Notoginsenoside R1 significantly promotes *in vitro* osteoblastogenesis

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Abstract. Notoginsenoside R1 (NGR1), one of the main effective components of *Panax notoginseng*, appears to be effective in promoting osteogenesis and treating osteoporosis. However, hitherto, whether NGR1 can directly promote osteoblastogenesis remains to be elucidated. In the present study, we hereby examined the effects of NGR1 on the osteoblastogenesis of a pre-osteoblast cell line (MC3T3-E1) in *in vitro* time-course and dose-dependent experiments. Its efficacy was evaluated by assessing cell viability (indicator of proliferation), alkaline phosphatase (ALP) activity (a marker of early osteoblastic differentiation), levels of osteocalcin (OCN; a marker of late osteoblastic differentiation), calcium deposition (a marker of final mineralization) and the expression of a series of osteoblastogenic marker genes (such as collagen Iα, Runx2, ALP and OCN) at different time points. When examining the proliferation of and ALP activity in the pre-osteoblasts, a bell-shaped dose-response pattern was observed when the cells were treated with various concentrations of NGR1, with a peak being observed at the concentration of 50 μg/ml. NGR1 markedly increased the expression of OCN at the concentration of 1,000 μg/ml in a dose-dependent manner. Furthermore, treatment with 1,000 μg/ml NGR1 resulted in the highest mineralization by 4.3- and 5.9-fold on the 21st and 28th day, respectively compared with the control group (no treatment). On the whole, our findings indicate that NGR1 significantly promotes the osteoblastogenesis of pre-osteoblasts, which suggests that NGR1 has potential for use as a bone regeneration agent.

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Introduction

Sufficient bone volume and adequate bone quality are of paramount importance to achieve the rapid establishment of implant functions in dentistry and orthopedics. However, various adverse bone conditions, such as bone defects, low bone density and compromised self-healing capacity can significantly compromise new bone regeneration and implant osteointegration, thus delaying the loading of implants (1). Autologous bone grafts are still regarded as the 'gold standard' to repair bone defects since they contain osteoinductive growth factors and osteogenic cells (2). However, theirs use is limited due to intrinsic disadvantages, such as limited availability and donor-site pain. In addition, the efficacy of autologous bone grafts can also be compromised when the patients have low bone density or a compromised healing capacity. As alternatives to autologous bone grafts, various bioactive agents that are used either alone or in combination with biomaterials have been resorted to promote bone regeneration (3). Bone morphogenetic proteins (BMPs), a group of proteinaceous growth factors, are the most extensively used agents for bone regeneration (4). However, the effective doses of homodimeric BMPs used clinically to induce bone formation are very high (e.g., up to several milligrams) (5,6), which leads to not only a substantial economic burden, but also to a series of potential side-effects, such as the overstimulation of osteoclastic activity

in the surrounding milieu and ectopic bone formation in unintended areas (7,8). Gene, cell and cytokine therapies have been reported to substitute autografting, but due to the high cost associated with their use, they have not been widely used clinically (9-11). In comparison, traditional Chinese medicine has attracted increasing attention, as it involves the use of agents that possess high bioactivity and have minimal side-effects.

One of such traditional Chinese medicine is *Panax notoginseng* saponins (PNS), a mixture of active compounds that are extracted from the *Panax notoginseng* root. *Panax notoginseng* has been widely used as a medicinal herb for over thousands of years in China and exhibits minimal side-effects, which is a great advantage. PNS has been widely used in the treatment of cardiovascular diseases (12). Recently, PNS was found to protect rabbit bone marrow stromal cells from oxidative stress-induced damage and apoptosis by scavenging ROS and regulating the Bcl-2/Bax pathway, thereby promoting bone formation (13). Another study also confirmed that PNS promoted the osteogenesis of bone marrow stromal cells by activating the ERK and p38 signaling pathways (14). PNS also promotes the proliferation and osteogenic differentiation of NIH3T3 fibroblasts by increasing the phosphorylation of ERK1/2 protein kinase (15). PNS can also promote the development of osteoblasts (16,17). These findings suggest that some effective components in PNS have the potential to be used clinically to promote osteogenesis. Notoginsenoside R1 (NGR1) is one of the main constituents of PNS. Unlike other pharmacologically active saponins in both PNS and other species of ginseng, NGR1 is found only in PNS (18,19). However, hitherto, whether NGR1 can directly affect osteoblastogenesis remains to be elucidated.

In this study, we aimed to examine the effects of NGR1 on the osteoblastogenesis of a pre-osteoblast cell line (MC3T3E1 cells) in time-course and dose-dependent experiments. We evaluated its effects by assessing cell viability (an indicator of proliferation), alkaline phosphatase (ALP) activity (a marker of early osteogenic differentiation), levels of osteocalcin (OCN; a marker of late osteogenic differentiation), calcium deposition (a marker of final mineralization) and the expression of osteoblastogenic marker genes (such as Runx2, collagen Iα, ALP and OCN) at different time points.

Materials and methods

Cell culture. MC3T3-E1 (subclone 14; ATCC CRL-2594) pre-osteoblasts were obtained from ATCC, Manassas, VA, USA. The cells were cultured in a α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) (Gibco®, Invitrogen, Grand Island, NY, USA). The medium was changed every 3 days. Triplicate experiments per group were performed for each parameter per time point. The exponentially growing cells were collected and seeded at a final concentration of $1x10^4$ cells/well in 24-well plates for the cell proliferation assay. For the ALP activity assay, OCN detection and polymerase chain reaction (PCR), the cells were seeded in 6-well plates at a final concentration of $2x10^5$ cells/well or in 48-well plates at a final concentration of $3x10^4$ cells/well for Alizarin red staining. Twenty-four hours post-incubation, the cells were subjected to a low-serum medium (α-MEM containing 2% FBS) for another 24 h. Thereafter, the cells were treated with various concentrations (0, 5, 50, 100, 20 and 1,000 μg/ml) of NGR1 (ZL140310529; Nanjing Zelang Medical Technological Co., Ltd., Nanjing, China.

Cell viability and proliferation assay. The viability and proliferation of the MC3T3-E1 cells in each treatment group (various concentrations of NGR1) were determined using the Alamar Blue cell viability reagent (Invitrogen, Carlsbad, CA, USA) following treatment with NGR1 for 1, 4 and 7 days. We used a fluorescence spectrometer (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA) to measure the fluorescence intensity with the excitation wavelength at 540 nm and the emission wavelength at 590 nm.

ALP activity assay. The ALP activity and total protein content were measured following treatment for 4 and 7 days to assess the early differentiation of the pre-osteoblasts. The ALP activity in the whole cell lysate was determined using a LabAssay™ ALP colorimetric assay kit (Wako Pure Chemicals, Osaka, Japan). The ALP activity was normalized by the total protein content that was measured using a commercial BCA protein assay kit (Beyotime, Haimen, China).

OCN expression assay. OCN expression in the cell culture medium after the 4- and 7-day treatments was determined to assess the terminal differentiation of the pre-osteoblasts. The OCN concentrations were determined using a mouse OCN EIA kit (Biomedical Technologies, Stoughton, MA, USA), as previously described (20).

Alizarin red staining. In order to assess the extracellular mineralization of the MC3T3-E1 cells, osteogenic medium (10% FBS, 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate; Sigma-Aldrich, St. Louis, MO, USA) containing various concentrations of NGR1 was used to treat the cells for 21 and 28 days (21). On the 21st and 28th day, mineralized nodules were stained with Alizarin red (Sigma-Aldrich) as previously described (22). The calcified areas were photographed and then quantified using Image-Pro Plus 6.0 software.

Isolation of total RNA and reverse transcription-quantitative PCR (RT-qPCR). On the 4th and the 7th day, total RNA was extracted using a RNeasy mini kit and purified with RNase-Free DNase set reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using PrimeScript® RT Master Mix (Perfect Real-Time; Takara, Otsu, Japan). A PrimeScript® RT reagent kit (Perfect Real-Time; Takara) was used to perform RT-qPCR. The primers for detecting mRNA transcripts of the collagen Iα, Runx2, ALP, OCN and β-actin genes were designed as previously published and are shown in Table I (1,4). The transcripts were normalized to the β-actin transcript levels. The n-fold upregulation for each gene over the internal control gene (β-actin gene) was calculated according to the ΔΔCt method using the formula following: $2^{-[(\text{CT gene of interest - CT internal control})\text{sample} - (\text{CT gene of interest - CT internal control})\text{control}]}$, as previously described (23).

Statistical analysis. Statistical comparisons among the results were made by one-way analysis of variance (ANOVA) with Bonferroni corrections for post hoc comparisons. The level of

Table I. Primers used for RT-qPCR.

Gene	Accession no.	Primer sequences
Akp2 (ALP)	NM_007431	F: 5'-TGCCTACTTGTGTGGCGTGAA-3'
		R: 5'-TCACCCGAGTGGTAGTCACAATG-3'
Osteocalcin (OCN)	NM_007541	F: 5'-AGCAGCTTGGCCCAGACCTA-3'
		R: 5'-TAGCGCCGGAGTCTGTTCACTAC-3'
Collagen I	NM_007742	F: 5'-ATGCCGCGACCTCAAGATG-3'
		R: 5'-TGAGGCACAGACGGCTGAGTA-3'
Runx2	NM_009820	F: 5'-CACTGGCGGTGCAACAAGA-3'
		R: 5'-TTTCATAACAGCGGAGGCATTTC-3'
β-actin	NM_007393	F: 5'-AGGAGCAATGATCTTGATCTT-3'
		R: 5'-TGCCAACACAGTGCTGTCT-3'

F, forward; R, reverse; ALP, alkaline phosphatase; OCN, osteocalcin.

significance was set at $p<0.05$. SPSS software (version 20) was adopted for the statistical analysis.

Results

Cell proliferation. The effect of NGR1 on cell proliferation exhibited a bell-shaped dose-dependent pattern. On the 1st day of treatment, a significant increase in the cell numbers was detected when the cells were treated with 50 μg/ml NGR1 (Fig. 1). In comparison with the control (no treatment), treatment with NGR1 at 5, 50 and 100 μg/ml NGR1 resulted in significantly increased cell viability (1.6, 2.2 and 1.9-fold of the control, respectively), while treatment with NGR1 at 200 and 1,000 μg/ml resulted in significant lower cell numbers. The cell numbers increased significantly with as time progressed with all NGR1 concentrations. On the 4th and 7th day of treatment, treatment with 50 and 100 μg/ml NGR1 led to significantly higher cell numbers, while treatment with 200 and 1,000 μg/ml NGR1 resulted in significantly lower cell numbers compared to the controls. The most prominent promoting effects of NGR1 on cell proliferation were observed at the concentration of 50 μg/ml, and the greatest inhibitory effects on proliferation were observed at the concentration of 1,000 μg/ml.

ALP activity. Similar to its effects on cell proliferation, the effect of NGR1 on ALP activity also exhibited a bell-shaped dose-dependent pattern. On the 4th day of treatment, NGR1 at the concentrations of 5 to 200 μg/ml significantly enhanced ALP activity, with a peak being observed at the concentration of 50 μg/ml. Treatment with NGR1 at 1,000 μg/ml did exert a significant modulatory effect in comparison with the control. On the 7th day, treatment with only 50 μg/ml NGR1 resulted in significantly higher ALP activity in comparison with the control group (no NGR1). By contrast, treatment with NGR1 at 100, 200 or 1,000 μg/ml led to a significantly lower ALP activity, with the lowest value being observed at the concentration of 1,000 μg/ml.

OCN expression. After the 4-day treatment, NGR1-induced OCN expression exhibited a dose-dependent increasing trend. With NGR1, OCN expression in the pre-osteoblasts significantly increased from the 4th to the 7th day of treatment for each concentration (Fig. 3). Different from its effects on cell proliferation and ALP activity, the effect of NGR1 on OCN expression exhibited a dose-dependent increasing pattern. On the 7th day in particular, treatment with 1,000 μg/ml NGR1 significantly increased OCN expression by almost 5-fold in comparison with the control (no NGR1) (Fig. 3).

Cell matrix mineralization. In the control group, rare mineralization was observed (Fig. 4). On the 21st and 28th day of treatment, mineralization in the cell matrix was observed in

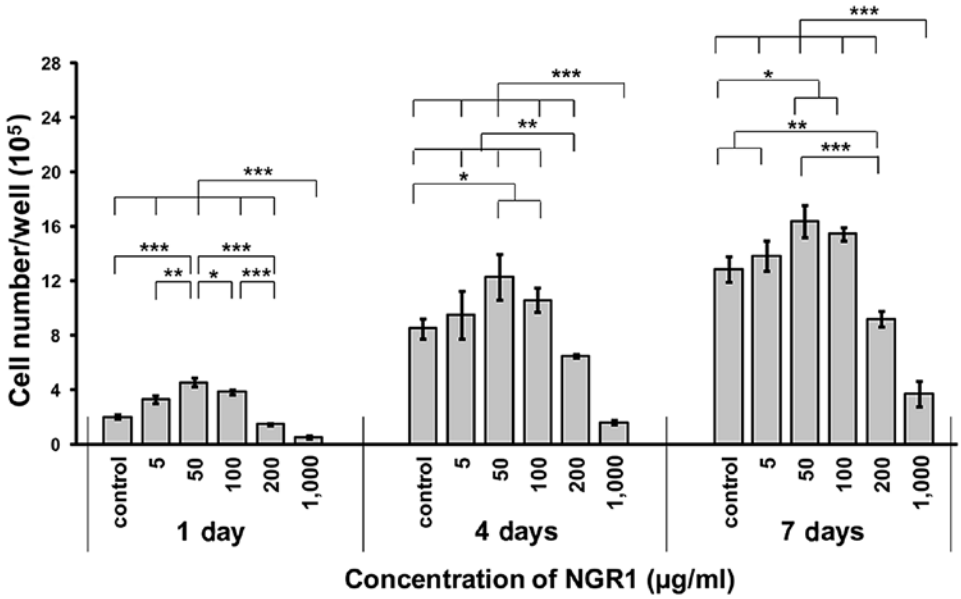

Figure 1. Numbers of murine calvarial pre-osteoblasts (MC3T3-E1 cell line) per well under the different treatment conditions: i) control (no treatment); ii) 5 μg/ml notoginsenoside R1 (NGR1); iii) 50 μg/ml NGR1; iv) 100 μg/ml NGR1; v) 200 μg/ml NGR1; vi) 1,000 μg/ml NGR1 for 1, 4 and 7 days. Data are presented as the mean values and the corresponding standard deviation (SD). $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

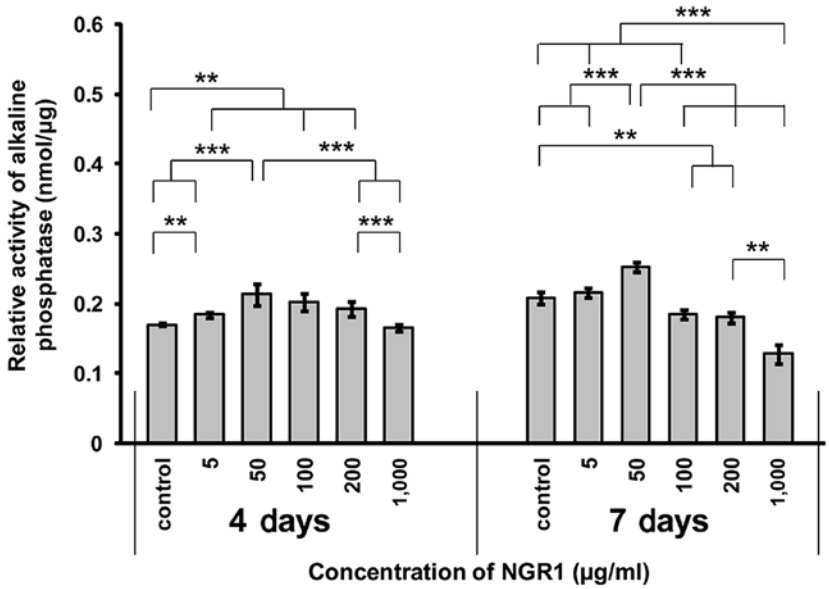

Figure 2. Activity of alkaline phosphatase (ALP) in murine calvarial pre-osteoblast (MC3T3-E1 cells) under the different treatment conditions: i) control (no treatment) ii) 5 μg/ml notoginsenoside R1 (NGR1); iii) 50 μg/ml NGR1; iv) 100 μg/ml NGR1; v) 200 μg/ml NGR1; vi) 1,000 μg/ml NGR1 for 4 and 7 days. Data are presented as the mean values and the corresponding standard deviation (SD). *p<0.05, **p<0.01 and ***p<0.001.

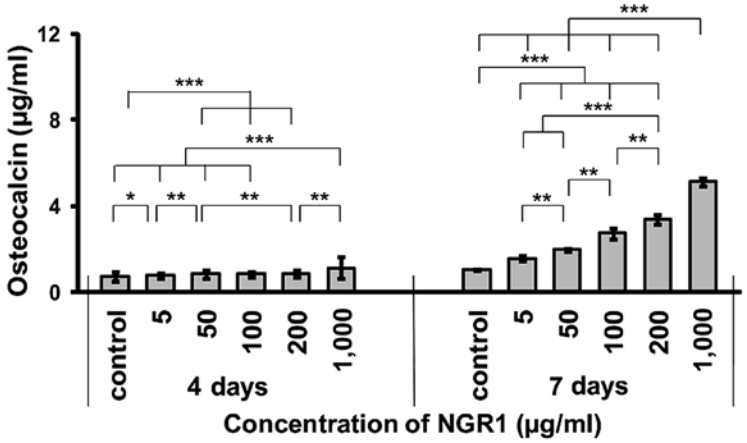

Figure 3. Expression of osteocalcin (OCN) in murine calvarial pre-osteoblasts (MC3T3-E1 cells) under the different treatment conditions: i) control (no treatment); ii) 5 μg/ml notoginsenoside R1 (NGR1); iii) 50 μg/ml NGR1; iv) 100 μg/ml NGR1; v) 200 μg/ml NGR1; vi) 1,000 μg/ml NGR1 for 4 and 7 days. Data are presented as the mean values and the corresponding standard deviation (SD). *p<0.05, **p<0.01 and ***p<0.001.

the cells treated with NGR1. Treatment with NGR1 at 100,200 and 1,000 μg/ml significantly enhanced mineralization in comparison with the control group on the 21st day. Treatment with NGR1 at 1,000 μg/ml resulted in the highest mineralization (4.3 and 5.9-fold on the 21st and 28th day, respectively) compared with the control group.

Expression of osteogenic genes. Runx2 gene is key to control the proliferation of osteoblasts and to promote the stage of cell proliferation to osteogenic differentiation (24,25). In this study, we found that on the 4th day of treatment, NGR1 at all the selected concentrations significantly enhanced the mRNA expression of Runx2, exhibiting a bell-shaped dose-dependent pattern. The highest expression of Runx2 mRNA was resulted from 50 μg/ml NGR1 (Fig. 5A). On the 7th day, in comparison with the control (no NGR1), treatment with 50, 100 and 200 μg/ml NGR1 resulted in a significantly higher mRNA expression of Runx2, whereas treatment with 1,000 μg/ml NGR1 resulted in a significantly lower expression.

On the 4th day of treatment, the expression of the collagen Iα gene was significantly enhanced by 50 μg/ml NGR1, while it was significantly suppressed by 1,000 μg/ml NGR1 (Fig. 5B). On the 7th day, NGR1 at 50 to 1,000 μg/ml significantly enhanced the mRNA expression of collagen Iα. Treatment with NGR1 at 50 μg/ml induced the greatest increase in collagen Iα at both time points.

Consistent with ALP activity, the highest mRNA expression of ALP was observed in the cells treated with 50 μg/ml NGR1 on both the 4th and 7th day (Fig. 5C). On the 7th day, treatment with 200 and 1,000 μg/ml NGR1 significantly downregulated the mRNA expression of ALP in comparison with the control group. Consistent with its effect on OCN expression, NGR1 exhibited a dose-dependent increasing pattern in promoting the mRNA expression of OCN (Fig. 5D). The mRNA expres-

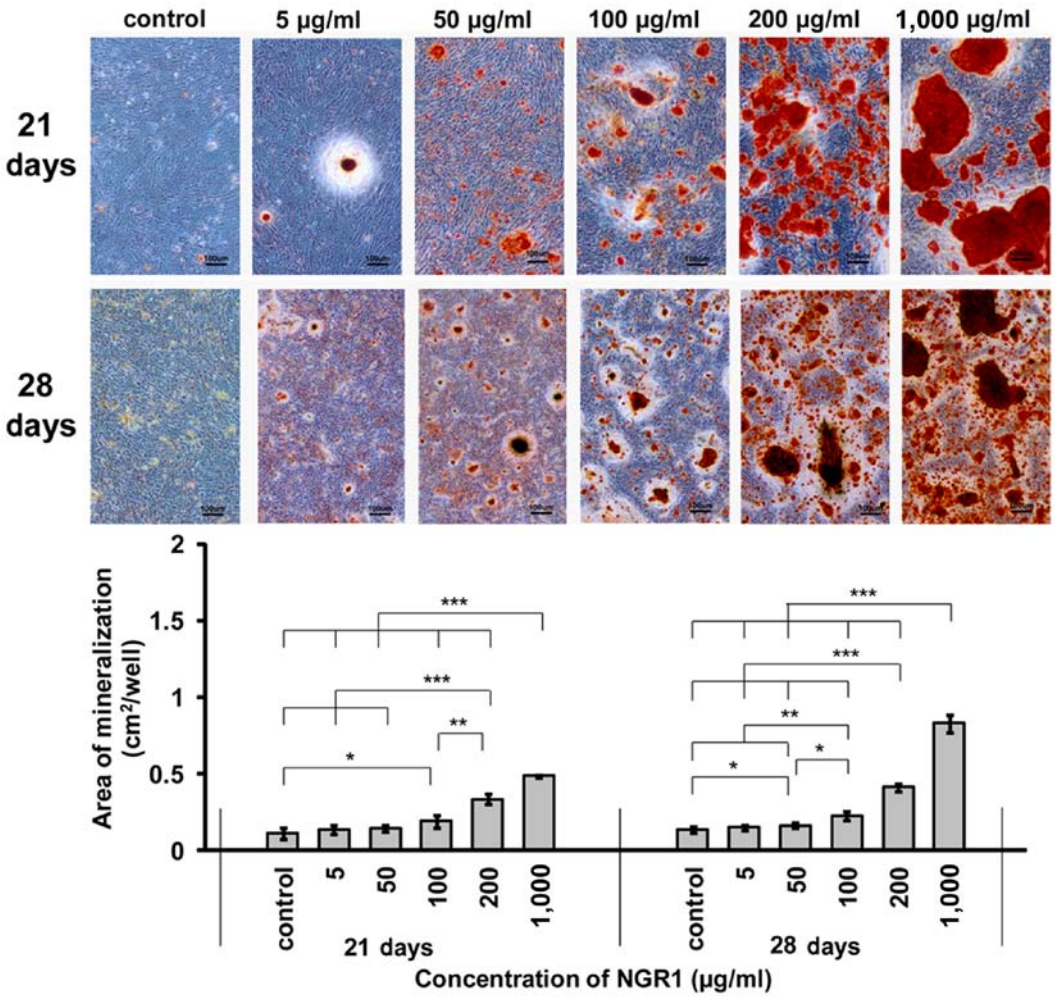

Figure 4. Mineralization of murine calvarial pre-osteoblasts (MC3T3-E1 cells) under the different treatment conditions: i) control (no treatment); ii) 5 μg/ml notoginsenoside R1 (NGR1); iii) 50 μg/ml NGR1; iv) 100 μg/ml NGR1; v) 200 μg/ml NGR1; vi) 1,000 μg/ml NGR1. (4-1) Light micrographs depicting Alizarin red staining on the 21st day and 28th day. (4-2) Graph depicting the calcification area on the 21st and 28th day. Data are presented as the mean values and the corresponding standard deviation (SD). $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

sion of OCN increased significantly as time progressed with all selected concentrations. NGR1 at 1,000 μg/ml resulted in the highest mRNA expression of OCN (1.8 and 1.7-fold on the 4th and 7th day, respectively) compared with the control.

Discussion

Bone regeneration is a delicately orchestrated activity of osteoblasts with coupling bone remodeling by osteoclasts. A number of Chinese medicinal herbs have been used to promote bone formation in fractures in China for over a thousand years. In modern traditional Chinese medicine, effective compositions are extracted from these herbs to treat bone diseases. For example, *Ligustri Lucidi Fructus*, *Drynaria fortunei*, Du-Zhong cortex and Icariin, can effectively correct pathological bone metabolism by promoting osteoblastogenesis and osteoblastic activity (26-28). Other extracts, such as *Cinnamomum zeylanicum*, can significantly inhibit RANKL-induced osteoclastogenesis and osteoclastic resorption (29). In a previous study using an *in vivo* rat model of osteoporosis, *Cervi Cornu Pantotrichum*, the main effective component of antlers, inhibited the progression of osteoporosis and promoted bone formation (30). All these findings indicate their promising potential for clinical application.

PNS, a traditional Chinese herbal medicine, has exhibited a wide range of pharmacological effects, such as angiogenetic, anti-neoplastic, neuroprotective, anti-inflammatory and immunomodulatory effects (31-35). These well-known effects confer that PNS has a strong ability to suppress pathological bone loss. PNS in combination with granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells has been shown to cure unreconstructable critical limb ischemia (36). As an immune modulator, PNS has been shown to significantly decrease lipopolysaccharide-induced alveolar bone loss and the expression of matrix metalloproteinase-9 in a model of periodontitis (37). Recently, PNS has been shown to directly enhance the proliferation and osteogenic differentiation of bone marrow stromal cells by modulating intercellular communication activities and activating the ERK and p38 MAPK signaling pathways (13,14,16). Canonical Wnt signaling is required for the PNS-induced suppression of the nuclear factor-κB ligand (RANKL)/osteoprotegerin (OPG) ratio in bone marrow stromal cells during osteogenic differentiation (38). NGR1, a unique and abundant component of PNS (39), has already

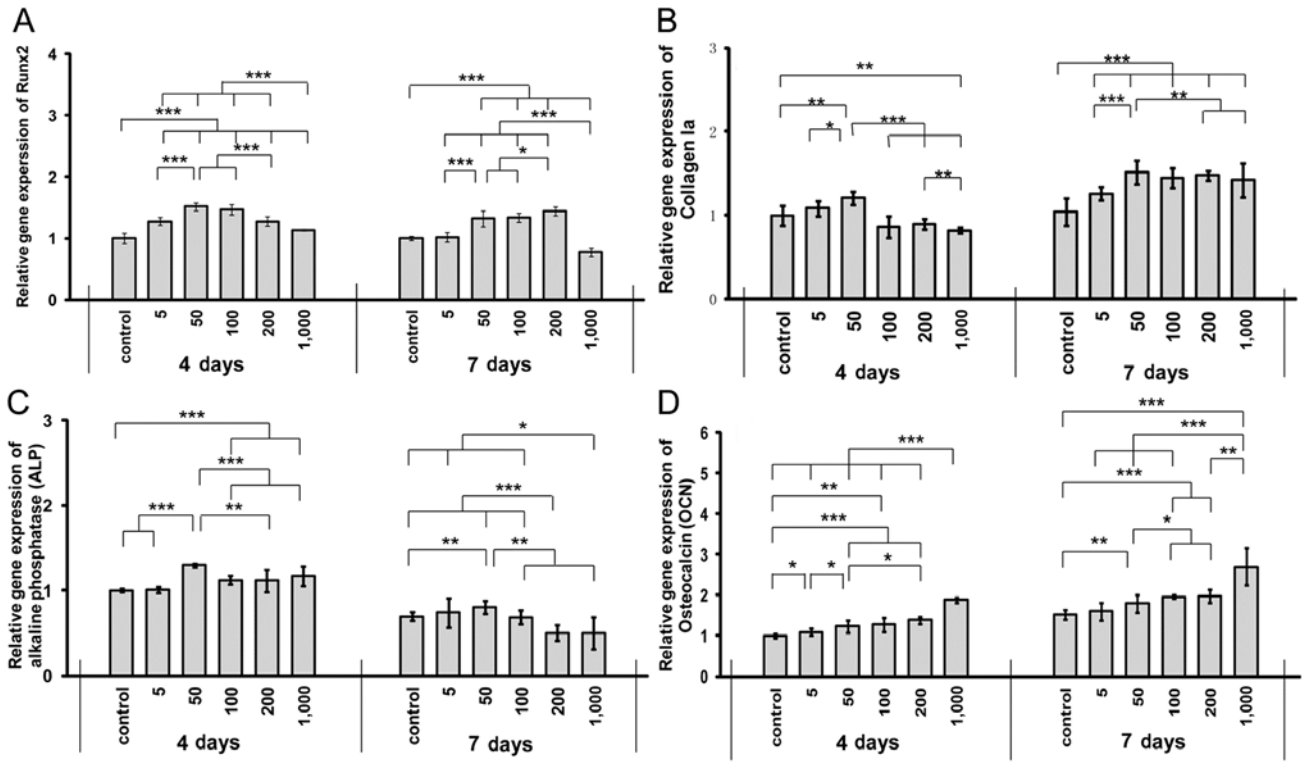

Figure 5. Relative expression of 4 osteogenic marker genes under the different treatment conditionss: i) control (no treatment); ii) 5 μg/ml notoginsenoside R1 (NGR1); iii) 50 μg/ml NGR1; iv) 100 μg/ml NGR1; v) 200 μg/ml NGR1; vi) 1,000 μg/ml NGR1 for 4 and 7 days. (A) Runx2; (B) collagen I; (C) alkaline phosphatase; (D) osteocalcin. The gene expression was first normalized to the corresponding β-actin gene expression for each sample. All the gene data in each group were then normalized to the gene data in control group on the 4th day. Data are presented as the mean values and the corresponding standard deviation (SD). *p<0.05, **p<0.01 and ***p<0.001.

been used to inhibit hypoxia-hypercapnia-induced vasoconstriction and protect cells against intestinal ischemia and reperfusion (40,41). Its clinical application includes treatment of vascular disorders and osteoporosis (42). However, whether NGR1 can directly modulate osteoblastogenesis is unclear. In this study, to the best of our knowledge, through dose-dependent and time course experiments, we demonstrated for the first time that NGR1 significantly promoted *in vitro* osteoblastogenesis, thus suggesting that NGR1 has potential for clinical use as a bone regeneration agent.

The osteoblast phenotype is acquired in two stages. In the first stage, the matrix matures, and specific proteins associated with the bone cell phenotype (e.g., ALP) are detected. In the second stage, the matrix becomes mineralized by calcium deposition. Consequently, layers of spongy bone are formed around the original cartilage (43). In this study, the effect of NGR1 on cell proliferation and ALP activity exhibited a dose-dependent, bell-shaped pattern (Figs. 1 and 2). The optimal effects were observed at the concentration of 50 μg/ml. NGR1 at 50 and 100 μg/ml significantly promoted cell proliferation (Fig. 1), while NGR1 at 200 and 1,000 μg/ml significantly inhibited cell proliferation in comparison to the control (no NGR1) (Fig. 1). Consistently, the expression of collagen Iα reached peak levels following treatment with 50 μg/ml NGR1 (Fig. 5B); collagen Iα is a primary gene product of osteoblasts during bone matrix formation and comprises 85-90% of the total organic bone matrix (44). On the 4th day, treatment with 5 to 200 μg/ml NGR1 exerted a significant promoting effect on ALP activity with peak levels being observed at the concentration of 50 μg/ml. On the 7th day, 100 to 1,000 μg/ml NGR1 of showed a significant inhibiting effect on ALP activities (Fig. 2). Analogous with ALP activity, peak levels in ALP gene expression were also observed at the concentration of 50 μg/ml NGR1 (Fig. 5C). On the 7th day, treatment with 200 and 1,000 μg/ml NGR1 inhibited ALP gene expression. Of note, the effect of NGR1 on OCN expression exhibited a dose-dependent increasing pattern (Fig. 3). A time-dependent and dose-dependent pattern was also observed in OCN gene expression (Fig. 5D).

After culturing these pre-osteoblasts in mineralization medium for 21 days, we observed that 1,000 μg/ml NGR1 produced more bone nodules than the other concentrations (Fig. 4). A time-dependent and dose-dependent pattern was also observed in mineralization (Fig. 4). Treatment with NGR1 at 1,000 μg/ml significantly enhanced mineralization 5.9-fold (Fig. 4), whereas ALP activity was inhibited at this concentration (Fig. 2). By contrast, the pattern of NGR1 in promoting mineralization (Fig. 4) was consistent with that in promoting OCN expression (Fig. 3). This result suggested that the promoting effect of NGR1 on mineralization may be partially attributed to its promoting effect on OCN, but not on ALP activity. These results suggested that high levels of NGR1 can significantly enhance osteoblastogenesis, thereby suggesting that NGR1 may hold promise and great potential for use as an agent to facilitate bone regeneration and implant osteointegration in patients.

Hitherto, the molecular mechanisms accounting for the promoting effects of NGR1 on osteoblastogenesis remain unveiled. Runx2, a key modulator of osteogenic differentia-

tion, controls osteoblast proliferation and promotes a transition from a proliferative to a post-proliferative stage prior to osteoblast differentiation (24,25). In this study, Runx2 expression significantly increased under the stimulation of 50 μg/ml NGR1, whereas it significantly decreased in the presence of 1,000 μg/ml NGR1 (Fig. 5A). This result suggested that the highest mineralization in response to 1,000 μg/ml NGR1 was not due to the upregulation of Runx2. A previous study demonstrated that the induction of ALP activity was mediated through the activation of a Smad-independent signaling pathway p38 MAPK (45). OCN is a late differentiation marker for osteoblastogenesis, which is modulated by osterix (46). It may be plausible that NGR1 at different concentrations can differentially modulate p38 MAPK and osteorix. Screening the gene expression pattern of pre-osteoblasts under the stimulation of NGR1 at different concentrations may help to further elucidate the signaling pathway involved.

One limitation in this study was that we only adopted one type of osteogenic cell line, which may behave differently from primary osteoblasts or bone marrow stromal cells. Studies using human primary mesenchymal cells may be more indicative for its clinical application potential. However, we demonstrate that NGR1 has potential for use as a bone regeneration agent.

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