

Noggin Suppression Decreases BMP-2-Induced Osteogenesis of Human Bone Marrow-Derived Mesenchymal Stem Cells *In Vitro*

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ABSTRACT

Numerous studies with rodent cells and animal models indicate that noggin inhibits osteogenesis by antagonizing bone morphogenetic proteins (BMPs); however, the effect of noggin on osteogenesis of human cells remains ambiguous. This study aims to examine the effects of noggin suppression on viability and BMP-2-induced osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs) *in vitro*. Noggin expression in human MSCs was suppressed by noggin-specific small interfering RNA (siRNA), and viability of human MSCs was determined by measuring the mitochondrial dehydrogenase activity, cellular DNA content and protein amount. The BMP-2-induced osteogenic differentiation of human MSCs was assessed by analyzing the expression levels of several osteoblastic genes, enzymatic alkaline phosphatase (ALP) activity and calcification. Our study showed that noggin suppression significantly decreased human MSC metabolism and DNA content on Days 3 and 6, and decreased total protein amount on Day 14. Noggin suppression also reduced the expression levels of osteoblastic genes, ALP, integrin-binding sialoprotein (IBSP), muscle segment homeobox gene (MSX2), osteocalcin (OC), osteopontin (OPN), and runt-related transcription factor-2 (RUNX2). Significantly decreased enzymatic ALP activity in noggin-suppressed group was evident. Moreover, noggin suppression decreased calcium deposits by BMP-2-induced osteoblasts. Collectively, this study showed that noggin suppression decreased viability and BMP-2-induced osteogenic differentiation of human MSCs, which suggests that noggin is stimulatory to osteogenesis of human MSCs. *J. Cell. Biochem.* 113: 3672–3680, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: NOGGIN; HUMAN MESENCHYMAL STEM CELLS; VIABILITY; OSTEOGENIC DIFFERENTIATION

After a fracture, mesenchymal stem cells (MSCs) migrate to the injured site and differentiate into osteoblasts, which produce bone matrix and repair the fractured bone [Granero-Molto et al., 2009]. This process is precisely regulated by various signals, including the potent osteoinductive signal elicited by bone morphogenetic proteins (BMPs) [Urist, 1965; Chen et al., 2004; Friedman et al., 2006; Tsuji et al., 2006]. BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 have been reported to induce osteogenic differentiation of MSCs both *in vitro* and *in vivo* [Friedman et al., 2006; Kang et al., 2009]. BMPs exert their activity by binding to type 1 receptors and type 2 receptors on the cell surface, and transduce

signals by activating intracellular Smad proteins [Shi and Massague, 2003; Lavery et al., 2008]. The activated Smad protein complex then regulates the expression of osteoblastic genes by interacting with various transcription factors. BMP-mediated osteogenic differentiation is closely regulated by extracellular BMP antagonists, including noggin, chordin, gremlin, and follistatin [Canalis et al., 2003; Chen et al., 2004].

The function of noggin in BMP-mediated osteogenic differentiation has been extensively investigated in rodent cells and animal models, and the results convincingly indicate noggin to be an inhibitor of osteogenesis. For instance, noggin blocked the

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stimulatory effects of BMPs on collagen I synthesis and alkaline phosphatase (ALP) activity in mouse bone marrow cells and rat osteoblasts [Gazzerro et al., 1998; Abe et al., 2000]. Over-expression of noggin impaired osteogenic differentiation of murine preosteoblastic cells and reduced bone formation in transgenic mouse model [Devlin et al., 2003; Wu et al., 2003; Okamoto et al., 2006]. Blocking the effect of noggin by noggin-neutralizing antibody or small interfering RNA (siRNA) increased osteogenic differentiation of murine bone marrow cells and preosteoblast cell lines, and accelerated bone formation in mice with critical-sized calvarial defects [Abe et al., 2000; Wan et al., 2007; Takayama et al., 2009]. These studies strongly show that noggin inhibits osteogenesis in rodent cells and animal models.

In comparison with the clear effect of noggin on osteogenesis of rodent cells and animal models, the influence of noggin on osteogenesis of human cells remains ambiguous. It has been observed clinically that the heterozygous mutations in the gene encoding noggin not only brought about abnormal joint formation [Gong et al., 1999], but also led to elevated blood ALP activity and accelerated osteogenesis [Rudnik-Schoneborn et al., 2010], although whether such mutations could diminish or boost noggin's activity has not been determined yet. A study by Rifas [2007] showed that recombinant mouse noggin increased ALP activity and mineralization of human bone marrow-derived MSC cultures, indicating a stimulatory effect of noggin on osteogenesis of human MSCs. In a recent study with human adipose-derived MSCs, transfection of noggin siRNA alone did not alter the expression of runt-related transcription factor-2 (RUNX2) and osteocalcin (OC), while co-transfection of noggin siRNA and BMP-2 expression plasmid elicited higher levels of RUNX2 and OC expression after 30 days of osteogenic differentiation than that elicited by BMP-2 transfection alone [Ramasubramanian et al., 2011]. Collectively, these studies show that the effects of noggin on BMP-induced osteogenesis of human MSCs are still elusive and further investigation on the role of noggin in osteogenesis of human MSCs is warranted. To this end, this study was conducted to examine the effects of noggin suppression on viability and BMP-2-induced osteogenic differentiation of primary human MSCs *in vitro*.

MATERIALS AND METHODS

ISOLATION AND EXPANSION OF HUMAN MSCs

Bone marrow samples were obtained with informed consent from five donor patients who underwent orthopaedic surgery (patient information in Supplementary Table I). The study was approved by the Research Ethics Committee at the University of Alberta. Mononuclear cells from the bone marrow samples of these five donor patients were separately isolated by centrifugation (400*g*, 25 min) with Ficoll-Paque (GE Healthcare, Piscataway, NJ, USA) and then were seeded at a density of 4×10^5 cells/cm² in MSC growth medium (MGM: high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ml Glutamax (all from Invitrogen, Burlington, ON, Canada), and 4 ng/ml FGF-2 (Millipore, Temecula, MA)). After a 3-day incubation, non-adherent cells were discarded. Adherent cells were washed twice with

phosphate buffered saline (PBS, Invitrogen) and expanded in MGM. After 7 days, the cells from each patient were either expanded for further experiments or frozen in 1 ml aliquots in liquid nitrogen. Characterization of these cells was presented in our previous study [Chen et al., 2012]. For all experiments, cells were incubated at 37°C in a 5% CO₂ incubator. All experiments were conducted in triplicates or quadruplicates on individual cell cultures from each patient. The data generated from the individual cell culture of five donor patients were pooled and presented in this article as combined data (mean ± SEM of *n* = 5).

BMP-2 TREATMENT

To determine whether BMP-2 induces noggin mRNA expression in human MSCs, a dose-response study and a time-course study were conducted. For the dose-response study, human MSCs were seeded in MGM in 35 mm tissue culture dishes at a density of 7×10^3 cells/cm². After 24 h, medium was replaced with basal medium (DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml Glutamax (all from Invitrogen)) containing various concentrations (0–50 μg/ml) of recombinant human BMP-2 (Medtronic, Brampton, ON, Canada). The cells were lysated with Trizol reagent (Invitrogen) and collected at 72 h after the BMP-2 treatment. Total RNA was extracted by using phenol/chloroform method and noggin mRNA expression was analyzed by quantitative reverse transcription-PCR (qRT-PCR). Noggin expression level was first normalized by β-actin, and then transformed to the ratio over the group without BMP-2 treatment. For the time-course study, human MSCs in 35 mm tissue culture dishes were treated with or without 0.1 μg/ml BMP-2, and cells samples were collected at 0, 24, 48, 72, and 96 h. Noggin expression level was first normalized by β-actin, and then transformed to the ratio over the noggin expression level at 0 h.

REVERSE TRANSCRIPTION AND qRT-PCR

Total RNA was quantified by using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and reverse transcribed to cDNA with the iScript cDNA synthesis Kit (Bio-Rad, Mississauga, ON, Canada). Reverse transcription was performed in 20 μl volume with the following protocol: 25°C for 5 min followed by 42°C for 30 min, and 85°C for 5 min. qRT-PCR was conducted in quadruplicates with the iQ5 system (Bio-Rad). The 25 μl reaction mixture contained 10 ng cDNA from reverse transcription, 200 nM of each primer, and 1 × iQ SYBR Green supermix (Bio-Rad). qRT-PCR was performed with the following protocols: one cycle of 95°C for 3 min, followed by 45 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 10 s. Human β-actin was used as the endogenous reference gene to normalize noggin gene expression. All primers (synthesized by Invitrogen) used in this study were listed in Supplementary Table II.

OPTIMIZATION OF siRNA TRANSFECTION CONDITION

BLOCK-iT Alexa Fluor Red fluorescent siRNA (Invitrogen) is an Alexa Fluor 555-labeled siRNA with the same length, charge and configuration as standard siRNA. In this study it was transfected into human MSCs with Lipofectamine RNAiMAX (transfection reagent from Invitrogen) to optimize the transfection condition. To identify

an effective condition for siRNA transfection, we selected several combinations of siRNA with Lipofectamine RNAiMAX (Supplementary Table III) and tested the transfection efficiency by flow cytometry. Human MSCs were seeded in 12-well plates (BD Biosciences, Franklin Lakes, NJ) at a density of 5.5×10^3 cells/cm². After 24 h, medium was replaced with 1 ml MGM without antibiotics. Fluorescent siRNA and RNAiMAX (see Supplementary Table III for final concentration/volume) were diluted separately in Opti-MEM I reduced serum medium (Invitrogen). Diluted fluorescent siRNA and diluted RNAiMAX were combined, gently mixed, and incubated for 20 min at room temperature. Then, 200 μ l fluorescent siRNA-RNAiMAX complexes were added to each well containing human MSCs. Twenty-four hours later, the cells were washed with cold PBS and detached with 0.05% trypsin (Invitrogen). The percentage and mean fluorescence intensity of transfected MSCs were analyzed by a Quanta SC Flow Cytometer (Beckman Coulter, Mississauga, ON, Canada) with the excitation light of 488 nm and emission peak of 575 nm. The transfection system that yielded the highest percentage and highest mean fluorescence intensity of transfected MSCs was chosen for the following experiments.

ASSESSMENT OF NOGGIN siRNA EFFICACY

Four synthetic siRNAs (Qiagen, Mississauga, ON, Canada), designed to target different regions of human noggin mRNA (Gene Accession Number: NM_005450), were obtained and their sequence information was listed in Supplementary Table IV. The above-mentioned BLOCK-iT Alexa Fluor Red fluorescent siRNA was used as the control siRNA since it has the same physical property as standard siRNA and it is not homologous to any known gene. To validate the knockdown efficacy of these noggin siRNAs, human MSCs were transfected with control siRNA and noggin siRNAs, respectively, under the previously optimized transfection condition. We set up six groups in triplicates: (1) non-transfected MSCs (named as NT group), (2) MSCs transfected with control siRNA (named as Ctrl siRNA group), (3) MSCs transfected with noggin siRNA1 (named as siRNA1 group), (4) MSCs transfected with noggin siRNA2 (named as siRNA2 group), (5) MSCs transfected with noggin siRNA3 (named as siRNA3 group), and (6) MSCs transfected with noggin siRNA4 (named as siRNA4 group). After a 24-h transfection, MSCs were exposed to basal medium containing 0.1 μ g/ml BMP-2. The total RNA was extracted at 72 h after BMP-2 treatment and noggin mRNA expression was assessed by qRT-PCR.

In addition, at 72 h after BMP-2 treatment, noggin protein in the culture supernatant was measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit for human noggin (Uscn Life Science Inc., Wuhan, Hubei, China). Briefly, the microtiter plate was pre-coated with a monoclonal antibody specific for human noggin. Standards and samples were added with a biotin-conjugated polyclonal antibody preparation specific for noggin. Then avidin-conjugated horseradish peroxidase (HRP) was added to each well and incubated. Next, a TMB substrate solution was added to each well and the enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution. The color was measured spectrophotometrically at a wavelength of 450 nm. The concentration of noggin in the samples was then determined by comparing the O.D. of the samples with the standard curve. The most effective

noggin siRNA to suppress noggin expression at both mRNA and protein levels was chosen for the following experiments.

To further understand the duration of noggin suppression by single transfection of noggin siRNA in the presence of BMP-2, we conducted a time-course study on noggin expression after siRNA transfection. Before transfection, we collected samples to determine the baseline of noggin expression (named as Day 0). For the transfection, we set up three groups in triplicates: one group without transfection (NT), one group transfected with control siRNA (Ctrl siRNA) and one group transfected with the most effective noggin siRNA. After a 24-h transfection, medium was replaced with basal medium containing 0.1 μ g/ml BMP-2 and medium was changed twice a week. Cell samples were collected on Days 3, 7, and 10. Total RNA was extracted and qRT-PCR was performed to assess the expression levels of noggin at the indicated time points.

WATER SOLUBLE TETRAZOLIUM SALT-8 (WST-8) ASSAY

The human MSCs were seeded in quadruplicates in 48-well plates at a density of 5.5×10^3 cells/cm². Twenty-four hours later, human MSCs in 300 μ l medium were transfected with control siRNA and the most effective noggin siRNA, separately. After 24 h, medium was replaced with basal medium containing 0.1 μ g/ml BMP-2. The metabolism of human MSCs was then assessed on Days 0, 3, and 6. The WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; Cedarlane, Burlington, ON, Canada) reagent was used to assess the mitochondrial dehydrogenase activity of human MSCs. WST-8 is reduced by mitochondrial dehydrogenases in viable cells to yield a soluble yellow formazan, which is directly proportional to the reduction activity of the cells and used as a measure of total cellular metabolic activity [Ishiyama et al., 1997]. Briefly, 30 μ l of the WST-8 solution was added to the human MSCs in 300 μ l medium, and incubated for additional 3 h. Then the absorbance was measured with an absorbance microplate reader (BioTek, Winooski, VT) at 450 nm with a reference wavelength of 650 nm. Data from individual patient were first transformed to the ratio over the non-transfection group and then data from all five patients were combined.

DNA CONTENT ANALYSIS

The human MSCs were seeded in quadruplicates in 96-well plates at a density of 3×10^3 cells/cm². Twenty-four hours later, human MSCs were transfected with control siRNA and the most effective noggin siRNA selected, respectively. After 24 h, medium was replaced with basal medium containing 0.1 μ g/ml BMP-2. Cells were lysated and samples were collected on Days 0, 3, and 6. The DNA content was measured by using a CyQUANT Cell Proliferation Assay Kit (Invitrogen) with the excitation wavelength of 450 nm and emission wavelength of 530 nm. Data from individual patient were transformed to the ratio over the non-transfection group and then data from all five patients were combined.

INDUCTION OF OSTEOGENIC DIFFERENTIATION OF HUMAN MSCs

To understand the effect of noggin suppression on osteogenic differentiation of human MSCs, we knocked down noggin expression in human MSCs and then induced human MSCs to undergo osteogenic differentiation with osteogenic medium (DMEM

containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.29 mg/ml L-glutamine (all from Invitrogen), 10 nM dexamethasone, 5 mM β -glycerolphosphate, and 50 mg/L ascorbic acid-2-phosphate (all from Sigma, St. Louis, MO) containing 0.1 μ g/ml BMP-2 in 12-well plates. There were three study groups: one group without transfection (NT), one group transfected with control siRNA (Ctrl siRNA) and one group transfected with the most effective noggin siRNA selected. All these three groups were treated with osteogenic medium containing 0.1 μ g/ml BMP-2 after transfection. According to the suggestion of the manufacturer (Qiagen), siRNA transfection was repeated every 7 days for prolonged silencing of noggin protein. Assessment of osteogenic differentiation was conducted in the following experiments.

qRT-PCR ANALYSIS OF OSTEOLASTIC GENES

On Day 14 of osteogenic induction, cell samples were collected, total RNA was extracted and reverse transcription was performed as described above. Expression levels of osteoblastic genes, ALP, integrin-binding sialoprotein (IBSP), muscle segment homeobox gene (MSX2), OC, osteopontin (OPN), and RUNX2 were examined by qRT-PCR. Human β -actin was used as the endogenous reference gene to normalize the osteoblastic gene expression. After normalization, data from individual patient were transformed to the ratio over the non-transfection group and then data from all five patients were combined. Expression of noggin was measured as well to confirm the suppression of noggin on Day 14. All primers were listed in Supplementary Table II.

ALP STAINING

On Day 14 of osteogenic induction, ALP staining was performed by using a Fast Blue B Kit (Sigma). Briefly, cells were washed three times with PBS and fixed by citrate-acetone-formaldehyde fixative for 30 s. After a brief rinse with deionized water, samples were stained by sodium nitrite/FBB alkaline solution in the dark for 15 min, and counterstained by neutral red solution for 2 min. Finally samples were washed three times with tap water to remove the dissociative dye.

ALP ASSAY

On Day 14 of osteogenic induction, ALP activity was quantitatively measured by using a commercial phosphatase assay kit (BioAssay Systems, Hayward, CA). The cells in triplicates were lysated with lysis buffer containing 0.5% Triton, 50 mM Tris-HCl, and 5 mM MgCl₂ (Sigma). The lysate was then transferred to 96-well plates, incubated with ALP substrate at 37°C for 30 min and then the reaction was halted by the addition of stop buffer. The *p*-nitrophenol product formed by enzymatic hydrolysis of *p*-nitrophenylphosphate (pNPP) substrate was measured at 405 nm by using an absorbance microplate reader (BioTek). The protein concentration of samples was measured by using a DC Protein Assay Kit (Bio-Rad) and bovine serum albumin (Bio-Rad) was used to create a standard curve to transform data. ALP concentration was normalized by the total protein amount. Data from individual patient were transformed to the ratio over the non-transfection group and then data from all five patients were combined.

ALIZARIN RED STAINING

On Day 28 of osteogenic induction, cells and the extracellular matrix of all groups were fixed in 75% ethanol at 4°C for 1 h, rinsed rapidly in distilled water, and stained by Alizarin red S solution (Sigma) until orange-red color. Samples were then washed with deionized water for three times, followed by washing once with PBS.

CALCIUM ASSAY

On Day 28 of osteogenic induction, the cells and extra cellular matrix secreted by the cells were demineralized by adding 600 μ l of 0.5 N hydrochloric acid solution to each well (12-well plates) and incubating at 4°C overnight. The supernatant containing calcium extracts was collected after centrifugation at 10,000g for 10 min. The calcium concentration was measured by using a QuantiChrom Calcium Assay Kit (BioAssay Systems). Data from individual patient were transformed to the ratio over the non-transfection group and then data from all five patients were combined.

STATISTICAL ANALYSIS

Data were presented as the mean with the standard error and analyzed by Student's *t*-test or one-way ANOVA followed by Bonferroni post hoc test, as appropriate. All tests were two-sided with *P* < 0.05 considered as the level of significance. All statistical analyses were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, IL).

RESULTS

INDUCED NOGGIN mRNA EXPRESSION BY BMP-2

Noggin mRNA expression was induced by BMP-2 in a dose-dependent manner, with the peak expression being induced by 1 μ g/ml BMP-2 (Fig. 1A). Within the range of 0 to 1 μ g/ml BMP-2, higher concentration of BMP-2 induced higher noggin expression, while the induction of noggin expression was diminished as the concentration of BMP-2 increased from 1 to 50 μ g/ml. There was no significant difference in noggin expression levels between the group without BMP-2 treatment and the group treated with 50 μ g/ml BMP-2 (Fig. 1A).

Noggin transcript was also induced by BMP-2 in a time-dependent manner. Induced by 0.1 μ g/ml BMP-2, noggin mRNA levels were gradually increased with time and significant increases were evident at 48, 72, and 96 h after the BMP-2 treatment (Fig. 1B).

SUPPRESSION OF BMP-2-INDUCED NOGGIN EXPRESSION BY NOGGIN siRNA TRANSFECTION

Among the transfection conditions examined, the optimal condition turned out to be 16.6 nM (final concentration) of siRNA duplex with 3 μ l Lipofectamine RNAiMAX in 1200 μ l medium for each well of 12-well tissue culture plates, which yielded the highest percentage of siRNA-positive cells and highest mean fluorescence intensity (i.e., siRNA concentration) in transfected cells (Supplementary Fig. 1A,B). This optimal condition was used for subsequent siRNA transfection studies.

Transfection of control siRNA did not alter noggin mRNA and protein levels, compared with the non-transfected group (Fig. 2A,B). In contrast, transfection of noggin siRNA1 and siRNA3 significantly

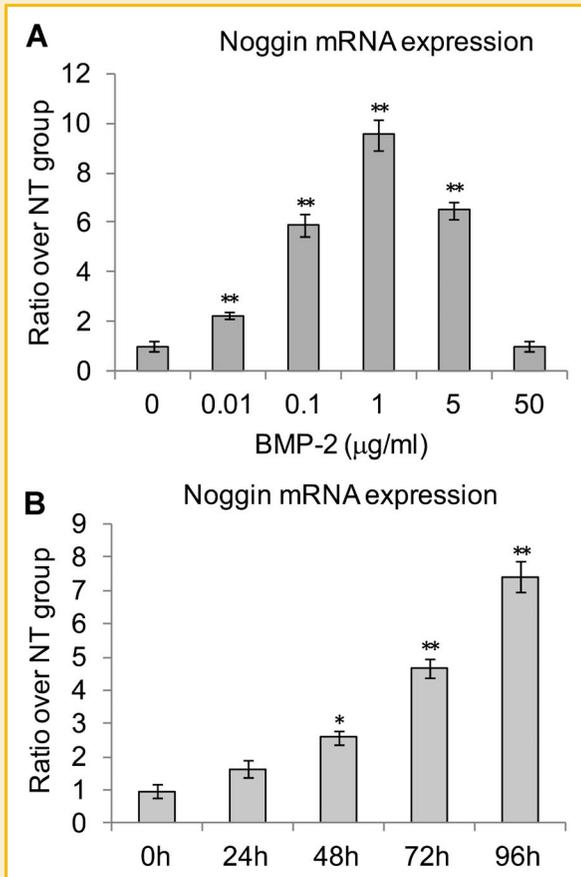


Fig. 1. Noggin mRNA expression induced by BMP-2 in human MSC cultures. A: Noggin mRNA expression was dose-dependently induced by BMP-2 after 72 h treatment. ** $P < 0.01$ versus the group without BMP-2 treatment. B: Noggin mRNA expression was time-dependently increased by 0.1 µg/ml BMP-2. * $P < 0.05$ and ** $P < 0.01$ versus the noggin expression level at 0 h.

reduced noggin mRNA expression level in human MSCs (Fig. 2A) and noggin protein level in the culture supernatant (Fig. 2B). While transfection of noggin siRNA2 and siRNA4 did not change noggin expression at mRNA level (Fig. 2A), they slightly increased the noggin protein level in the culture supernatant (Fig. 2B). Since noggin siRNA3 achieved the best suppression effect on noggin expression (Fig. 2A,B), we conducted the following experiments with noggin siRNA3 only.

In the time-course study, with BMP-2 treatment, increased noggin mRNA expression was observed from Day 0 to 7 in the non-transfection group and the control siRNA-transfected group. Single noggin siRNA3 transfection was able to significantly suppress noggin expression on Days 3 and 7 after siRNA transfection, while the suppression effect was not evident on Day 10 after siRNA transfection (Fig. 2C).

DECREASED VIABILITY OF HUMAN MSCs BY NOGGIN SUPPRESSION

The WST-8 assay indicated that MSC metabolism was significantly inhibited by noggin siRNA3 on Days 3 and 6 after siRNA transfection (Fig. 3A). Similar pattern was observed in the DNA assay. The total DNA content was significantly decreased by noggin

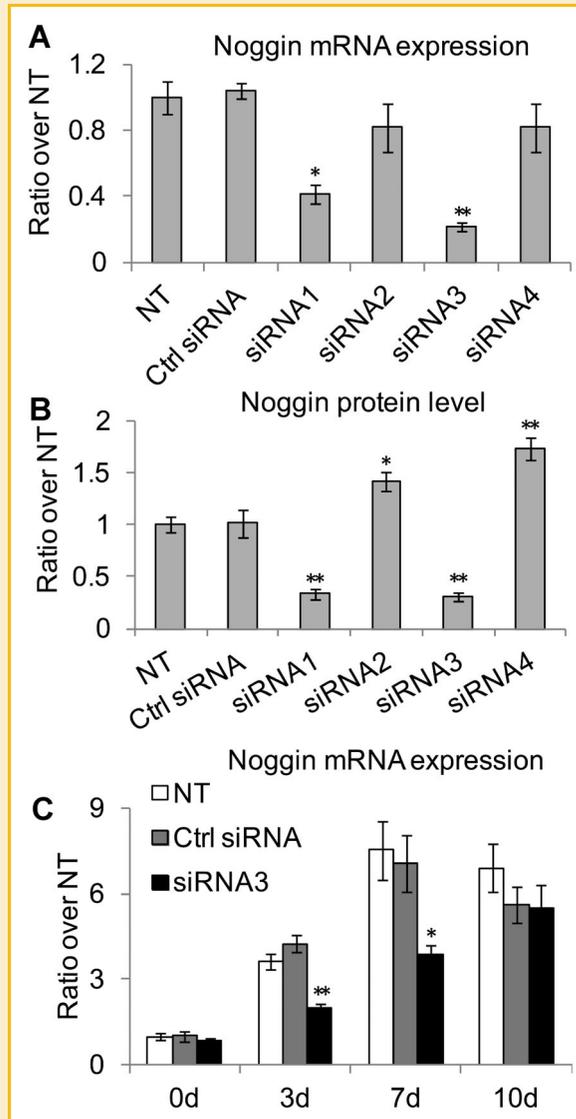


Fig. 2. Efficacy of noggin siRNAs in suppressing noggin expression. A: Noggin mRNA expression at 72 h after transfection of different siRNAs. B: Noggin protein concentration in the culture supernatant at 72 h after transfection of different siRNAs. C: Noggin mRNA expression in human MSCs. Cells were treated with 0.1 µg/ml BMP-2 after transfection. * $P < 0.05$ and ** $P < 0.01$ versus both NT and Ctrl siRNA groups at the same time point.

siRNA3 on Days 3 and 6 after siRNA transfection (Fig. 3B). In addition, the total protein amount in noggin siRNA3-transfected group was significantly lower than that in the control groups on Day 14 (Fig. 3C).

DECREASED OSTEOGENIC DIFFERENTIATION OF HUMAN MSCs BY NOGGIN SUPPRESSION

Since the suppression effect of single transfection of noggin siRNA3 lasts for a maximum of 7 days (Fig. 2C), we performed siRNA transfection every 7 days during the osteogenic differentiation of human MSCs. On Day 14 of osteogenic induction, effective noggin suppression by noggin siRNA3 was confirmed (Supplementary

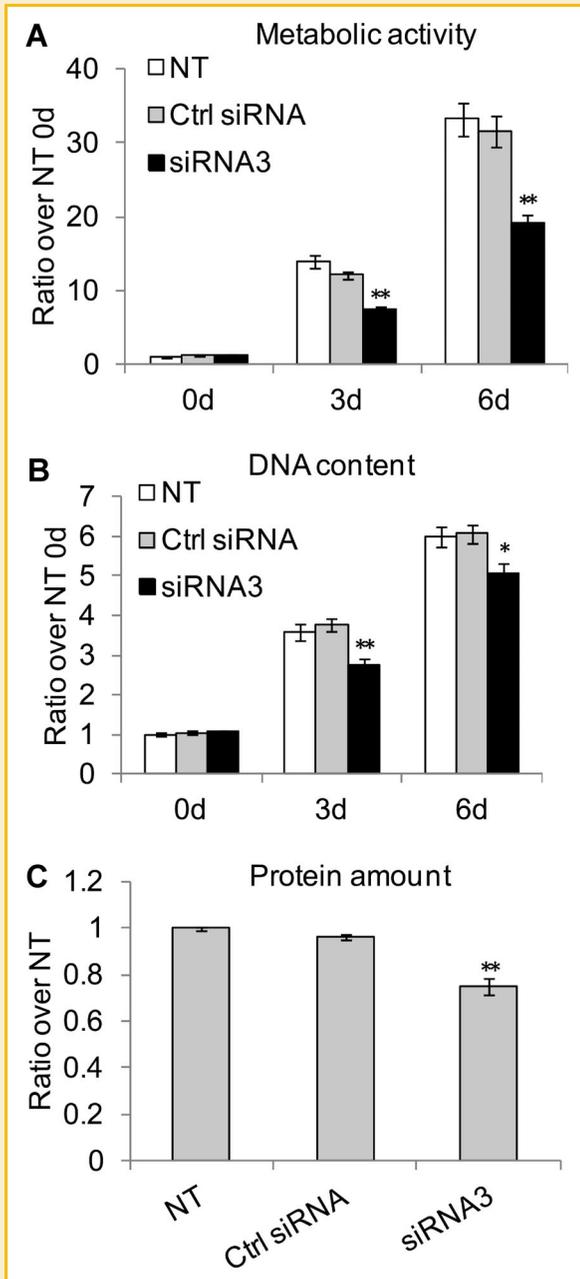


Fig. 3. Decreased viability of human MSCs by noggin suppression. Both metabolism (A) and total DNA content of human MSCs (B) were decreased by noggin suppression on Days 3 and 6 after siRNA3 transfection. C: Total protein amount of human MSCs was also decreased by noggin suppression on Day 14. * $P < 0.05$ and ** $P < 0.01$ versus both NT and Ctrl siRNA groups.

Fig. 2). At the same time, noggin siRNA3 significantly decreased expression levels of all select osteoblastic genes, including ALP, IBSP, MSX2, OC, OPN, and RUNX2 (Fig. 4A–F).

Quantitative Fast Blue B staining showed that noggin siRNA3 transfection decreased the ALP activity of the cells after a 14-day osteogenic induction (Fig. 5A). Similar result was obtained in the quantitative ALP assay, which showed that noggin siRNA3 transfection significantly decreased the ALP activity of the cells (Fig. 5B).

Qualitative Alizarin Red staining showed less calcium deposits were produced by the group that was transfected with noggin siRNA3, compared with two control groups (Fig. 6A). Quantitative calcium assay also showed that transfection of noggin siRNA3 significantly decreased the calcium deposits in the cell culture (Fig. 6B).

DISCUSSION

In the present study, we found that noggin suppression decreased viability and BMP-2-induced osteogenic differentiation of human MSCs. This study extends our understanding of the role of noggin in osteogenesis of human MSCs, revealing that noggin might be beneficial to human bone formation.

We observed that noggin expression was induced by BMP-2 in human MSC cultures in a dose- and time-dependent manner, which is in agreement with the previous observations in both mouse cells [Takayama et al., 2009] and human MSCs [Diefenderfer et al., 2003ab]. However, the biphasical nature of dose-dependent noggin induction by BMP-2 was observed for the first time. Noggin induction was enhanced by BMP-2 at the concentrations from 0.01 to 1 $\mu\text{g/ml}$, while the induction was diminished when the concentrations of BMP-2 shifted from 1 to 50 $\mu\text{g/ml}$ (Fig. 1). Interestingly, the concentrations of BMP-2 that were reported to induce noggin expression in the previous studies all fell into the range from 0.01 to 1 $\mu\text{g/ml}$ [Takayama et al., 2009; Diefenderfer et al., 2003ab]. However, we do not know why further increase in BMP-2 concentration above 1 $\mu\text{g/ml}$ diminished noggin induction. One possibility is that the high concentrations of BMP-2 may have some cytotoxic effects on the cells. Regardless of specific mechanism(s), by showing that the high concentration BMP-2 of 50 $\mu\text{g/ml}$ failed to induce noggin expression and noggin suppression decreased osteogenesis, our data suggest that high dose of BMP-2 might not be beneficial to new bone formation.

We observed that noggin suppression decreased cellular metabolism, DNA content and protein amount of human MSCs. These effects were unlikely to be brought by transfection system since the transfection of control siRNA did not alter these features of human MSCs in culture (Fig. 3A–C). It has been reported that noggin accelerated proliferation rate of human embryonic stem cells (ESCs) [Chaturvedi et al., 2009]. Noggin and basic FGF synergistically sustained the proliferation of human ESCs [Wang et al., 2005; Xu et al., 2005]. In addition, noggin retained the self-renewal and increased the proliferation of mouse neural stem cells [Bonaguidi et al., 2008]. These data indicate that noggin plays important roles in proliferation of stem cells. Therefore, it is logical that noggin suppression resulted in decreased viability of human MSCs in this study. Although our data indicate noggin suppression did decrease the cell viability of human MSCs, we do not think the decreased ALP induction and expression levels of osteoblastic genes on Day 14 were solely the secondary inhibitory effects of noggin suppression on cell viability, since the ALP concentration presented in this article was normalized by the total protein amount and expression levels of osteoblastic genes were normalized by β -actin of the corresponding group. After such normalizations, a decrease in osteogenic activity

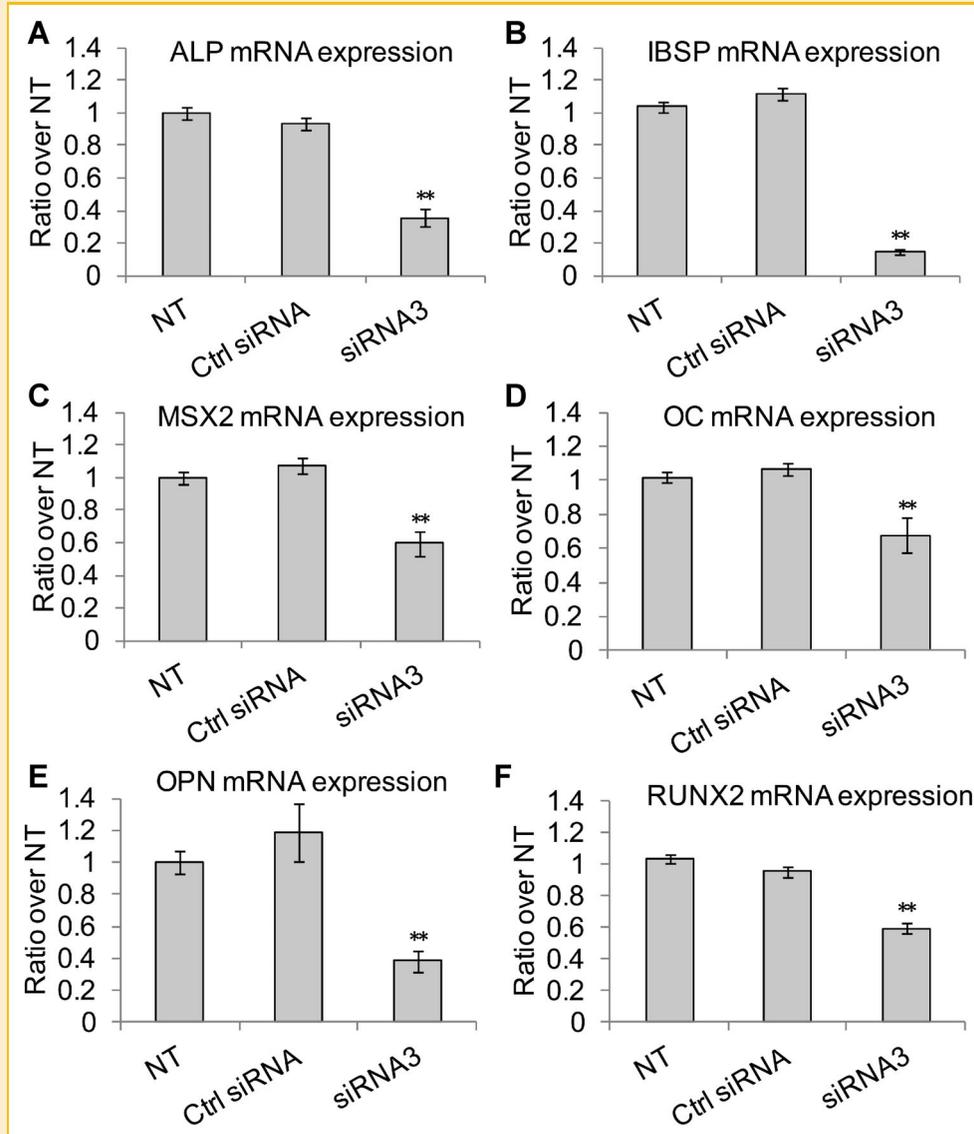


Fig. 4. Expression levels of osteoblastic genes, ALP (A), IBSP (B), MSX2 (C), OC (D), OPN (E), and RUNX2 (F) on Day 14 during osteogenic induction by osteogenic medium containing 0.1 μ g/ml BMP-2. ** $P < 0.01$ versus both NT and Ctrl siRNA groups.

was still evident, so that stagnated cell growth alone cannot explain the differences. In other words, the decreased ALP production and expression levels of osteoblastic genes by noggin suppression represent a suppression of osteogenic differentiation per cell basis. However, we did not normalize calcium deposits; therefore, the decreased calcium deposits by noggin suppression might be partly due to the stagnation of cell growth. The suppression of noggin expression by single transfection of noggin siRNA3 lasted for a maximum of 7 days in this study (Fig. 2C). Based on this observation, we transfected cells with noggin siRNA3 every 7 days during the osteogenic induction, in order to constantly knock down noggin expression. The repeated transfections of noggin siRNA3 turned out to be effective to continuously suppress noggin expression, as demonstrated by Supplementary Figure 2, in which the expression of noggin was still suppressed on Day 14.

Our data are not in agreement with several studies with rodent cells and animal models, in which noggin was demonstrated to be inhibitory to osteogenic differentiation [Gazzerro et al., 1998; Wan et al., 2007; Takayama et al., 2009]. In these studies, skeletal-specific over-expression of noggin in mice decreased expression of osteoblastic genes, trabecular bone volume, and bone formation rates [Devlin et al., 2003; Wu et al., 2003]. Noggin suppression by siRNA enhanced osteogenesis of mouse cells in vitro and accelerated bone formation in vivo [Wan et al., 2007; Takayama et al., 2009]. Noggin blocked the osteoinductive activity of BMPs in mouse and rat cells [Gazzerro et al., 1998; Garrett et al., 2003; Zhu et al., 2006]. Collectively, these observations from rodent cells and animal models indicate that noggin expression is detrimental to bone formation. However, our observation is in agreement with a previous observation that the addition of noggin to human MSC culture

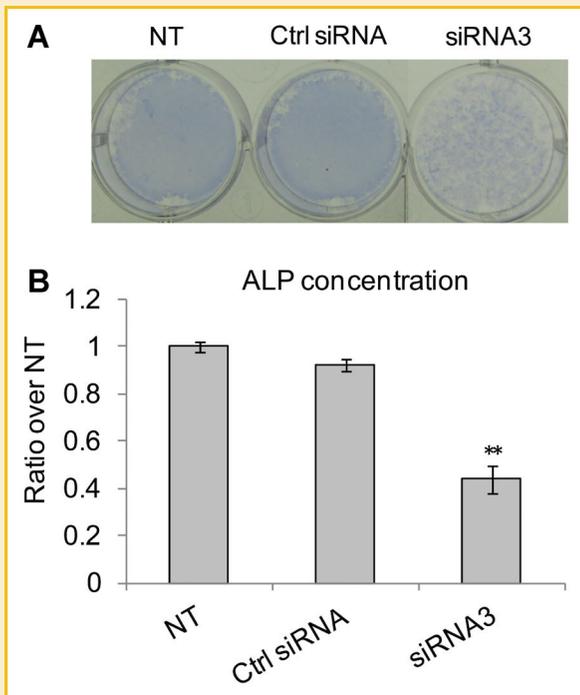


Fig. 5. ALP activity was decreased by noggin suppression, as determined by Fast Blue-B staining (A) and enzymatic ALP assay (B) on Day 14 during osteogenic induction by osteogenic medium containing 0.1 $\mu\text{g/ml}$ BMP-2. ** $P < 0.01$ versus both NT and Ctrl siRNA groups. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jcb>]

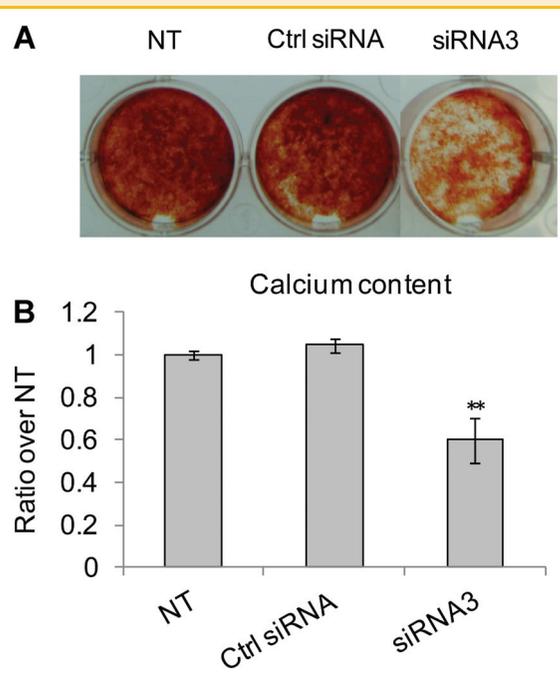


Fig. 6. Calcium deposits were decreased by noggin suppression, as determined by Alizarin Red staining (A) and calcium assay (B) on Day 28 during osteogenic induction by osteogenic medium containing 0.1 $\mu\text{g/ml}$ BMP-2. ** $P < 0.01$ versus both NT and Ctrl siRNA groups. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jcb>]

increased ALP activity and calcification, and up-regulated expression of several osteoblastic genes [Rifas, 2007], which indicates that noggin is a stimulator of osteogenesis of human MSCs. Instead of studying the effects of adding exogenous noggin, our study showed the effect of endogenous noggin on BMP-2-induced osteogenesis of human MSCs, by knocking down the endogenous noggin expression. These inconsistent observations from studies with rodent cells/animal models and human MSCs, suggest that there might be a species-specific difference in the role of noggin during BMP-2-induced osteogenesis of MSCs. This conjecture is partially supported by several studies. BMP-2 and dexamethasone exerted different osteoinductive effects on MSCs from humans, rats, and mice [Diefenderfer et al., 2003b; Osyczka et al., 2004]. Although rat MSC expressed mRNA for ALK-6 (type 1 receptor for BMPs), human MSCs lacked this particular receptor [Osyczka et al., 2004]. BMP-2 could up-regulate *Msx-2* up to 10 folds in human MSCs, but BMP-2 barely changed *Msx-2* expression in rat MSCs [Osyczka et al., 2004]. These observations, along with our data, indicate that there might be a species-specific difference during BMP-2-induced osteogenesis of MSCs.

Our study indicates that noggin is beneficial to osteogenic differentiation of human MSCs in the presence of BMP-2. One possible mechanism might be that upon binding to BMP-2, noggin does not inactivate BMP-2 in human MSC cultures, but rather makes it more available to the receptor. Noggin is known to bind to cell-surface proteoglycans [Paine-Saunders et al., 2002] and may act as a

“bait” for extracellular BMP-2, facilitating its binding to cell surfaces. As for the possible intracellular signaling, we speculate that noggin regulates osteogenic differentiation, at least partly, through modulating the intracellular canonical BMP/Smad signaling, then RUNX2, MSX2, or other unidentified transcription factors, and the subsequent gene expression of ALP, IBSP, OC, and OPN, based on the following observations. It has been reported that noggin actively stimulates BMP-2 and its type 2 receptor production [Rifas, 2007]. Previous studies also showed that noggin orchestrated BMP signaling and subsequent osteoblastic differentiation through regulating the transcription, phosphorylation and translocation of Smad 1/5 [Wrinkler et al., 2004; Wan et al., 2007]. Our data suggest that noggin suppression decreased the expression of RUNX2 and MSX2, the crucial transcription factors for osteogenic differentiation. In addition, expression levels of osteoblastic markers, ALP, IBSP, OC and OPN were all decreased by noggin suppression (Fig. 4). Further studies on the intracellular signaling pathways through which noggin regulates osteogenic differentiation of human MSCs are warranted.

We conclude that noggin expression in human MSCs culture was up-regulated by BMP-2 in a dose-dependent and time-dependent manner. Noggin suppression decreased viability and BMP-2-induced osteogenic differentiation of human MSCs, which suggests a stimulatory effect of noggin on osteogenesis of human MSCs. The underlying mechanism(s) for these observations are unclear, and further studies on the intracellular signaling pathways through

which noggin regulates osteogenic differentiation of human MSCs are warranted. In addition, similar studies on human MSCs with a larger sample size are of importance, to reveal if there is any difference between genders in response to noggin suppression.

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