TANG [HUMANITAS MEDICINE]

Original Article

Effects of the Fraction of Sambucus Williamsii, NNMBS 246, on Osteoblastic Differentiation

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ABSTRACT

In the field of osteoporosis, there has been growing interest in anabolic agents that enhance bone formation. The purpose of this study was to examine the effects of NNMBS 246 osteoblastic differentiation with associated signaling pathways. NNMBS 246 markedly increased alkaline phosphatase (ALP) activity and calcium nodule formation. Stimulation with NNMBS 246 not only increased the differentiation markers (ALP, OPN, OCN) level and transcription markers (RUNX2, Osterix) mRNA expression but also upregulated the ECM molecules and OPG mRNA expression. Treatments of NNMBS 246 downregulated MMPs (MMP-1, MMP-2, MMP-9), but RANKL mRNA expression. Furthermore, NNMBS 246 activated osteoblastic differentiation markers and formed calcium nodules in human periodontal ligament cells (hPDLCs) and cementoblast cells. NNMBS 246 induced phosphorylation of MAPKs, Akt, nuclear p65 and IkB-α. BMP-2/Smad and β-catenin signaling pathways were activated by NNMBS 246. Sirtinol (SIRT1 inhibitor) inhibited NNMBS 246-induced osteoblastic differentiation markers mRNA expression. These results suggested that NNMBS 246 has the potential to enhance osteoblastogenesis probably through the activation of BMP/Smad and β-catenin signal pathways, and SIRT1 plays as critical mediator in bone anabolic effect of NNMBS 246.

Keywords NNMBS 246, Osteoblast, Differentiation, BMP Pathway, SIRT1 Pathway

INTRODUCTION

The periodontium is a complex anatomical structure composed of both hard (bone and cementum) and soft (periodontal ligament) connective tissues. Periodontal tissue regeneration requires the restoration of both hard and soft connective tissues to their original functional architecture in a spatially defined microenvironment. In particular, to restore functional resistance to masticatory load, periodontal ligament fibers must insert perpendicularly to cementum (Bartold PM and McCulloch CA, 2000). Currently, there are a number of approaches utilized to induce periodontal regeneration, for example guided tissue regeneration (Karring T and Nyman S, 2000). Recently, a number of therapeutic agents, such as the basic fibroblast growth factor (bFGF), polyphosphate (poly(P)), platelet-derived growth factor and bone morphogenetic protein, have been used with tissue engineered techniques to enhance the bone regeneration (Lieberman JR and Daluiski A, 2002; Ripamonti U and Reddi AH, 1994). However, these approaches are associated with unpredictable and variable outcomes.

The integrity of bone tissue depends on maintaining a delicate balance between bone resorption caused by osteoclasts and bone deposition caused by osteoblasts (Silva TA and Garlet GP, 2007). Recently, non-invasive pharmacological treatments using proteinaceous biologicals and natural products for periodontal disease have been studied. For example, SBD.4A – a novel, stable multicomponent growth factor isolated from a medicinal plant *Angelica sinensi*, revealed beneficial effect of PDL cells as well as bone regeneration in the calvarial model (Zhao H and Alexeev A, 2008). In addition, PPAR-gamma agonist (rosiglitazone) suppressed the bone resorption by inhibiting RANKL-mediated osteoclastogenesis elicited during the course of experimental periodontitis in rats (Hassumi MY and Silva-Filho VJ, 2009). Green tea polyphenol (-)-epigallocatechin gallate also prevent the alveolar bone resorption that occurs in periodontal diseases by inhibiting the expression of MMP-9 in osteoblasts and the formation of osteoclasts (Yun JH and Pang EK, 2004). Previously we reported that sappanchalcone isolated from *Caesalpinia sappan L* inhibit LPS-induced inflammatory mediators in PDL cells (Jeong GS and Lee DS, 2010). In addition, we demonstrated that apigenin downregulated NO, PGE2 and pro-inflammatory cytokines responses in periodontal pathogens and nicotine-stimulated PDL cells (Jeong GS and Lee SH, 2009). Sirtuin 1 (SIRT1) is known to deacetylate histones and non-histone proteins including transcription factors thereby regulating metabolism, stress resistance, cellular survival, cellular senescence/aging, inflammation- immune function and apoptosis (H.A. Tissenbaum and L. Guarente, 2001; S.C. Dryden and F.A. Nahhas, 2003). Recently, SIRT1 has been shown to play a critical role in differentiation of muscle, fat cells, pig pre-adipocytes, mesenchymal stem cells (MSCs) and primary rat bone marrow stromal cells (Bäckesjö CM and Li Y, 2006; Bai L and Pang WJ, 2008; Blander G and Bhimavarapu A, 2009; Fulco M and Schiltz RL, 2003; Hisahara S and Chiba

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S, 2008; Picard F and Kurtev M, 2004).

The herb *Sambucus williamsii* (*S. williamsii*) is a folk medicinal plant, which is widely distributed in Europe, North Africa, and Asia including Korea, Japan, and China (T.B. Lee, 1998). *S. williamsii* been used in traditional medicine as an analgesic, antivirus, anti-inflammatory, homoeostatic, and diuretic drugs which act on bruises, fractures, and edema (F. Xie and C.F. Wu, 2005; R.H. Zhang and H.M. Qiu, 1997; T.B. Lee, 1998). Previous phytochemical investigation regarding *S. williamsii* has reported the presence of terpenes, fatty acids, phenolic compounds, and lignans (H. Xiao and Y. Dai, 2014; Wang Z and Han H, 2011). However, it is not known whether SIRT1 regulates periodontal regeneration-related cells including osteoblasts, cementoblasts and PDL cells in response to natural products NNMBS 246.

To investigate the osteogenic potential of NNMBS 246 in periodontal regeneration as well as the underlying mechanism involved, we studied the effect of NNMBS 246 on the osteoblastic differentiation in human osteoblasts, cementoblasts and PDL cells. In addition, we examined the potential involvement of SIRT1 in the differentiation -inducing activity elicited by NNMBS 246.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagent were purchased from Gibco BRL Co. (Grand Island, NY). Anti-SIRT1 antibody (Ab) was purchased from Abcam (Cambridge, UK). Antibodies against ERK, phospho-ERK (p-ERK), p38, phospho-p38 (p-p38), JNK, phosphor-JNK (p-JNK), Akt, phosphor-Akt (p-Akt), AMPK, phosphor-AMPK (p-AMPK), NF-Kb p65, Nrf-2, and I-kBα were purchased from Cell Signaling (Beverly, MA). All other chemicals were obtained from Sigma Chemical Co. unless otherwise stated.

Plant Material and Preparation of NNMBS246

The dried stem of *Sambucus latipinna* (*S. Lappa*) were purchased from the University Oriental Herbal Drugstore, Iksan, Korea, in August 2010, and a voucher specimen was deposited at the Herbarium of the College of Pharmacy at Wonkwang University, Iksan, Korea. The dried stem of *S. latipinna* (50 g) were extracted twice with hot 70% ethanol (1 L) for 2 h at room temperature. The 70 % ethanol extract was suspended in distilled water (100mL), followed by filtration. The filtrate was then evaporated *in vacuo* to produce a standard fraction of S. Lappa (NNMBS246, 2.27 g, 4.5 w/w%). NNMBS246 was deposited at the Standardized Material Bank for New Botanical Drugs, College of Pharmacy at Wonkwang University.

Cell culture

We used human osteosarcoma cell (MG-63) line. Cells were cultured in DMEM supplemented with 10% FBS, 100 U/Ml penicillin and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. To induce differentiation, MG-63were cultured with osteogenic medium (OM)-DMEM supplement with 10% FBS, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, and 10-7 M dexamethasone (DEXA) as described previously (Bäckesjö CM and Li Y, 2006; Blander G and Bhimavarapu A, 2009; Bolander ME, 1992; Kawai T and Matsuyama T, 2006). The culture medium was 72 °C for 30 s. PCR products were resolved on 1.5 % agarose gels and stained with ethidium bromide.

replaced every 3 days during the incubation period. This study was approved by the institutional review board and ethical committee at Wonkwang University.

Alkaline phosphatase (ALP) activity

ALP, a marker of osteoblast activity, was assayed using a buffer containing 2-amino-2-methylpropanol and p-nitrophenyphosphate (Sigma-Aldrich Inc). Absorbance was measured at 415 nm using an enzyme-linked immunosorbent assay reader (Beckman Coulter, Fullerton, CA). MG63 cells were incubated with NNMBS246 and cell growth was measured. Based on this preliminary observation (non-cytotoxic up to a concentration of 50 μg/mL), the differentiation-inducing activities of NNMBS 246 on MG-63 cells were assessing for intracellular ALP activity, RT-PCR and Alizarin red staining.

RNA isolation and reverse transcriptase-polymerase chain reaction

Total RNA was isolated using the Trizol reagent (Invitrogen, Carsbad, CA) according to the manufacturer's instructions. It was then reverse transcribed using AccuPower RT Premix (Bioneer, Daejeon, Korea). PCR amplification of the resulting cDNA samples was performed using a GeneAmp PCR System 2400 thermal cycler (PerkinElmer, Wellesley, MA). The primers used were: ALP, 5'-ACGTGGCTAAGAATGTCATC-3' and 5'-CTGGTAGGCGATGTCCTTA-3' (476 bp); OPN, 5'-CCCACAGACCCTTCCAAGTA-3' and 5'-GGGGACAACTGGAGTGAAAA (244 bp); OCN, 5'-GTGCAGCCTTTGTGTCCAAGCAGGA-3' and 5'-CCGTAGAAGCGCCGATAGGCC-3' (196 bp); osterix, 5'-CTCTGCGGGACTCAACAACTCTG-3' and 5'-GAGCCATAGGGGTGTGTCATGTC-3' (316 bp); Runx2, 5'-AGACCAACAGAGTCAGTGAGTGCT-3' and 5'-TGGTGTCACTGTGCTGAAGAGGC-3' (317 bp); MMP-1, 5'-GAAGGTGATGAAGCAGCCCA-3'- and 5'-CAGTTGTGGCCAGAAAACAG-3' (721 bp); MMP-2, 5'-ATCCGTGGTGAGATCTTCTT-3' and 5'-AGCCAGGATCCATTTTCTTC-3' (364 bp); MMP-9, 5'-TGCCAGTTTCCATTCATCTT3' and 5'-CTGCGGTGTGGTGGTGGTT-3' (519 bp); collagen 1, 5'-GGACACAATGGATTGCAAGG-3' and 5'-TAACCACTGCTCCACTCTGG-3' (461 bp); elastin. 5'-GTTGGTGTCGGCGTCCCTGG-3' and 5'-AGCGGCTGCAGCTGGAGGTA-3' (354 bp); fibronectin, 5'-TGGAACTTCTACCAGTGCGAC-3' and 5'-TGGAACTTCTACCAGTGCGAC-3' and 5'-TGTCTTCCCATCATCGTAACAC-3' (451 bp); OPG, 5'-TCAAGCAGGAGTGCAATCG-3' and 5'-AGAATGCCTCCTCACACAGG-3' (342 bp); RANKL, 5'-GAGGCCAGAGACCAGCCCGA-3' and 5'-CTTGCTCCTGCACCGGCCTC-3' (373 bp); CEMP1, 5'-CAGGATCCACATCCGTC-3' and 5'-CTGAACAGCTTCGAGGC-3' (434 bp); IBSP, 5'-AGAGACCGGAAGGCAGGGCA-3' and 5'-CCCCACGAGGTTCCCCGTTC-3' (218 bp); CAP, 5'-GCGGAACTCTGAGGTGGTCC-3' and 5'-CAGGCCAGTCCGGTGGTATG-3' (643 bp); BMP-2, 5'-CCACGACACAGTTCCCTGCA-3' and 5'-CACGGCTTCTAGTTGATGGA-3' (356 bp); and SIRT1, 5'-TCAGTGTCATGGTTCCTTTGC-3' and 5'-AATCTGCTCCTTTGCCACTCT-3' (820 bp). The following PCR conditions were used: 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 51~60 °C for 30 s, and extension at

Western blot

Cells from each of experiments were harvested and washed twice in 1xPBS. Cells were solubilized in PRO-PREPTM (iNtRON, Biotechnology) lysis buffer. After 20minutes on ice, the lysates were clarified by centrifugation. Proteins (30 μg per lane) were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and proteins were then transferred to a polyvinylidinedifluoride membrane. The membranes were blocked with 5% skim milk (1 hr), rinsed, and incubated with primary antibodies against with 1:1000 dilution. This was followed by incubation for 1 hr with a secondary antibody (horseradish peroxidase-conjugated). Following three washes in TBS, bands were visualized by chemiluminescence and exposed to X-ray film.

Alizarin red staining

After incubation for 7 and 14 days, cell mineralization was determined using alizarin red staining. Cells were washed in PBS (pH 7.2) and fixed by incubation in ice-cold 70 % (v/v) ethanol for 40 min. Then, they were rinsed in deionized water and stained with alizarin red (dissolved in deionized water, pH 4.2) for 20 min at room temperature. Following staining, excess dye was removed through gentle washing in PBS.

Statistical Analysis

Differences among group were analyzed using one-way analysis of variance combined with the Bonferroni test. All values are expressed as means ± standard deviation; differences were considered significant at $p<0.05$.

RESULTS

NNMBS 246 promoted cell differentiation and mineralization of MG-63 human osteoblast-like cells

To elucidate whether NNMBS 246 has an anabolic effect on bone, we first examined its effects on cell differentiation and mineralization of MG-63 human osteoblast-like cells. Cells were cultured in DMEM supplemented with OS medium (10 mM b-glycerophosphate and 50 μg/mL ascorbic acid), 10-7M dexamethasone (DEXA), and NNMBS 246 for 7 and 14 days. Results showed that NNMBS246 increased ALP enzyme activity in culture in a concentration-dependent manner. The increase in ALP activity was significant at concentrations of 25- 50 μg (Fig. 1A). In Figure 1B, C, ALP is considered to be of osteoblastic differentiation, while OPN, OCN are intermediate and late bone differentiation that NNMBS 246 had stimulated osteoblastic differentiation in MG-63. Next, calcium nodule formation by alizarin red staining and the expression of early, intermediate, and late differentiation markers by RT-PCR were examined. As shown in Figure 1B, C, expression of markers increased concentration-dependently on 7 and 14 days. The essential transcriptional factors Runx2 and osterix also increased (Fig. 1B, C). Mineralization, and hence osteoblastic differentiation, was confirmed by positive alizarin red staining concentration-dependently on 14 days (Fig. 1D).

Effects of NNMBS 246 on the expression of MMPs and ECM molecules

We examined MG-63 for MMPs and ECM molecules mRNA expression induced by NNMBS 246, because the induction of MMPs and degradation of ECM molecules by tissue destruction has been implicated in inflammation. As shown in Fig. 2A, NNMBS 246 in a concentration dependent manner had a down-regulated effects on MMPs expression in MG-63. In contrast, NNMBS 246 increased the levels of the ECM molecules mRNA, including collagen I, elastin and fibronectin in MG-63 in a dose-dependent fashion (Fig. 2A). Osteoblasts can secret OPG to protect the skeleton from excessive bone resorption by binding to RANKL and preventing it from binding to its receptor, RANK. Thus, the OPG/RANKL ratio is an important determinant of bone mass and skeletal integrity. We found that NNMBS 246 treatment markedly increased the expression of OPG mRNA in a concentration-dependent manner on 7, 14 days: a significant in

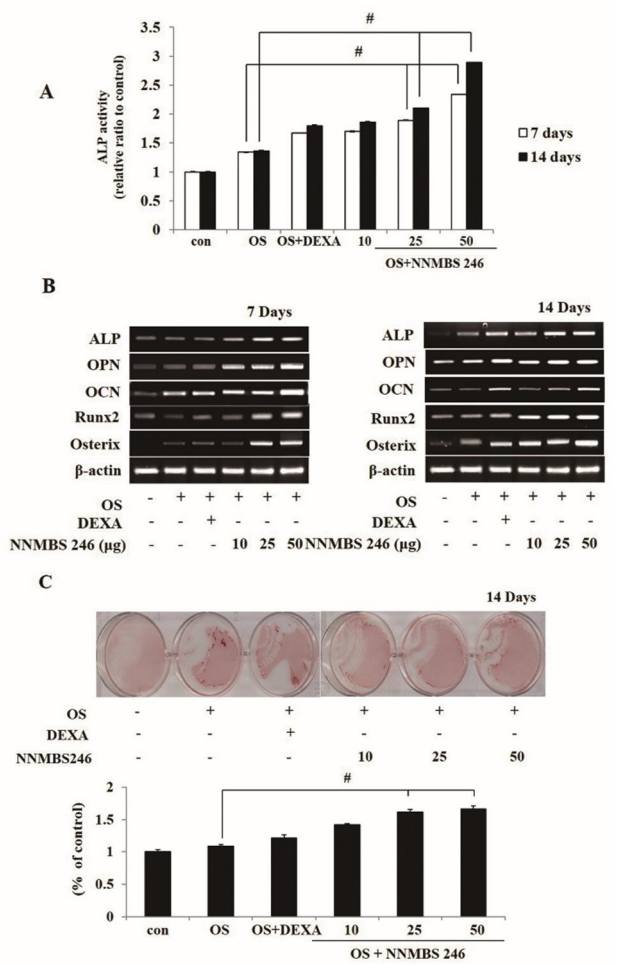

Fig 1. Effects of NNMBS 246 on the ALP activity (A), the mRNA expression of differentiation markers and transcription factors (B) and the calcified nodule formation (C) in MG-63 human osteoblast-like cells. Cells were cultured in DMEM supplemented with OS medium (10 mM b-glycerophosphate and 50 μg/mL ascorbic acid), 10-7M dexamethasone (DEXA), and NNMBS246 for 7 and 14 days. The intensity of Alizarin red staining was determined by relative to the control (optical density, C). Each results were obtained in three independent experiments. # means $P < 0.05$ compared with control without NNMBS246 treatment.

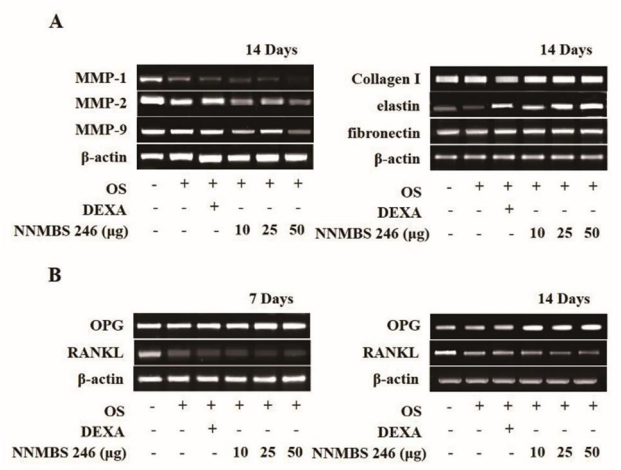

Fig 2. Effects of NNMBS246 on mRNA expression of MMPs, ECM molecules (A), OPG and RANKL (B) in MG-63 human osteoblast-like cells. Each results were obtained in three independent experiments.

-crease in OPG mRNA was observed at 10 μg and peaking at 50 μg NNMBS 246 (Fig. 2B). NNMBS 246 was tended to repress the expression of RANKL mRNA. Generally, the OPG/RANKL ratio was upregulated by NNMBS 246.

Effects of NNMBS 246 on osteoblast differentiation and mineralization in hPDLCs and Cementoblasts.

To confirm whether osteoblast differentiation in hPDLCs and cementoblasts were influenced by the presence of NNMBS 246, an experiment was performed. Treatment of hPDLCs and cementoblasts with NNMBS 246 increased the expression of mRNA encoding the cementoblasts's differentiation markers CEMP1, IBSP, CAP, the hPDLCs's differentiation markers ALP, OPN, OCN, and the transcriptional factors Runx2 and osterix (Fig. 3A, B, C, D). HPDLCs and cementoblasts cultured at 10 to 50 μg NNMBS 246 showed significantly increased matrix mineralization concentration-dependent manner on 14 days (Fig. 3E).

Involvement of Akt, AMPKα, MAP kinases, and NF-kB in osteoblastic differentiation

To investigate the molecular basis of the osteoblastic differentiation of MG-63, the effects of NNMBS 246 on the phosphorylation of three mitogen-activated protein kinase (MAPKs; p38, extracellular signal-related kinase (ERK), and c-Jun N-terminal kinase (JNK)), Akt, and adenosine monophosphate kinase (AMPKα) were examined. NNMBS 246 treatment increased the phosphorylation, but not overall levels, of p38, ERK, JNK, Akt, or AMPKα (Fig. 4A). Maximal expression of p-p38, p-ERK, p-JNK, and p-Akt was detected following incubation of MG-63 in OS supplemented NNMBS 246 50 μg for 90 min, while p-AMPKαlevel reduced time-dependent manner. The effects of NNMBS 246 on NF-kB activation by measuring IkBα degradation and nuclear translocation of p65 were examined. Maximum degradation of IkBα and expression of p65 protein were detected in MG-63 that had been incubated in OS supplemented NNMBS 246 50 μg for 90 min (Fig. 4B).

Effects of NNMBS 246 on the expression of BMP-2 mRNA and involvement of Smad1/5/8 and active β-catenin

Because BMP-2 has been shown to play an important role in bone formation, confirm whether the level of BMP-2 expression was influenced by the presence of NNMBS 246. The results indicated that NNMBS 246 caused a significant increase in BMP-2 mRNA levels in MG-63. The maximum expression was observed in OS supplemented NNMBS 246 50 μg (Fig. 5A). This indicated that the BMP pathway is required in NNMBS 246-mediated osteoblastic differentiation. We therefore exam-ined whether the Smad signal could be activated by NNMBS 246. Results showed that the stimulation of cells with NNMBS 246 50 μg for 90 min induced a strong activation of Smad 1/5/8, as revealed by increased phosphorylation (Fig. 5B). NNMBS 246 (50 μg) significantly activated β-catenin in the MG-63 after a 90-

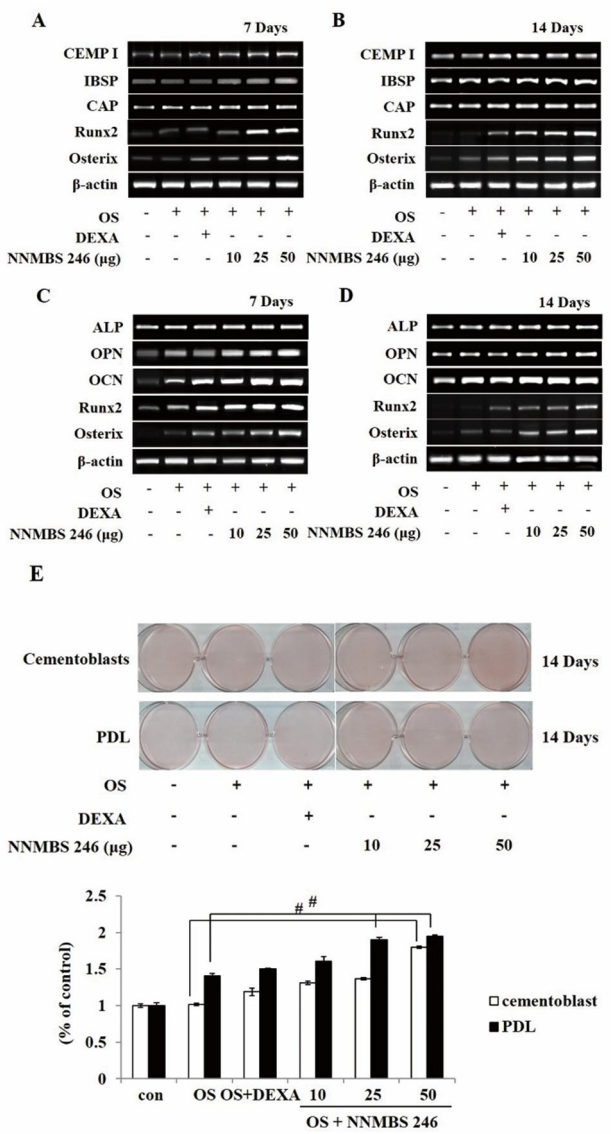

Fig 3. Effects of NNMBS246 on differentiation of cementoblasts (A, B, E) and periodontal ligament cells (C, D, E) by RT-PCR (A-D) and alizarin red staining (E). These cells were incubated with or without NNMBS246 for 7 and 14 days. Respect mRNA expression were measured by using RT-PCR (A, B, C, D). Cultures were fixed with 70% ethanol on day 14 and stained for mineral by the alizarin red staining. The intensity of Alizarin red staining was determined by optical density measurement. The alizarin red staining fold are calculated relative to the control by optical density. Each results were obtained in three independent experiments.

-min culture period, as determined by using an antibody to detect β-catenin that is dephosphorylated at serine 37 and threonine 41 (Fig. 5B). This active form of β-catenin has been shown to be increased in response to Wnt stimulation and by

mechanical loading. Furthermore, to understand the signal pathways involved in the regulation of cell differentiation by NNMBS 246, MG-63 were pretreated with a BMP inhibitor noggin 0.1μg/mL for 1h, then co-incubated with 50 μg NNMBS 246 for a further 90 min. As shown results, NNMBS 246-induced cell differe-ntiation was abolished by noggin treatment, suggesting that a BMP-dependent mechanism may be involved (Fig. 5C).

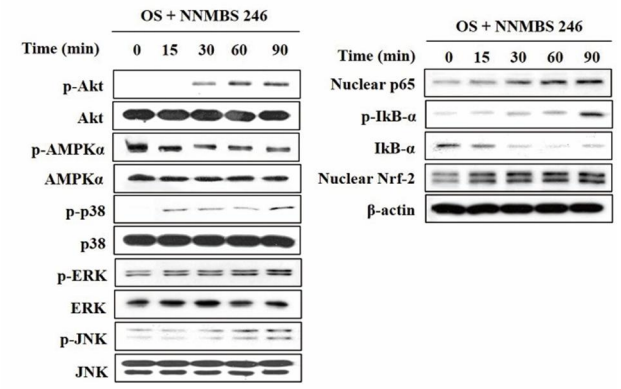

Fig 4. Effects of NNMBS246 on the activation of Akt and MAPK (A), and NF-kB and Nrf-2 (B) in MG-63 human osteoblast-like cells. Cells were treated with NNMBS246 50 μg for indicated times. The results shown are representative of three independent experiments

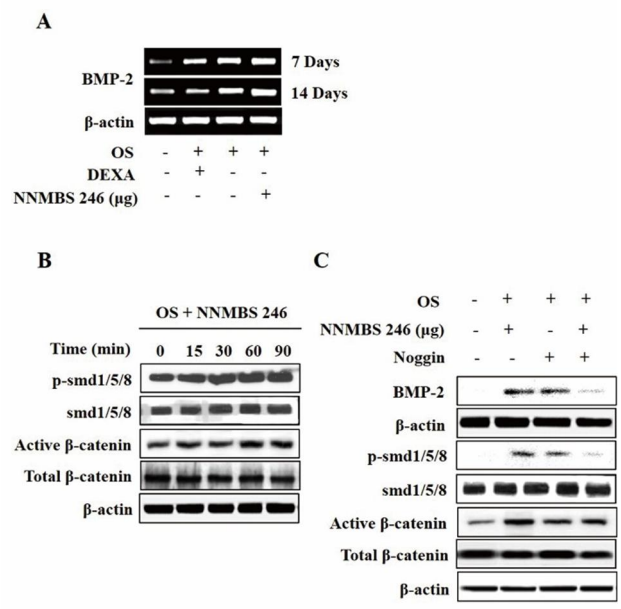

Fig 5. Effects of NNMBS246 on expression of BMP-2 mRNA (A), smad 1/5/8 phosphorylation and, active β-catenin (B) in MG-63 human osteoblast-like cells. Effects of BMP-2 inhibitor, noggin, on NNMBS246-induced BMP-2, Smad1/5/8, and β-catenin upregulation (C). Cells were treated with NNMBS246 50 μg for indicated times. Cells were pre-treated with noggin (0.1 μg/ml) for 3 h and then stimulated by NNMBS246 (50 μg) for 90 min. Similar results obtained in three independent experiments.

Effects of SIRT1 inhibitor on NNMBS 246-induced differentiation in MG-63

To investigate the expression of SIRT1 mRNA and protein durin

g osteoblastic differentiation of MG-63, MG-63 were cultured for 7, 14 days and samples collected were analyzed by Western blotting (Fig. 6A), conventional RT-PCR (Fig. 6A). Expression of SIRT1 mRNA and protein was detected in unstimulated MG-63; it increased in a concentration-dependent manner following exposure to OS supplement NNMBS 246 on 7, 14 days. The increase in SIRT1 protein expression in MG-63 correlated with a parallel increase in SIRT1 mRNA levels (Fig. 6A). In contrast, treatment with the SIRT1 inhibitor, sirtinol (pretreated for 16 h, 50 μM), reduced the expression of SIRT1 protein and mRNA levels (Fig. 6B). Next, the effects of using sirtinol on differenti-ation following OS supplement NNMBS 246 were accessed. Treatment of MG-63 with sirtinol blocked NNMBS 246-induced upregulation of ALP, OPN, OCN, Runx2, and osterix mRNA expression on 7, 14 days (Fig. 6C). As shown above, p38, ERK, JNK, and Akt were activated by NNMBS 246

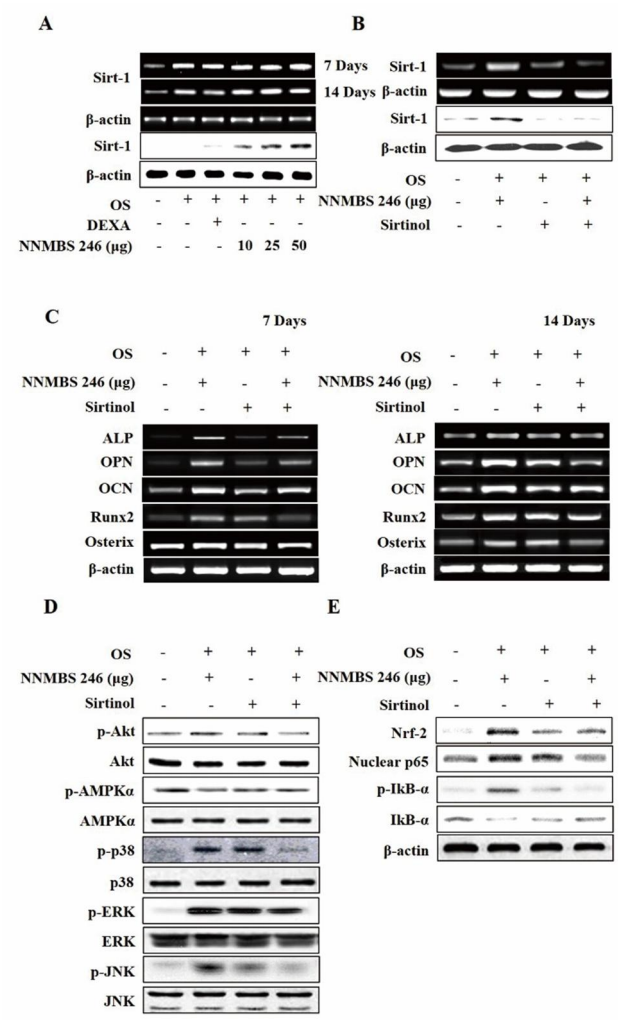

Fig 6. Effects of NNMBS246 on Sirt1 mRNA expression in mg-63 osteoblast-like cells (A). Effects of SIRT1 inhibitor by sirtinol on NNMBS246-induced Sirt1 (B), osteoblast differentiation (c), and signal transduction (D, E). Cells were incubated with 50 μg NNMBS246 for 7 and 14 days. Sirtinol (50 μM) was pretreated for 16hr and then cells were stimulated by NNMBS246 (50μg) for 7 and 14 days (C), and 90 min (D, E). Similar results obtained in three independent experiments.

in MG-63, we next tested whether SIRT1 inhibitor might influence the phosphorylation of these same signaling molecules. Pretreatment with the SIRT1 inhibitor, sirtinol, decreased NNM BS 246-induced phosphorylation of Akt, p38,

ERK, and JNK (Fig. 6D). In an attempt to determine whether SIRT1 inhibition influenced NNMBS 246-induced activation of NF-kB, we tested the effects of treatment with sirtinol on NNMBS 246-induced degradation of IkBα and nuclear translocation of p65. As shown in Figure 6E, sirtinol inhibited these processes.

DISCUSSION

Osteoporosis is a systemic metabolic bone disease characterized by a reduction in bone mass, bone quality, and microarchitectu-ral deterioration. The ideal strategy for treating osteoporosis is to inhibit bone resorption by osteoclasts and/or increase bone formation by osteoblasts. However, most of the current therapies for treating osteoporosis focus on inhibiting bone resorption, and there are only few agents available that promote bone formation. An imbalance in bone remodeling that is caused by more osteoclast-mediated bone resorption than osteoblast-mediated bone formation results in such pathologic bone disorder.

Osteogenic cell proliferation and differentiation play a central role in adequate fracture healing to increase extracellular bone matrix production (Bolander ME, 1992). In this study, we found that NNMBS 246 has the ability to stimulate osteoblast differentiation/mineralization. During differentiation in vitro, osteoblast phenotypic markers appear in the following order: accumulation of collagenous matrix, expression of ALP, secretion of OPN and OCN, and finally mineralization of bone nodules. Our results showed that NNMBS 246 indeed markedly raised the levels of ALP, OPN and OCN, three bone matrix proteins which are implicated in osteoblast differentiation. Certain bone formation agents such as statins have been shown to induce osteoblast differentiation by stimulating the expression of BMP-2 in MC3T3-E1 cells, leading to positive effects on bone formation (Mundy G and Garrett R, 1999). BMP has been proven to induce bone formation both in vivo and in vitro (De Biase P and Capanna R, 2005). Its signaling is initiated by receptor binding, propagated by the phosphorylation of Smad1/5/8 complex, and finally translocated into the nucleus to regulate the transcription of target genes (Nohe A and Keating E, 2004; Yamamoto N and Akiyama S, 1997). Canonical Wnt/β-catenin is another signal pathway that regulates bone mass increase through a number of mechanisms (Krishnan V and Bryant HU, 2006). In the present study, we found that the adding of noggin significantly repressed NNMBS 246 evoked differentiation, suggesting that NNMBS 246 may influence osteoblast functions potentially through the BMP and Wnt/β-catenin pathway.

Among the downstream targets of BMPs are Runx2 and other osteoblast-related transcription factors such as osterix (Lee KS and Kim HJ, 2000). Runx2 is the main transcription factor required for the activation of osteoblast differentiation and is crucial for the regulation of genes responsible for the production of bone specific proteins such as collagen type I, OCN, OPN, and bone sialoprotein (Prince M and Banerjee C, 2001). Our results showed that the stimulation of MG-63 cells with NNMBS 246 resulted increases in Runx2 and osterix expression; this might be helpful in elevating its binding level to enhancer regions in target genes. Therefore, it is possible that upregulation of Runx2 and osterix expression participated in the bone anabolic effect by NNMBS 246. In the remodeling process, the members of the molecular triad OPG/RANK/RANKL are closely linked to each other. RANKL is synthesized either in membranous or soluble form, primarily by the osteoblastic lineage cells, the immune cells, and some cancer cells. The binding of RANKL to the extracellular RANK leads to the activation of specific signaling pathways involved in the formation and survival of osteoclasts, hence bone resorption (Miyamoto T and Suda T, 2003). OPG is secreted by the stromal cells and other cell types, including osteoclasts, and acts as a soluble decoy receptor for RANKL. OPG, by interacting with RANKL, inhibits the binding of RANKL to RANK, thereby preventing RANK activation and subsequent osteoclastgenesis, and, as a result, inhibits bone resorption. Results obtained from Figure 2 indicated that NNMBS 246 reciprocally regulated OPG and RANKL levels especially significantly reduced in RANKL expression.

In the bone, the main extracellular matrix (ECM) macromolecules are collagenous proteins especially type I and III collagen, non-collagenous proteins such as fibronectin, tenascin and glycosaminoglycans. The degradation of the ECM was reported in suppurative pulpitis. MMPs are endopeptidases that play a primary role in the degradation of ECM proteins. Collagenases, including MMP-1, -8, -13, and -14, hydrolyze native collagens to generate ¼ and ¾ fragments, which are substrates for gelatinases and stromelysin. Stimulation of both inflammatory cytokines and bacterial extracts may induce production of MMPs and cell mediated collagen degradation in pulp cells. In the present study, we found that NNMBS 246 treatment concomitantly up-regulated the expression of ECM molecules such as type I collagen, elastin and fibronectin and down-regulated the expression of metalloproteinase-1 (MMP-1), MMP-2, and MMP-9 mRNA in time and dose-dependent manner. This suggesting that NNMBS 246 may influence both hard and soft connective tissues in periodontal tissue regeneration.

To assess the role of SIRT1 in the osteoblastic differentiation of human osteosarcoma cells, we tested the effects of SIRT1 inhibition on the expression of key differentiation markers. Our results indicate that activation of sirtinol in human osteosarcoma cells down-regulated the expression of mRNAs encoding the osteoblastic markers ALP, osteopontin, osteocalcin, osterix and Runx2 in a dose-dependent manner. These results are consistent with previous data obtained using mouse mesenchymal stem cells and rat primary bone marrow stromal cells (Bäckesjö CM and Li Y, 2006), osteoblastic MC3T3-E1 cells (Mizutani K and Ikeda K, 1998) and human bone marrow-derived mesenchymal stem cells (Dai Z and Li Y, 2007). Our results suggest that osteoblastic differentiation is stimulated by SIRT1 activation and repressed by inhibition of SIRT1. Thus, the differentiation of human osteosarcoma cells into osteoblasts appears to be controlled, at least in part, by a SIRT1-dependent mechanism.

We next investigated whether SIRT1 influenced the phosphorylation of kinases, because activation of Akt (Fujita T and Azuma Y, 2004) and MAP kinases, including p38, ERK and JNK (Matsuguchi T and Chiba N, 2009), has been reported to contribute to osteoblastic differentiation in bone cells and osteoblasts. In Figure 4, NNMBS 246-induced phosphoactivation of Akt, p38, ERK, and JNK, but not AMPKα, was significantly enhanced. The results of the Figure 6 show that sirtinol attenuated NNMBS 246-induced phosphorylation of p38, ERK, JNK and Akt. Consistent with these findings, sirtinol was shown to attenuate epidermal growth factor and insulin-like growth factor 1-stimulated phosphorylation of ERK, JNK and p38 in MCF-7 and H1299 cells (Ota H and Tokunaga E, 2006). Activation of NF-kB is dependent on the degradation of IkB, an endogenous inhibitor of NF-kB signaling that binds to NF-kB in the cytosol (Lee FS and Hagler J, 1997). Recent studies demonstrated that resveratrol inhibits NF-kB activation (Kumar A and Sharma SS, 2010; Youn J and Lee JS, 2009). As this

transcription factor is strongly linked to differentiation responses (Eliseev RA and Schwarz EM, 2006; Inanc B and Elcin AE, 2006), we hypothesized that NNMBS 246 and/or sirtinol influence differentiation, at least in part, by enhancing or suppressing NF-kB activation. Our results indicate that NNMBS 246 increased the degradation of IkB and the nuclear translon-cation and DNA binding activity of p65 NF-kB in human osteosarcoma cells (Figure 4). In contrast, treatment with sirtinol reduced NNMBS 246-induced NF-kB activation. These findings suggest that sirtinol influence the osteoblastic differentiation of human osteosarcoma cells by modulating the activation of NF-kB and degradation of IkB.

In summary, the present study is the first report of the expression of SIRT1 mRNA and SIRT1 protein on NNMBS 246-induced in human osteosarcoma cells during the early phase of osteoblastic differentiation. These findings support the hypothesis that SIRT1 may play an important regulatory role in NNMBS 246-induced osteoblastic differentiation for periodontal regeneration.

CONCLUSION

In conclusion, the present study indicates that NNMBS 246 promotes growth and osteoblastic differentiation in periodontal regeneration related cells via BMP and SIRT1 pathways. The impact of NNMBS 246, on periodontal regeneration, in humans also needs to be studied further, as it may prove to be useful in the treatment of periodontal disease.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests

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