Noggin Prevents Osteogenesis but Induces Chondrogenesis in a Human Mesenchymal Cell Line (USAC)

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Abstract: The differentiation of mesenchymal stem cells (MSCs) into chondrocytes and osteoblasts is minutely regulated by bone morphogenetic proteins (BMPs) and various factors. There is no study describing the role of noggin in the differentiation from MSCs to chondrocytes or osteoblasts. We thus examined the effect of noggin on chondrogenesis and osteogenesis in a human cell line that expresses chondrocytic phenotypes, and the role of noggin in these processes. Noggin induces chondrogenic differentiation, and its repression induces osteogenic differentiation through BMP effects.

Noggin is reported to bind several BMPs and to inhibit their functions, but its exact role is not yet clarified. USAC cells usually show chondrogenic phenotypes and also have the potential to differentiate into osteoblasts and adipocytes under some circumstances. We investigated the effects of noggin on chondrogenic and osteogenic differentiation of USAC cells in vitro and in vivo.

After USAC cells were treated with noggin antisense oligonucleotide (As-Noggin) or noggin protein (rhNoggin), RT-PCR was performed to detect mRNAs of type I collagen (Col I), type II collagen (Col II), Cbfal, aggrecan (AG), Sox9 and osteocalcin (OC) on days 3, 7 and 14. Western blot analysis was done to detect Col II, BMP-2, BMP-4, Cbfal and OC proteins. Toluidine blue staining and immunostaining for Col II, BMP-2, Cbfal and OC were also performed.

As-Noggin induced the up-regulation of Col I and OC mRNA expression in USAC cells time-dependently, while it down-regulated the expression of Col II and AG mRNAs. As-Noggin stimulated BMP-2, Cbfal and OC production, but it reduced the production of Col II and BMP-4. Moreover, OC mRNA expression and BMP-2 production were down-regulated by the addition of rhNoggin. The same results were obtained by immunostaining. These results suggest that noggin may regulate chondrogenic and osteogenic differentiation through the production of BMP-2 and BMP-4.

Key words: noggin, bone morphogenetic protein, chondrogenesis, osteogenesis, human mesenchymal cells.

Noggin is an antagonist of bone morphogenetic proteins (BMPs) expressed in Spemann’s organizer.1) Noggin was reported to bind to several BMPs with very high affinity and prevented their activity by competitively inhibiting interaction with their receptors.2) Noggin is expressed in primordial cartilage in most skeletal tissue. In the in vitro differentiation of C1 mesodermal cells, it was reported that noggin was expressed during chondrogenic differentiation, but was not expressed during osteogenic differentiation.3) In embryonic calvaria, where membranous bone formation occurs, noggin expression is not detected by in situ hybridization.4) On the other hand, noggin is
required for growth and patterning of the neural tube and somite. Lim et al. indicated that the expression of ectopic noggin was accelerated in neurogenesis by inhibiting BMP-2 and BMP-4. Additionally, Seminon et al. reported noggin mutation in three Spanish families with fibrodysplasia ossificans progressiva. These data indicate that noggin may create an environment for the initiation of differentiation of mesenchymal cells. BMPs are members of the TGF-β superfamily, which have been described for osteogenic protein, including BMP-2, -4, -6 and -7.8) BMPs are known for their role in embryonic development and differentiation. They also have modest myogenic properties for skeletal cells, induce the differentiation of mesenchymal cells into osteogenic cells, and enhance the differentiation of osteoblasts.9) In addition, BMP-4 enhances chondrogenic differentiation and increases chondrogenic proteins, such as aggrecan and type II collagen, while recombinant human BMP-2 (rh-BMP-2) prevents the differentiation of rat osteoblastic cells to myoblasts and adipocytes, and promotes the maturation of these cells.10) From the above knowledge, noggin and BMPs play a morphogenetic role in skeletogenesis during the differentiation of mesenchymal cells into chondrocytes or osteoblasts; however, there is no report describing the role of noggin and BMPs in the chondro-osteogenic differentiation of mesenchymal stem cells (MSCs). This study was thus undertaken to examine the effect of noggin on chondrogenesis and osteogenesis in a human cell line that expressed chondrocytic phenotypes, and the role of noggin in these processes.

Materials and Methods

1. Cell culture
USAC cells were cultured in Dulbecco’s modified α-minimum essential medium (α-MEM; GIBCO BRL Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; GIBCO BRL, Gaithersburg, MD, USA), 50 U/ml of penicillin (Meiji Seika, Tokyo, Japan), and 100 mg/ml of streptomycin (Meiji Seika) as described previously.11) Cells were plated onto 6 cm dishes (Corning, Acton, OH) at a density of 5×10^5/dish in 5% FBS/α-MEM under 5% CO_2. After subconfluence, recombinant human noggin (rhNoggin, Wako Chemical, Osaka, Japan) was added to evaluate the effect of noggin on chondro-osteogenic differentiation in USAC cells.

2. Transfection of anti-sense noggin into USAC Cells
Lipofectin (Invitrogen, Japan K.K., Tokyo, Japan) was used in the transfection of oligonucleotides according to the manufacturer’s methods. One day before transfection, USAC cells were counted and plated at a density of 5×10^5 cells per 10 cm dish. After seeding overnight, OPTIMEM (Invitrogen, Japan K.K., Tokyo, Japan) plus Lipofectin and OPTIMEM plus FITC-labeled antisense noggin (As-Noggin) oligomer (100 pmol) were incubated for 15 min at room temperature separately and then combined in one polystyrene tube. The combined solution was incubated for 20 min at room temperature. The solution was then mixed gently and pipetted onto USAC cells. The cells were checked for noggin expression after 3 to 5 days of culture as described in the RT-PCR and Western blot method. The following DNA oligonucleotide was designed from 5' cap to 25 mer downstream of human noggin sequence (GI: HUS31202, 1557 bp) for anti-sense experiment. As-Noggin; 5'-CGATAACAGAGTCTCAGGG-3', control; 5'-CGAUAACAGAGUCUCAGGGdTdT-3'.

3. Immunohistochemical analysis
The production of proteoglycans was determined using 0.1% toluidine blue staining. The expressions of type II collagen, BMP-2, Cbfa1, and osteocalcin were examined immunohistochemically according to the method previously described. Briefly, cells on days 7 and 14 were collected and indirect immunostaining was performed using a streptavidin-peroxidase system (Seikagaku Kogyo, Tokyo, Japan). Anti-human type II collagen (Col II) antibody, anti-human BMP-2 antibody, anti-human Cbfa1 antibody, and anti-human osteocalcin antibody (OC) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4. RT-PCR analysis
Total RNA was extracted from USAC cells by ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer’s protocol. First-strand cDNA was synthesized from one mg of total RNA with 2.5 mM random primer using AMV reverse transcriptase XL (Takara, Tokyo, Japan) at 45°C for 30 min. Subsequent amplification was perfor-
med with an RNA PCR kit version 2.1 (Takara) for 29 cycles under the following conditions: 94°C for 30 s, 62°C for 30 s, and 72°C for 90 s. PCR products were evaluated by ethidium bromide staining after gel electrophoresis. The following human specific 5' and 3' primers were used: aggrecan32; 5'-AGGCCCAAGCTACCAAGTGG-3' and 5'-TTCTGGAAGCTCTTCTCAGT-3', Col II31; 5'-AACTGGCAAGCAAGGAGACA-3' and 5'-AGTTTCAGGTCTCTGCAGGT-3', Col X31; 5'-AGCCAGGGTTGCCAGGAACA-3' and 5'-TTTTCCACTTCCAGGAGGC-3', Sox9 (GenBank accession No. X65665), 5'-AGAACCCCAAGATGCACAAC-3' and 5'-TTTGCTTCGTCAAACTGGAACAAGGAGAAGGAGGTG-3', Oc (GenBank accession number X004126); 5'-GACTGTGACGAGTTGGCTGA-3' and 5'-GGGAAGAGGAAAGAAAGGGTG-3', Cbfal (Cbfal /Runx2; GenBank accession number XM 004126); 5'-CTCTTCCAAAGAGTGCTTGAGCTT-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3', GAPDH31; 5'-GACTGTGACGAGTTGGCTGA-3', and 5'-CATGTGGGCCATGAGGTCCACCAC-3'.

5. Western blot analysis

Cells were lysed in 0.5% TritonX-100 buffer (50mM Tri-HCl pH 8.0, 0.5% TritonX-100, 300 mM NaCl, 5 mM EDTA) with 100 mM sodium o-vanadate and complete mini protease inhibitor (Roche Diagnostics, Mannheim, Germany). Lysate was clarified by centrifugation for 15 min at 14,000×g at 4°C, and the protein concentration was measured according to the Bradford assay. Twenty milligrams of protein were loaded and separated by SDS-PAGE, transferred to Hybond PVDF membranes (Amersham Biosciences Corp, Piscataway, NJ, USA), and after incubation for 90 min in 5% skim milk blocking buffer at room temperature, reacted with primary antibodies followed by an HRP-conjugated secondary antibody. Protein bands were visualized with ECL plus a Western blot detection system (Amersham Biosciences Corp).

6. In vivo analysis of USAC cells in athymic mice using a diffusion chamber

We have reported that recombinant human bone morphogenetic protein-2 (rhBMP-2) promotes chondro-osteogenic differentiation of USAC cells both in vivo and in vitro. Therefore, in vivo expression of noggin during chondro-osteogenesis was examined using a diffusion chamber. USAC cells were transplanted into the peritoneal cavities of athymic mice using diffusion chambers with rhBMP-2 (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan) as described previously. Briefly, 5×106 cells suspended in 200 ml of α-MEM containing 10% FBS were loaded into diffusion chambers. Diffusion chambers were then implanted into the peritoneal cavities of 6-week-old athymic mice (BALB/c, nu/nu, male) under anesthesia. In some chambers, 5 mg of rhBMP-2 was coated on the inner surface of the membrane filter. The chambers were removed from the mice at 3 weeks after implantation under anesthesia. Chambers were fixed with neutral buffered formalin at 4°C for 24 h and embedded in paraffin.

Results

1. Transfection efficiency of As-Noggin in USAC cells

In the first experiment, we determined whether As-Noggin was transfected into USAC cells. As shown in Figure 1, noggin mRNA expression and protein production was inhibited by As-Noggin transfection on day 5 in USAC cells. There was no difference in GAPDH mRNA expression and β-actin protein production during culture (Figures 6 and 7).

2. Noggin increased chondrocyte-related protein production and decreased osteoblast-related protein production in USAC cells

Next, we studied the effects of noggin and
As-Noggin on the extracellular matrix synthesis of USAC cells. Figures 2, 3 and 4 summarize the histochemistry of chondrogenic or osteogenic markers. Proteoglycan and Col II were produced in a USAC cell control culture. However, the production of proteoglycan and Col II was decreased by As-Noggin, while Cbfa1 and OC production was increased. In contrast, rhNoggin in-

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Figure 2 Toluidine blue staining in USAC cells transfected with anti-sense noggin. USAC cells on days 7 and 14 in 3 cm dishes culture were fixed with 10% formalin and stained with toluidine blue. Proteoglycan was strongly detected in USAC cells transfected with anti-sense noggin in a time-dependent manner.

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Figure 3 Expressions of type II collagen and BMP-2 proteins in USAC cells transfected with anti-sense noggin. USAC cells on days 7 and 14 in 3 cm dishes culture were fixed with 10% formalin and used for immunohistochemical examination. Col II was time-dependently down-regulated, whereas BMP-2 was time-dependently up-regulated by anti-sense noggin.

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Figure 4 Expression of osteocalcine (OC) and Cbfa1 proteins in USAC cells transfected with anti-sense noggin. USAC cells on days 7 and 14 in 3 cm dishes culture were fixed with 10% formalin and used for immunohistochemical examination. OC was time-dependently up-regulated, and Cbfa1 was also up-regulated by anti-sense noggin.

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Figure 5 Western blot analysis of chondrocyte- and osteoblast-related proteins products. USAC cells at a density of $5 \times 10^4$/ml were cultured and transfected with anti-sense noggin in sub-confluent culture. Products of chondrocyte- and osteoblast-related proteins were examined. Proteins were extracted from samples on days 3, 7, and 14 of culture. Western blot analysis was performed using type II collagen, BMP-2, Cbfa1 and OC antibodies.
creased proteoglycan and Col II production. The production of BMP-2, OC and Cbfal was completely blocked by rhNoggin on days 7 and 14. These results suggested that noggin regulated the chondrogenic or osteogenic differentiation of USAC cells.

Furthermore, we investigated changes in chondrogenic- and osteogenic-related protein production in USAC cells by As-Noggin with Western blot analysis (Figure 5). Col II was time-dependently decreased by As-Noggin. In contrast, OC and Cbfal were time-dependently increased by As-Noggin.

As shown in Figure 6, we next conducted RT-PCR analysis to confirm the expression of various mRNAs in USAC cells with As-Noggin or rhNoggin. The expressions of Sox9, aggrecan, and Col II mRNA were inhibited by As-Noggin, and Col X mRNA was expressed in the early stage when compared with the control culture. Interestingly, USAC cells treated with As-Noggin expressed a high level of Col I and OC mRNA on day 7 and their expression continued on day 14, whereas USAC cells untreated with As-Noggin did not express these mRNAs. Cbfal mRNA, an important transcription factor regulating the differentiation of osteoblasts and chondrocytes, was slightly increased by As-Noggin.

The effects of rhNoggin on USAC cells were contrary to the above. The expressions of Sox 9, aggrecan and Col II mRNA were enhanced by the addition of rhNoggin. Col X mRNA expression was increased from day 7. On the other hand, the expressions of osteoblast-related genes, Cbfal, Col I and OC mRNA were decreased by treatment with rhNoggin.

3. Noggin regulates the expression of BMP-2 and BMP-4

The above results suggested that USAC cells differentiated into chondrocytes in the presence of noggin, and that osteoblastic differentiation was induced by the suppression of noggin. We next examined the effect of noggin on the expression of BMP-2 and BMP-4 during USAC cell differentiation. Figure 7 shows the expression of BMP-2 and BMP-4 by treatment with As-Noggin or rhNoggin in USAC cells. The expression of BMP-2 was increased by As-Noggin, but was decreased by rhNoggin. The expression of BMP-4
was decreased by As-Noggin, but increased by rhNoggin.

4. **In vivo localization of noggin in USAC cells**

To determine the *in vivo* localization of noggin in tissues, diffusion chambers were transplanted into athymic mice. Morphologically, the tissue developing in the diffusion chambers showed marked metachromasia, as indicated by toluidine blue staining (Figure 8A). When compared with diffusion chambers not coated with rhBMP-2, cartilaginous tissue formation was more prominent in diffusion chambers coated with rhBMP-2. Noggin was localized on the opposite side of the diffusion chamber membrane (Figure 8B, arrows). Namely, noggin was localized in flat cells or immature cells of inside diffusion chambers.

**Discussion**

This study revealed that noggin induced chondrogenesis and the inhibition of noggin induced the osteogenesis of USAC cells *in vitro*. The expressions of osteoblast-related genes, Col I and OC, were increased by As-Noggin, whereas the expression of chondrocyte-related gene, Sox 9, aggrecan and Col II, were decreased by As-Noggin. The expressions of chondrocyte-related genes, aggrecan and Col II, were increased by rhNoggin, whereas the expressions of osteoblast-related genes, Cbfa 1, Col I and OC, were decreased by rhNoggin. Furthermore, As-Noggin increased BMP-2 and rhNoggin increased BMP-4. These data suggest that the deletion of noggin induces osteogenic differentiation in USAC cells. On the other hand, the exogenous administration of rhNoggin induces chondrogenic differentiation.

The exact role of noggin in chondro-osteogenesis is yet undetermined. There have been some reports describing that noggin was a negative factor for bone formation through the impairment of osteoblast differentiation, using noggin+/- transgenic mice and noggin mutein in rats, whereas Nifuji et al. reported that noggin overexpression did not affect osteogenic differentiation in mesodermal stem cells, because osteoblast-related gene expression levels were not changed. Tsumaki et al. reported that noggin-transgenic mice lacked most of their cartilaginous components. Nifuji et al. reported that the overexpression of noggin reduced chondrocyte-related gene expression levels in mesodermal stem cells, which differentiated into chondrocyte lineage by culturing in differentiation medium. Human embryonic stem cell differentiation was regulated by BMP-2 and its antagonist noggin. Canalis et al. reported that noggin chiefly bound to BMP-2 and BMP-4. However, noggin was not affects completely chondrogenic maturation process via BMP-4 signaling on USAC cells. There were other stimulate factors might be induce chondrogenic maturation of USAC cells, as GDF-5 or TGF-b. RhBMP-2 stimulated osteoblastic maturation and inhibited the myogenic differentiation of C2C12. Grimrud et al. also indicated that BMP signaling stimulated chondrocyte maturation. These data suggest that the interaction of
noggin with BMP-2 and BMP-4 may be necessary in chondro-osteogenesis.

Specific BMP signals were activated in the skeleton by the targeted expression of BMP-4 in cartilage, and the overactivity of BMP-4 in the skeleton caused an increase in cartilage production and chondrocyte differentiation. Furthermore, noggin and BMP-4 coordinately regulate the expression of chondrogenic differentiation in mouse clonal ES cells, ATDC-5. Our results revealed that rhNoggin up-regulated BMP-4 production when USAC cells expressed aggrecan, Col II collagen and Col X collagen. In addition, Sox 9 mRNA expression was up-regulated by rhNoggin and was down-regulated by As-Noggin in USAC cells. Furthermore, the expression of osteoblast-related genes, OC and Col I mRNAs, was down-regulated when BMP-4 production was down-regulated by rhNoggin and As-Noggin decreased the production of BMP-2. Together, an excess amount of noggin might dramatically reduce the activity of BMP-2 and might enhance BMP-4 production during chondro-osteogenesis.

The expression of noggin during chondrogenesis was found in the tissue in diffusion chambers. Noggin was highly expressed in immature cells of the tissue on the opposite side of the membrane coated with rhBMP-2; however, this expression disappeared with the maturation of chondrocytes. Namely, noggin was not expressed in mature cells (prehypertrophic to hypertrophic areas) of the tissue near the membrane. This finding is consistent with the report that noggin was not expressed in hypertrophic chondrocytes and was expressed in immature cells in the spine of mice. These data suggest that the in vivo chondro-osteogenic differentiation of USAC cells was also regulated by noggin. In conclusion, we demonstrated the effect of noggin on chondrogenesis and osteogenesis in a human cell line USAC, which has multi-potency that differentiates into chondrocytes, osteoblasts and adipocytes, and the role of noggin in these processes. Noggin might induce chondrogenic differentiation and its repression induces osteogenic differentiation through the modulation of BMP-2 and BMP-4. Thus, noggin may work in the initiation of differentiation of mesenchymal cells.

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