% by dnNotch1 expression (Fig. **3A**, panel b). It is noteworthy that forced expression of dnNotch1 alone also slightly reduced S/G2 phase in both OS lines compared with that of the GFP-treated cells. Furthermore, ComE was shown to reduce the BMP9-promoted S/G2 phase from 60.4% to 55.1% in 143B cells (Fig. **3B**, panel a), and 76.6% to 61.7% in MG63 cells (Fig. **3B**, panel b), respectively. Thus, we demonstrated that BMP9 effectively promotes the S/G2 progression of OS cells, which can be blocked by inhibiting Notch signaling.

BMP9-Augmented OS Tumor Growth and Invasiveness Is Inhibited by the Forced Expression of dnNotch Mutant

We next tested if the BMP9-promoted OS tumor growth *in vivo* could be inhibited by the blockade of Notch signaling. Consistent with our previous findings [8], BMP9 significantly promoted OS tumor growth in the orthotopic tumor model, which was inhibited by the forced expression of dnNotch1 mutant in 143B cells (Fig. **4A**). Using the Xenogen bioluminescence imaging technique, we confirmed that BMP9 effectively promoted OS tumor growth compared with that of GFP or dnNotch1 group at weeks 2 and 4 (Fig. **4B**, panels a & b). However, the BMP9-promoted OS tumor growth was significantly blunted by the forced expression of dnNotch1 at the indicated time points (Figs. **4C** & **4D**).

At the endpoint, the retrieved tumor-bearing animal samples were subjected to microCT analysis. The tibia and fibula bones of the animals injected with BMP9-transduced 143B cells exhibited rather extensive osteolytic lesions, compared with that of the GFP or dnNotch1 treated cells group. However, a forced expression of dnNotch1 was

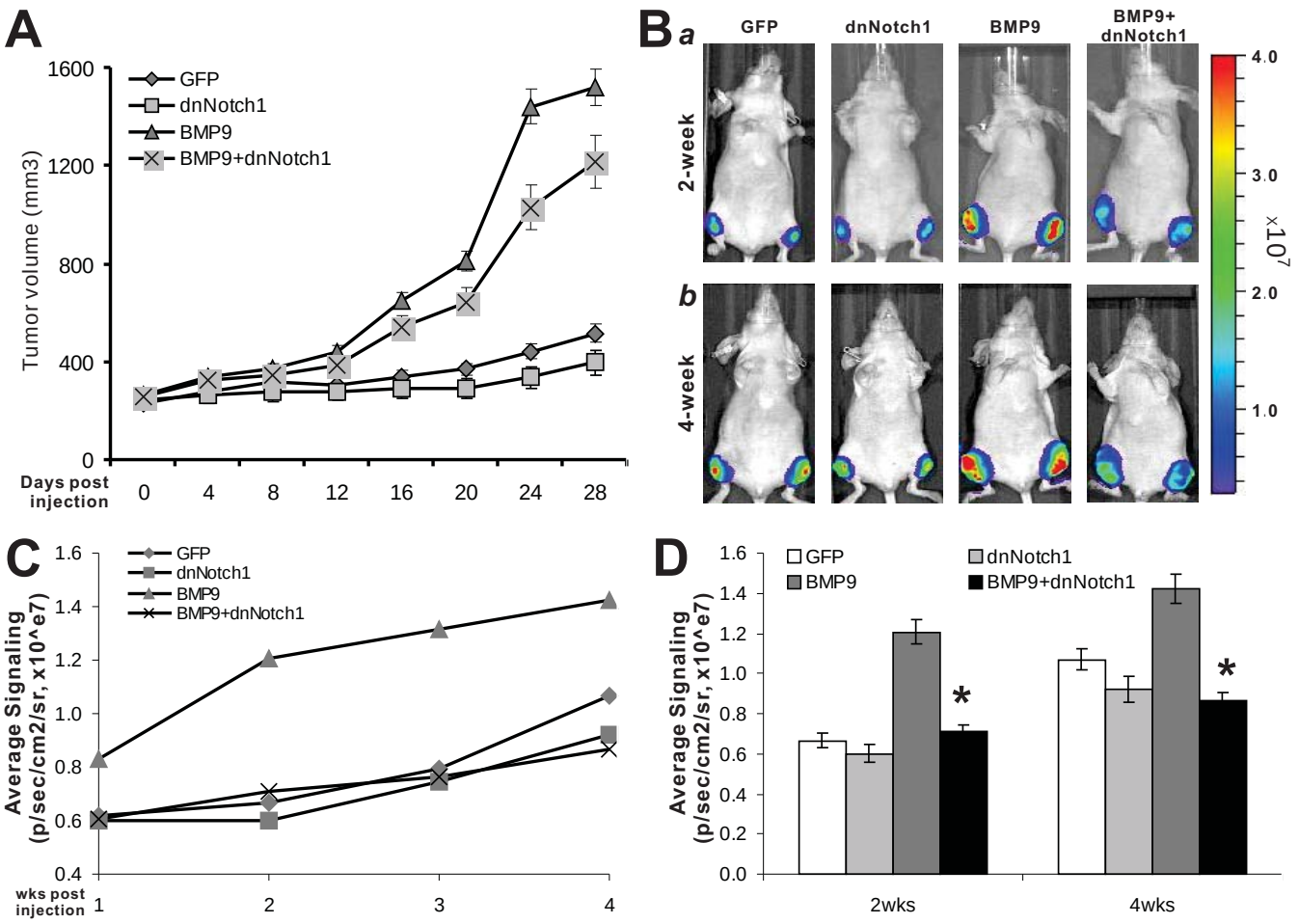

Fig. (4). BMP9-augmented OS tumor growth is inhibited by exogenous expression of dnNotch mutant. 143B-Luc cells were infected with GFP, AdBMP9 and/or AdR-dnNotch1 for 16h. The infected cells were collected and used for tibia subperiosteal injection into athymic nude mice ($1x10^6$ cells/injection; 5 mice per group). Tumor volumes were measured every 4 days (**A**).The injected mice were also subjected to weekly Xenogen optical images (**B**). Representative of the nude mice injected with treated 143B-Luc cells at 2wk and 4wk is shown. **(C** and **D)** Tumor sizes (in photons/sec/cm^2/steradian) were analyzed using Xenogen's Living Image software. "*" $p<0.05$ when compared with BMP9-treated groups at corresponding time points.

shown to at least partially block the development of osteolytic lesions (Fig. **5A**, panel a). In fact, BMP9 treatment group had the lowest average bone volumes of the tibia and fibula bones among the four groups, while dnNotch1 was shown to partially restore the bone volume (Fig. **5A**, panel b). The above results were consistent with the gross findings of the retrieved tumor samples (Fig. **5A**, panel c). Furthermore, the H & E histologic evaluation confirmed that BMP9 promoted aggressive tumor growth and caused extensive osteolytic lesions, which were partially restored by exogenous expression of dnNotch1 (Fig. **5B**, panels c vs. a, b, and d). Immunohistochemical staining using the antibody against cell proliferation marker PCNA revealed that the dominant-negative mutant of Notch1 effectively blocked BMP9-promoted proliferation of OS tumors (Fig. **5C**, *c vs. d*). Thus, these *in vivo* studies have further demonstrated that BMP9 can effectively promote OS tumor growth and aggressiveness, which is inhibited by the inactivation of Notch signaling in OS cells.

BMP9 Up-regulates Notch Signaling in OS Cells

To investigate how BMP9 regulates Notch signaling in OS cells, we analyzed if BMP9 could induce the expression of Notch receptors and/or ligands. We found that BMP9 up-regulated the expression of Notch1, Notch3, DLL1, and JAG1 in 143B (Fig. **6A**, panel a) and MG63 cells (Fig. **6A**, panel b), although other Notch receptors and ligands were not significantly induced by BMP9 (data not shown). The hallmark of Notch pathway activation is the γ-secretase-mediated cleavage and nuclear translocation of the Notch intracellular domain or NICD. We found that, when OS cells were effectively transduced with GFP or BMP9 (Fig. **6B**, panel a), the nuclear level of NICD was significantly elevated in BMP9-stimulated cells, compared with that of the GFP-treated cells (Fig. **6B**, panels b & c). Furthermore, BMP9-induced NICD accumulation was effectively blocked by ComE although ComE was also shown to block the basal level of nuclear NICD (Fig. **6B**, panels b & c). These results

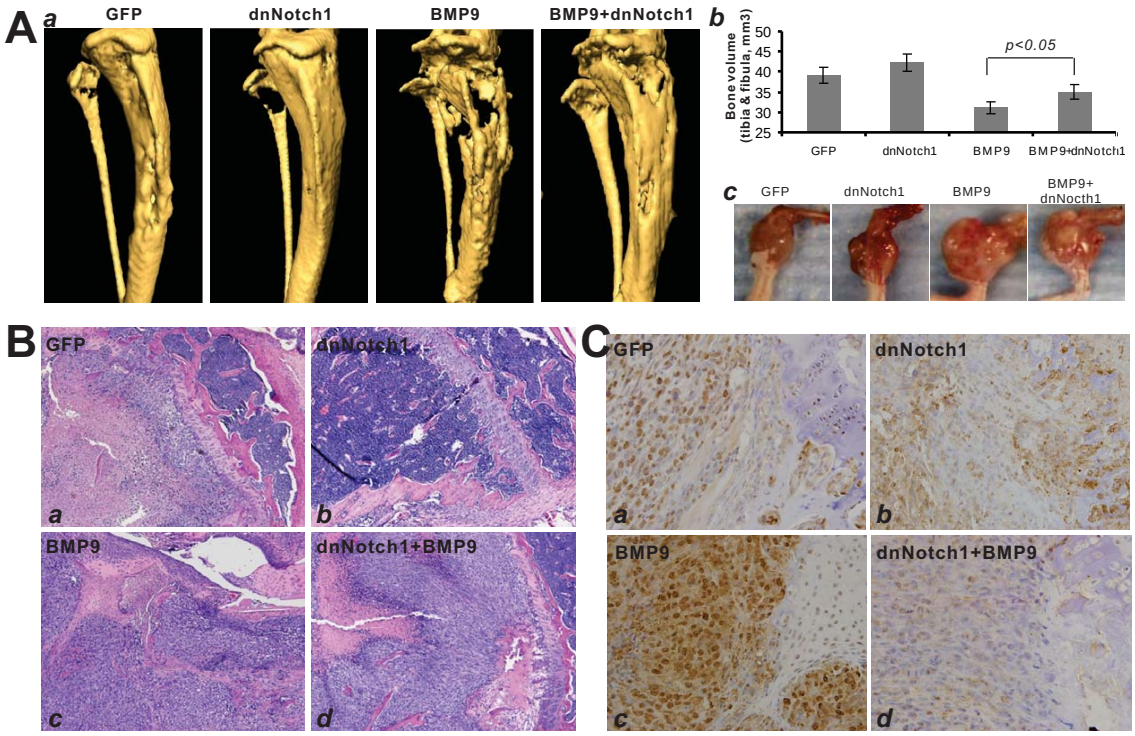

Fig. (5). Dominant-negative mutant of Notch1 can block BMP9-promoted invasiveness and proliferation of OS tumors *in vivo*. (**A**) OS cell-injected athymic mice were sacrificed at 4wk and subjected to micro-CT imaging for 3D reconstruction (**a**) and calculation of the average volumetric data of tibia & fibula bones (**b**) using Amira software. Representative images of resected tumor-bearing legs are shown in (**c**). (**B**) Retrieved tumor samples were fixed, decalcified, and subjected to paraffin-embedding and H & E staining. Representative images of H & E staining are shown (x100). (**C**) Proliferative activity of the OS tumor cells. Embedded tumor samples were sectioned and stained with PCNA antibody. Control IgG was used as a negative control (not shown). Representative images of anti-PCNA staining of tumor samples are shown.

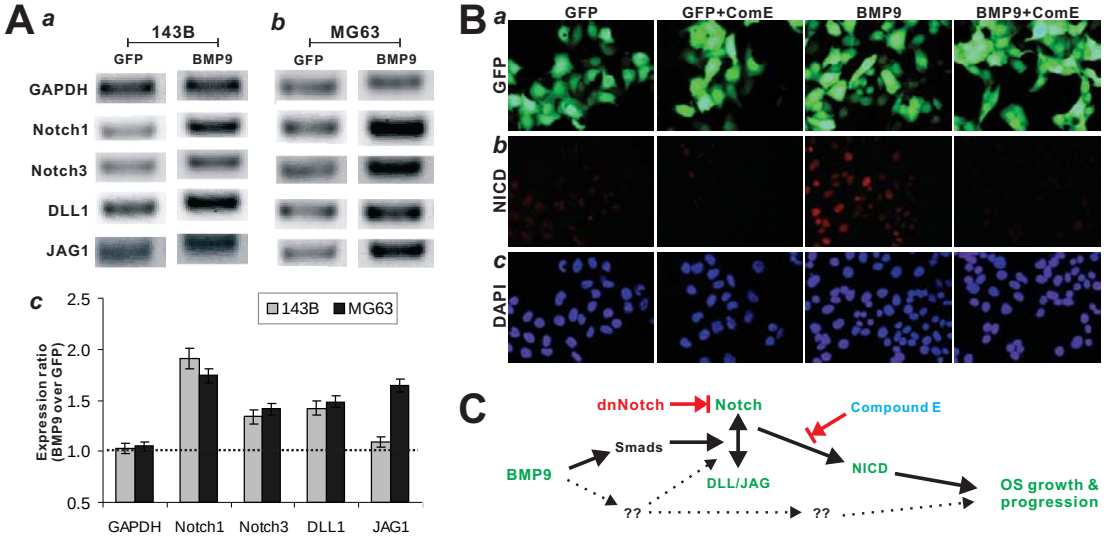

Fig. (6). BMP9 up-regulates Notch signaling in OS cells. (**A**) BMP9 induces the expression of Notch receptors and ligands in OS cells. 143B (**a**) and MG63 (**b**) cells were infected with AdGFP or AdBMP9. At 36h, the cells were collected for RNA isolation, followed by sqPCR analysis. The cDNA levels of all samples were normalized with GAPDH expression. The PCR analysis was repeated at in three independent experiments. The dotted line indicates the ratio of BMP9/GFP =1. Representative results are shown. (**B**) BMP9 activates Notch signaling in OS cells. 143B cells were infected with AdGFP or AdBMP9, and treated with DMSO or ComE (1μM). Adenoviral infection efficiency was confirmed by fluorescence microscopy at 24h (**a**). Cells were fixed at 48h and subjected to immunofluorescence staining using anti-NICD (Notch1) antibody (**b**). Cell nuclei were counter-stained with DAPI (**c**). Control IgG was used as a negative control (not shown). Representative results are shown. (**C**) The working mode for BMP9-regulated OS growth. BMP9 can promote OS cell proliferation and tumor growth at least in part through the activation of Notch signaling, possibly by up-regulating the expression of Notch receptors and ligands in OS cells. Conversely, dnNotch1 or downstream γ-secretase inhibitors, such as ComE, can block BMP9's effect on OS proliferation and tumor progression.

strongly suggest that Notch may play an important downstream role in mediating BMP9 signaling in OS cells.

DISCUSSION

We previously found that the highly osteogenic BMP9 is unable to induce osteogenic differentiation in human OS cells, but rather promotes OS cell proliferation and tumor growth [8]. Here, we demonstrate that BMP9-promoted OS cell proliferation, migration, and cell cycle S/G2 progression are blocked by a dominant-negative mutant of Notch1 (dnNotch1) or the γ-secretase inhibitor ComE *in vitro*. The forced expression of dnNotch1 mutant is shown to inhibit BMP9-promoted OS tumor growth and osteolytic lesions in the subperiosteum OS tumor model. Mechanistically, BMP9 is shown to up-regulate the expression of Notch receptors and ligands, and to induce the nuclear accumulation of NICD in OS cells. Based on these results, a mode of BMP9 action on OS cells is proposed (Fig. **6C**). In this mode, BMP9 can promote OS cell proliferation and tumor growth, at least in part, through the activation of Notch signaling, possibly by up-regulating the expression of Notch receptors and ligands in OS cells. A dominant-negative mutant form of Notch1 or downstream γ-secretase inhibitors, such as ComE, can effectively block BMP9's effect on OS proliferation and tumor progression. Thus, our results strongly suggest that BMP9-promoted OS proliferation and tumor growth may be mediated by Notch signaling. Thus, the targeted inhibition of Notch pathway may be considered as a possible adjuvant treatment of OS.

BMP9 (also known as growth differentiation factor 2, or GDF2) was originally identified in developing mouse liver [13, 50]. In addition to its function as an osteogenic factor [9, 11-13], BMP9 plays certain roles in inducing and maintaining embryonic basal forebrain cholinergic neurons, inhibiting hepatic glucose production and inducing the expression of key enzymes of lipid metabolism, stimulating hepcidin 1 expression, and regulating angiogenesis [13]. We have demonstrated that TGFβ/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in MSCs [47]. Gene expression profiling analysis identifies several early downstream targets, including Hey1, of BMP9-induced osteoblast differentiation [9, 13, 15, 33, 34, 48, 51]. Interestingly, some of the early target genes, such as Ids and CTGF [33, 34, 51], are involved in progenitor cell propagation and up-regulated in many cancers [8, 13]. Nonetheless, the exact roles of BMP9 in OS tumorigenesis remain to be fully elucidated.

Our studies strongly suggest that Notch pathway may play an important role in mediating BMP9 signaling. Notch signaling plays a critical role in bone and skeletal development as Notch knockout mice exhibit severe skeletal abnormalities [52-54]. DLL3 Mutations cause spondylocostal dysostosis in humans [17, 54]. A forced expression of Notch ligands and receptors impairs both osteoblastic and osteoclastic differentiation of the progenitor cells [17, 53, 54].

Aberrant activation of Notch signaling has been reported in many types of human cancers, such as T-cell leukemia, lung cancer, colorectal cancer, prostate cancer and breast cancer [55], although Notch signaling was also shown to inhibit tumorigenesis in B-cell leukemia, neural tumors, and skin cancer, suggesting that the effect of Notch signaling on tumorigenesis may depend on cellular context [56]. Notch receptor expression has been detected in OS tumor samples, OS cell lines and osteoblastic cells [19, 20]. It was reported that Notch inhibition by γ-secretase inhibitors or by the expression of dominant-negative Mastermind-like protein (DN-MAML) inhibits OS cell proliferation and tumor growth [18], possibly through inhibition of cell cycle regulator expression in OS cells [21]. Notch signaling has recently shown to play an important role in controlling the invasiveness and metastasis of OS tumors [22, 23]. These reported results are supportive of the current findings. Moreover, our studies may provide further evidence about the possible upstream regulators of Notch signaling in OS cells. It is conceivable that pharmacologic intervention of Notch signaling (e.g., using γ-secretase inhibitors) may be considered a new therapeutic strategy for human OS tumors.

CONCLUSIONS

We investigate if Notch signaling mediates BMP9-promoted OS cell proliferation and tumor growth. Our results indicate that the expression of Notch receptors and ligands is readily detected in most of the tested 11 OS cell lines. BMP9-promoted OS cell proliferation, migration, and cell cycle S/G2 progression are effectively blocked by a dominant-negative mutant of Notch1 (dnNotch1) or the γ-secretase inhibitor Compound E. BMP9-promoted OS tumor growth and osteolytic lesions are significantly inhibited by the forced expression of dnNotch1 mutant. Mechanistically, BMP9 is shown to up-regulate the expression of Notch receptors and ligands, and to induce the nuclear accumulation of NICD in OS cells. Taken together, our results suggest that BMP9-promoted OS proliferation and tumor growth may be mediated by Notch signaling. Thus, targeting Notch pathway may provide an important alternative to OS therapies.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

LIST OF ABBREVIATIONS

BMP	=	bone morphorgenetic protein
DAPI	=	4',6-diamidino-2-phenylindole
DMSO	=	dimethyl sulfoxide
FACS	=	fluorescence-activated cell sorting
GAPDH	=	glyceraldehyde 3-phosphate dehydrogenase
GFP	=	green fluorescent protein
MSC	=	mesenchymal stem cell
NICD	=	Notch intracellular domain
OS	=	osteosarcoma
PCNA	=	proliferating cell nuclear antigen
RFP	=	red fluorescent protein
TGFβ	=	transforming growth factor β

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