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Original

Neural-inducing Factor Netrin-1 is Regulated in Chondrogenesis and Osteogenesis by BMP or Noggin

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Abstract : This is the first report describing neurogenic factor of Netrin-1 related to chondrogenesis or osteogenesis in a human cells. Netrin is a morphogenetic factor that induces a growth cone of an axial filament of the nervous system. However, the roles of Netrin in chondrogenesis or osteogenesis are not yet understood. We analyzed the relationship between Netrin and bone morphogenetic protein-2 (BMP-2) in chondrogenesis or osteogenesis, using a human chondrocyte-like cell line (USAC), which also retains multi-potency to differentiate into osteoblasts and adipocytes. Netrin-1 mRNA was decreased in USAC cells, though the expression was increased during osteogenic differentiation at the stage when osteocalcin mRNA were increased by BMP-2. Furthermore, inhibition of Netrin-1 gene increased Cbfa1 mRNA expression, and decreased Sox9 mRNA expression. We also found that Netrin-1was strongly expressed in immature chondrocytes of cartilage-like tissues that were formed in an exo vivo experiment with diffusion chambers. These findings indicate that Netrin-1 and BMP-2 regulates in the stage dependent process of mesenchymal cell differentiation to chondrocytes or osteoblasts.

Key words: Netrin-1, BMP-2, Noggin, Chondrogensis, Osteogenesis

Introduction

Numerous tissue regeneration methods for postoperative patients with malignant tumor and trauma has been established in the skin, blood vessels, bone and cartilage. However, the method is still undeveloped with regard to regenerating the nervous system during bone reconstruction. The differentiation of nerve cells and the formation of neurons develop in parallel with bone or cartilage is indispensable for total tissue regeneration. Netrin was discovered as another morphogenetic factor to induce the growth cone of an axial filament apart from bone morphogenetic proteins (BMPs) and *Hedgehogs* (*Hhs*)[1, 2], which Netrin is included in a family of laminin-related secreted proteins. The function of this gene has not yet been defined; however, Netrin is thought to be involved in axon guidance and cell migration during development[3, 4]. Mutation and the loss of expression of *Netrin-1* were involved in cancer development as a survival factor via its receptors, UNC5H and DCC[5]. Furthermore, it was reported that the overexpression of *Netrin-1* enhanced tumor development by inhibiting apoptosis.

Recently, BMP-2 has been employed for the reconstruction or augmentation of bone defects or deformities caused by disease or operation. BMPs have particular relevance to skeletal development, since they are expressed in the areas of cartilage and bone formation[6-8]. Cell differentiation and specialization for chondrocyte or osteoblasts after mesenchymal cell condensation are delicately regulated by various cytokines and transcription factors, such as BMPs, Hhs and core-binding factor 1 (Cbfa1)[9,10].There were reported BMP-2 or BMP-4 inhibits cyto-differentiation and -growth of neurons of nerve cells[11, 12]. BMPs appear to act as chemorepellents that guide the early trajectory of the C-axons of neurons in the developing spinal cord, and serve an essential function in the repulsion of C-axons[13]. Bjurholm et al.[14-16] demonstrated that some neuropeptides, such as neuroendocrine and neuropeptide Y, were involved in osteoblasts and exonogenic bone formation in rats. These findings suggested the extension of axons of peripheral sensory and sympathetic

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neurons to osteoblastic and osteoclastic cells and the possible neural regulation of bone metabolism in osteogenic cells. However, there have been no reports about the presence and function of Netrin in osteogenesis or chondrogenesis.

We hypothesize that neurogenesis regulated by BMPs caused chondro-osteogenic cells differentiation, and the related regeneration and restoration of the nervous system would be slow. In the present study, we investigated whether commitment would occur for *Netrin-1* expression on chondro-osteogenic differentiation of USAC cells, which retain the potential to differentiate into chondrocytes, osteoblasts and adipocytes by BMP-2[10].

Materials and Methods

Transplantation of human osteo-chodrogenic tumor

Subculture of transplanted tumor of human osteo-chondrogenic sarcoma with nude mouse was performed by methods shown previously[17]. The tumour formed only the cartilage tissue which was a characteristic during subculture process. Tumor was transplanted into the peritoneal cavities of athymic mice (6-week-old BALB/c nu/nu male) under ether anesthesia, and were then removed from the mice 3 month after implantation. The tumor tissue were fixed with 4% paraformaldehyde and embedded in paraffin. The samples were sectioned for immunological staining and 0.1% toluidine blue staining. All surgical procedures and care of animals were carried out in accordance with the guidelines of the National Institutes of Health for care and use of animals. The study protocol was approved by the Animal Experimentation Committee of Matsumoto dental University and the Researches Ethical Review Board of Matsumoto Dental University.

Cell culture

A cell line, USAC, established from transplanted tumor of a human osteo-chondrogenic sarcoma[17], was used in the present study. Cells were maintained 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), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Meiji Seika, Tokyo, Japan) at 37 ℃ in a humidified atmosphere of 5%CO_2. Cells (1 x 10^6/dish) were plated into 10 cm dishes (Corning, Acton, OH, USA) in 5% FBS/α-MEM and cultured for 0-21 days in the presence or absence of 0-500 ng/ml of recombinant human bone morphogenetic protein-2 (BMP-2), which was kindly donated from Yamanouchi Pharmaceutical Co. Ltd., Tsukuba, Japan. After the period of each culture day, the cells were harvested, and protein and mRNA were extracted by the method previously described[10].

Immunological detection and alkaline phosphatase activity and extracellular matrix synthesis

Netrin-1 expression of USAC cells treated with BMP-2 was studied using high-density micro-mass culture. USAC cells were seeded onto 96-well plates at a density of 2.5x10^5/well, and 0-100 ng/ml of BMP-2 was added to the wells after 24 hours. After the experiment, the cells were rinsed with PBS and fixed with 4% paraformaldehyde for 15 minutes at 4 ℃. Indirect immunostaining was preformed using a streptavidin-peroxidase system (Seikagaku Kogyo, Tokyo, Japan) with specific anti-human antibody were used for immunological detection. The antibody signal was detected using a peroxidase detection kit (Nichirei, Tokyo, Japan). To identify the activity of alkaline phosphatase (APase), fixed samples were rinsed twice in saline and incubated in 50 mM Tris-HCl (pH 9.5) containing 0.5 mg/ml of naphthol AS-BI phosphate (Sigma, St. Louis, USA) and 1 mg/ml of fast red trisodium salts (Sigma, St. Louis, USA) for 2 min at 37°C. Toluidine blue and von Kossa's staining were performed as method of previously[10].

Western blot analysis

The production of Netrin-1, type II collagen and osteocalcin were confirmed by Western blot analysis. Medium was collected from each culture at each experimental period. Protein was collected by protein A-Sepharose CL-4B with anti-human anti-body for overnight at 4℃ by the modified method, as previously described[15]. The samples were solubilized in Laemmli sample buffer containing 2.5 mM EDTA, 5 mM benzamidine HCl, 10 mM N-ethylmaleimide and 2 mM phenylmethylsulfonyl fluoride. Twenty micro grams of protein was loaded, separated by 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and blotted onto nitrocellulose membranes (Bio-Rad Laboratories). The signal was detected using a FITC labeled alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:5000) (Nichirei, Tokyo, Japan).

Genes expression analysis by quantitative real-time polymerase chain reaction (qPCR)

USAC cells at a density of 1 x 10^5 cells/ml were cultured in α-MEM containing 2.5% FBS, and were treated with BMP-2 (100 ng/ml) for 0-14 days. Total RNA was prepared from USAC cells by ISOGEN® (Nippon gene, Toyama, Japan) according to the manufacturer's protocol. The first strand cDNA was synthesized from 1 μg of total RNA with 1 mM of random hexamer primer using AMV Reverse Transcriptase XL (Takara, Tokyo, Japan) at 45℃ for 30 min. Subsequent amplification was performed with an RNA PCR Kit Ver 2.1 (Takara, Tokyo, Japan) for 30 cycles under the following conditions: 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 90 sec. Real-time PCR was performed in triplicate using human specific primers-probes (Table 1), and analyzed by the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems Co.). The mean fold changes in gene expression

Table 1.Human specific primers for "RT-PCR"

Genes name	Primer sequence	GenBank No.
Netrin-1	5'-ACTATGCCGTCCAGATCCAC-3' 5'-TTCTTCATGGACGACGACCC-3'	NM_004822
Type II collagen	5'-AACTGGCAAGCAAGGAGACA-3' 5'-AGTTTCAGGTCTCTGCAGGT-3'	X16711.1
Osteocalcin	5'-GACTGTGACGAGTTGGCTGA-3' 5'-GGGAAGAGGAAAGAAGGGTG-3'	X04143
Sex-determining region on the Y chromosome-related high-mobility group box 9 (Sox9)	5'-AGAACCCCAAGATGCACAAC-3' 5'-TTTGCTTCGTCAATGAATGG-3'	X65665
Core binding factor alpha 1/runt-related transcription factor 2 (Cbfa1/Runx2)	5'-CTCTTCCCAAAGCCAGAGTG-3' 5'-CAGCGTCAACACCATCATTC-3'	XM_004126
Glyseraldehyde-3-phosphate dehydrogenase (GAPDH)	5'-TGAAGGTCGGAGTCAACGGATTT-3' 5'-CATGTGGGCCATGAGGTCCACCAC-3'	NM_000181

relative to GAPDH were calculated using the values obtained from untreated USAC cells as a calibrator by means of $^{2-\triangle\triangle CT}$ method[18].

Gene suppression analysis for Netrin-1 and Noggin

The suppression of *Netrin-1* or *Noggin* gene in USAC cells was achieved by the transfection of 100 pmol of anti-sense cDNA fragments for human *Netrin-1* (Genbank: NM004822) 5'-GGACGCAGCATGATGCGCGC-3' (*As-Netrin-1*) or human *Noggin* (Genbank: HSU31202) 5'-CGATAACAGAGTCTCAGGG-3' (*As-Noggin*). Transfection and determination of incorporation of anti-sense genes were proceeded by the manufacturer method of Lipofectin ® (Invitrogen, Carlsbad, CA, USA). cDNA/Lipofectin complexes was added to USAC cells in Opti-MEM-1 medium without serum. After being incubated for 24 hours at 37℃, the medium was replaced to 2.5%FBS/α-MEM medium and used for following experiments. mRNA expressions were determined by qPCR analysis described above.

Histological analysis of exo vivo

To exclude the invasion of the host cells to transplant cells *in vivo*, we employed diffusion chambers to study effects of rhBMP-2 on chondrogenesis and/or osteogenesis as described previously[16]. Five million USAC cells were loaded into diffusion chamber with or without 5 mg of rhBMP-2. The chambers were implanted into the peritoneal cavities of athymic mice (6-week-old BALB/c nu/nu male) under ether anesthesia, and were then removed from the mice 4 weeks after implantation. The chambers were fixed with 4% paraformaldehyde and embedded in paraffin. Fore micrometer of serial sections were performed for immunological staining by anti-human Netrin-1 antibody and 0.1% Toluidine blue staining.

Quantitative histological analysis was carried out on six areas (1.0 mm^2/area) of three specimens from three diffusion chambers per data point. The light microscopic images were measured using MacSCOPE Ver. 2.5 (Mitani Corporation, Tokyo, Japan). Each tissue was examined at 200 of magnification to ensure that it was identified properly.

Anti-bodies for histochemistry and Western blot

Anti-human antibody of Netrin-1 antibody (Santa Cruz Biotechnology Coporation, Santa Cruz, CA, USA), Noggin (Chemicon International Incorporation, Temecula, CA, USA) and osteocalcin antibody (Santa Cruz Biotechnology Coporation, Santa Cruz, CA, USA) were purchased, and used for immunohistochemistry and Western blot analysis.

Statistical analysis

Values were represented as means ± standard deviation (SD). Analysis of variance and Student's *t*-test were used to determine the significance of difference ($p<0.01$).

Results

Distribution of Netrin-1 in transplanted tumor and tissue in diffusion chamber

Perspective view of transplanted tumor (Fig. 1A and B) and tissue in diffusion chamber (Fig. 1G) which shows toluidine blue stained cartilage and von Kossa's stained calcified cartilage (*white arrow*). Netrin-1 was observed in a strongly positive in the small cell region (Fig. 1C and H, *arrow*) and decreased in the hypertrophic cell region (Fig. 1D and lower part of H). However, BMP-2 weakly expressed in small cell region, and strongly positive in hypertrophic cell layer (Fig. 1E and F). APase positive osteoblastic cells were located in near part of diffusion chamber (Fig. 1, I, *red stained region of O*).

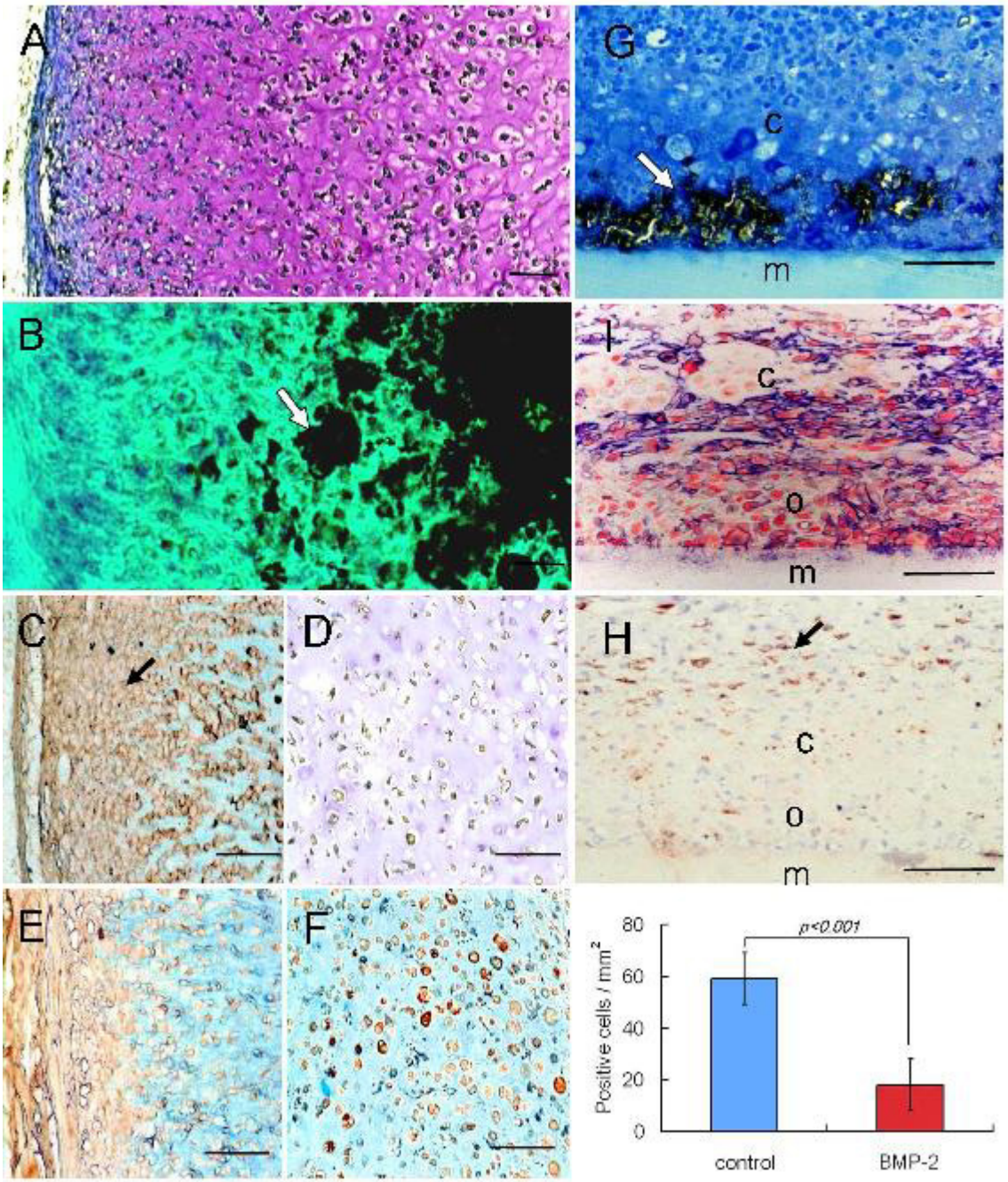

Figure 1. Histological findings of transplanted tumor (A-F), tissue of diffusion chamber (G-I) and quantification of Netrin-1 positive cells (J). Sections stained for Toluidine blue (A and G), von Kossa's (B and G, *white arrow*), Netrin-1 antibody (C, D and H) and BMP-2 (E and F) and APase (I). Sections were counter stained with alcian blue (C-F) to specifically determined for cartilage. c: chondrocytic area, o: osteoblastic area, m: membrane filter, Bar: 100 μm. Histogram shows comparison of the number of Netrin-1 positive cells in the sections per unit area (mm^2) between control and BMP-2 treated diffusion chambers at 4 weeks after transplantation (J). Netrin-1 positive signal were analyzed by light microscopy (Olympus, Tokyo, Japan) with an FX-500 digital-camera (Fujifilm, Tokyo, Japan). *P<0.001 compared with a. Values are means ± SD.

Localization of Netrin-1 in the tissue formed in diffusion chamber

To determine the appearance of Netrin-1 during chondrogenesis or osteogenesis in vivo, USAC cells were transplanted into athymic mice by diffusion chamber. Morphologically, cartilage was developed in the diffusion chambers coated with BMP-2 showed marked metachromasia, as indicated by toluidine blue staining at 4 weeks after transplantation (Fig. 1G). The numbers of Netrin-1 positive cells in the tissue formed in diffusion chamber were higher in BMP-2 treatment than control (Fig. 1J, p<0.001).

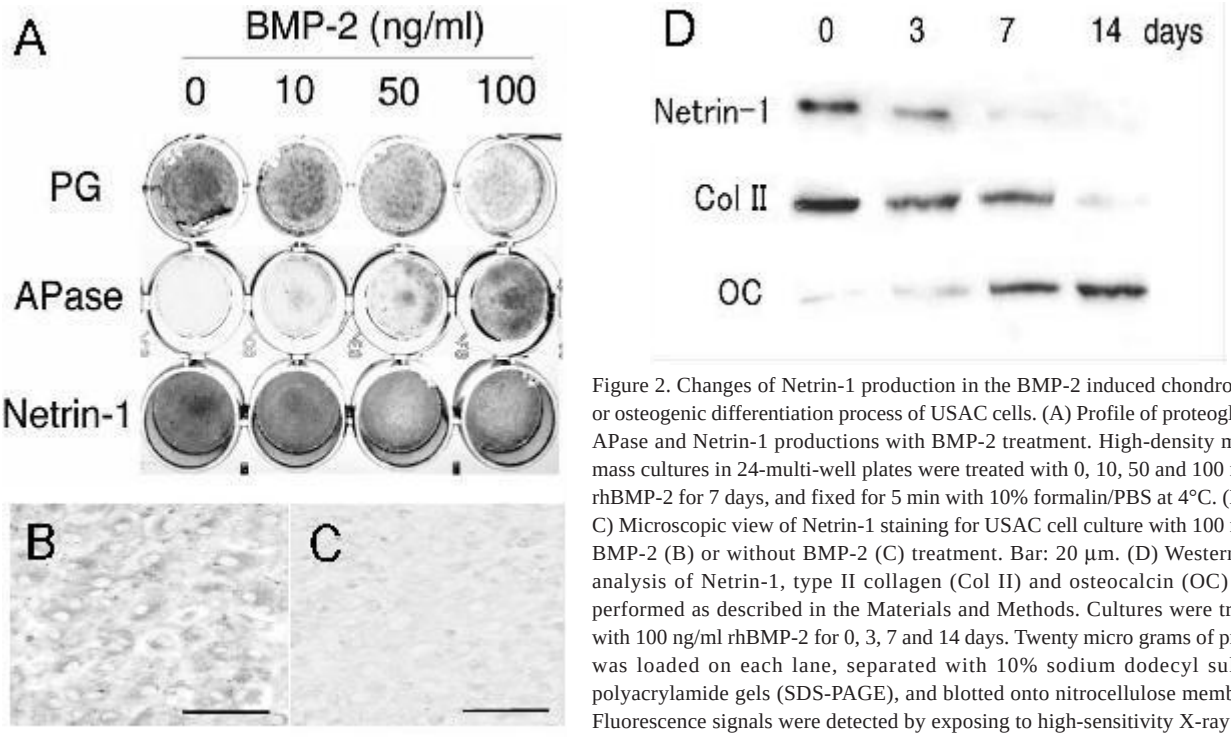

Figure 2. Changes of Netrin-1 production in the BMP-2 induced chondrogenic or osteogenic differentiation process of USAC cells. (A) Profile of proteoglycan, APase and Netrin-1 productions with BMP-2 treatment. High-density micro-mass cultures in 24-multi-well plates were treated with 0, 10, 50 and 100 ng/ml rhBMP-2 for 7 days, and fixed for 5 min with 10% formalin/PBS at 4°C. (B and C) Microscopic view of Netrin-1 staining for USAC cell culture with 100 ng/ml BMP-2 (B) or without BMP-2 (C) treatment. Bar: 20 μm. (D) Western blot analysis of Netrin-1, type II collagen (Col II) and osteocalcin (OC) were performed as described in the Materials and Methods. Cultures were treated with 100 ng/ml rhBMP-2 for 0, 3, 7 and 14 days. Twenty micro grams of protein was loaded on each lane, separated with 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and blotted onto nitrocellulose membrane. Fluorescence signals were detected by exposing to high-sensitivity X-ray film.

Effects of Netrin-1 production of USAC cells during chondrocytic or osteoblastic differentiation

Changes of expressions of Netrin-1 and proteoglycan, and alkaline phosphatase activity in the differentiation process of USAC cells were determined by high-density micro-mass culture with 0-100 ng/ml of BMP-2. The results revealed that alkaline phosphatase staining was increased; while Netrin-1 and alcian blue staining staining were decreased dose-dependently (Fig. 2A). Netrin-1 was localized at cytoplasm and matrix in USAC cells in control culture (Fig. 2B), but only a few signals were expressed in the small round cells in BMP-2 treated culture (Fig. 2C).

Changes in Netrin-1 production by BMP-2

The effects of BMP-2 on the production of Netrin-1, type II collagen and osteocalcin in USAC cell culture using Western blot analysis. USAC cells produce Netrin-1 at day 0 culture without

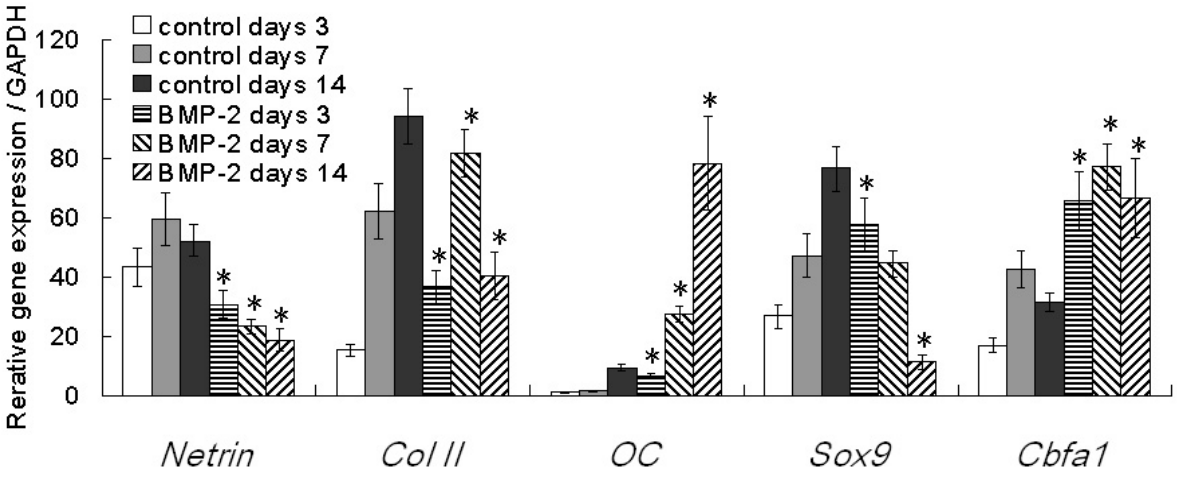

Figure 3. Changes of mRNA expressions in USAC cells by BMP-2.
Variability of the mRNA expressions of Netrin-1, Sox9, type II collagen, Cbfa1 and osteocalcin were detected in USAC cells with or without 100ng/ml rhBMP-2. Mean values ± SD derived from independent experiments (n=9) were shown. Significant differences: $p<0.001$.

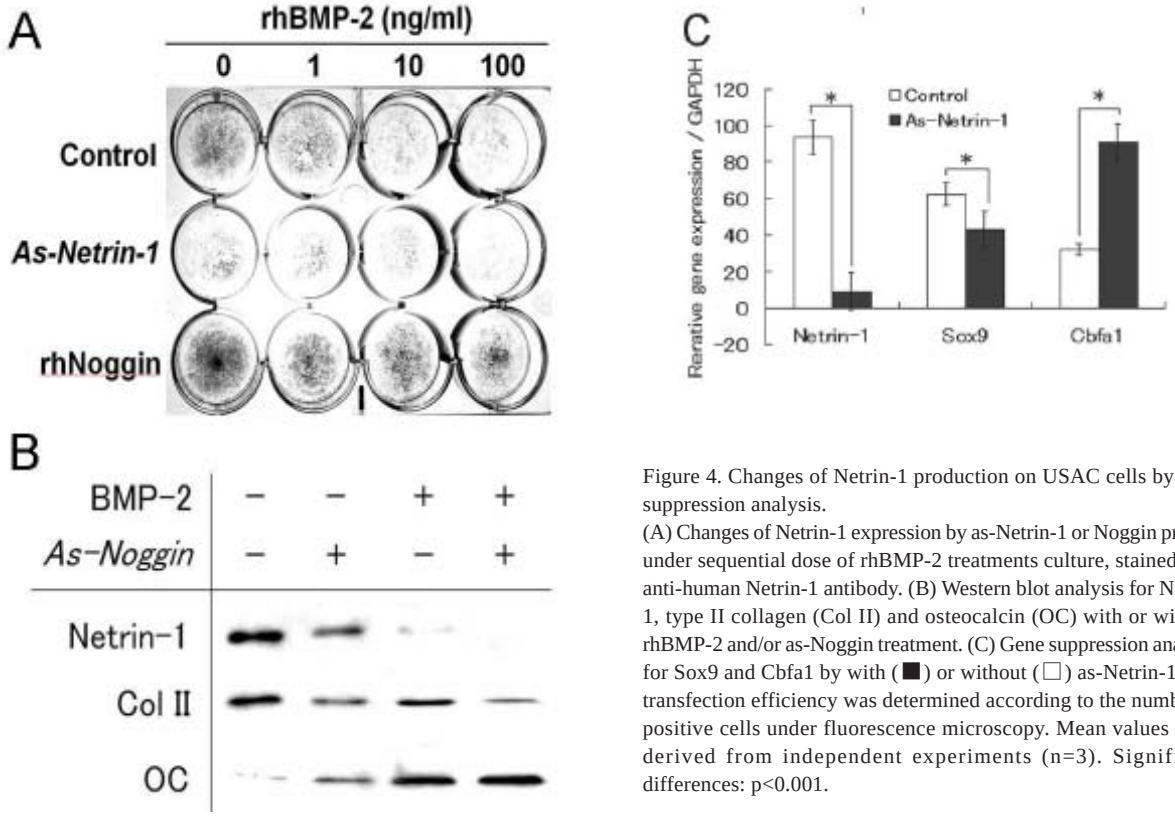

Figure 4. Changes of Netrin-1 production on USAC cells by gene suppression analysis.
(A) Changes of Netrin-1 expression by as-Netrin-1 or Noggin protein under sequential dose of rhBMP-2 treatments culture, stained with anti-human Netrin-1 antibody. (B) Western blot analysis for Netrin-1, type II collagen (Col II) and osteocalcin (OC) with or without rhBMP-2 and/or as-Noggin treatment. (C) Gene suppression analysis for Sox9 and Cbfa1 by with (■) or without (□) as-Netrin-1. The transfection efficiency was determined according to the number of positive cells under fluorescence microscopy. Mean values ± SD derived from independent experiments (n=3). Significant differences: $p<0.001$.

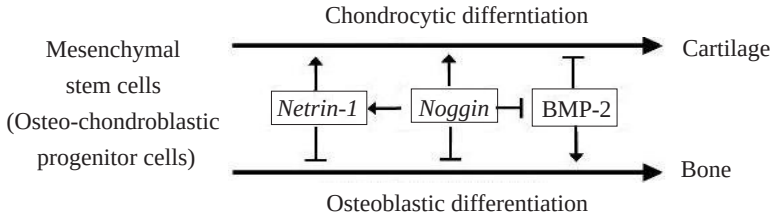

Figure 5. Schematic explanation of osteogenic and chondrogenic regulation through Netrin-1. Netrin-1 is regurated during maturation process for chondrocyte or osteoblast by BMP-2 and Noggin.

BMP-2. The production was decreased time-dependently by BMP-2 treatment from day 3 to day 14 (Fig. 2D, Netrin-1). Type II collagen was strongly detected from day 0 to day 7 (Fig. 2D, Col II) and osteocalcin was increased from day 7 to day 14 (Fig. 2D, OC) by BMP-2, indicating that chondrogenic or osteogenic differentiation was promoted by BMP-2.

mRNA expression of Netrin-1, and cartilage- and bone-related genes in USAC cells

Since the above described results suggested that USAC cells produced *Netrin-1* and decreased with the differentiation into chondrocytes and osteoblasts in response to BMP-2, we analyzed the changes in the expressions of *Netrin-1*, and cartilage- and bone-related genes due to BMP-2. Namely, the expressions of *Netrin-1, Sox9, type II collagen, Cbfa1* and *osteocalcin* mRNAs were determined by RT-PCR (Fig. 3). *Netrin-1* mRNA gradually increased with time in USAC cells culture, while the expression was time-dependently decreased by about half to one-third by BMP-2 treatment. The expression of *Sox9* mRNA, a master gene for chondrogenesis, increased time-dependently in the absence of BMP-2. In the presence of BMP-2, *Sox9* mRNA had already increased on day 3 and time-dependently decreased thereafter. Though *type II collagen* mRNA expression elevated more than two levels without BMP-2, it was increased on day 7 then was decreased on day 14 by BMP-2. *Cbfa1* mRNA expression was increased 2 to 3 fold by BMP-2 treatment during the culture period. *Osteocalcin* mRNA expressions were increased in time-dependent manner in the control culture, and increased from day 3 by BMP-2.

Effects of gene suppression of Netrin-1 and Noggin for USAC cell

An inhibition experiment was performed using *as-Netrin-1* or *as-Noggin* to study the role of Netrin-1 in the differentiation process to chondrocytes or osteoblasts in USAC cells. The production of Netrin-1 was inhibited in the presence of BMP-2 by *as-Netrin-1*, but the effect was reversed by Noggin (Fig. 4A). On the other hand, Netrin-1 and type II collagen were decreased by Noggin treatment, osteocalcin was slightly increased, and bmp accelerated the effort (Fig. 4B). ,

To verify the change of the transdifferentiation gene to cartilage and bone; the suppression of *Netrin-1* decreased *Sox9* mRNA but increased *Cbfa1*mRN*A* expressions (Fig. 4C).

Discussion

The present study is the first report describing that Netrin-1 involve with in chondrogenesis and osteogenesis on a human mesenchymal cell line. USAC cells have the potential to be differentiated into osteoblasts or adipocytes other than chondrocytes by BMP-2[10]. We demonstrated that the expression and production of Netrin-1 were decreased in the process whereby mesenchymal cells differentiate into osteoblasts by BMP-2. This has been proven by the presented results; that is, 1) though increasing of osteogenic markers, Nerin-1 production was decrease by BMP-2, 2) increasing of *Cbfa1* mRNA expression but decreasing of *Sox9* mRNA expression were observed by *Netrin-1* gene suppression and 3) the localization of *Netrin-1* in early cartilage tissue formation by BMP-2 in *exo vivo* and *in vivo*.

BMPs have many actions in the nervous system, including patterning, cell fate determination, apoptosis, and cell proliferation, which are modulated by extracellular binding proteins, such as Noggin, Chordin, Cerberus and Follistatin[19]. Chalazonitis et al.[11] reported that the number of neurons was increased by a low concentration and decreased by a high concentration of BMP-2 in primordial bowel embryonic cells. In addition, Lim et al.[20] presented that Noggin produced neurogenic environment by blocking endogenous BMP signaling. BMP-2 enhanced not only chondrogenesis but also osteognesis of USAC cells[9,10]. Progression of cartilage and osteogenic differentiation and decrease of the production of Netrin-1 of USAC cells were confirmed in diffusion chamber (Fig. 1G-J). *Netrin-1* mRNA expression was detected suddenly before when *type II collagen* mRNA increased after *Sox9* mRNA was increased of USAC cells. The reduction of *Netrin-1* was observed with osteogenic differentiation at the time when *Cbfa1* mRNA and *type II collagen* mRNA were increased by BMP-2. Thus, it was indicated that *Netrin-1* was down-regulated with chondrogenic differentiation by the treatment with BMP-2. The expression of *Netrin-1* in USAC cells was inhibited by 10-100ng /ml of rhBMP-2 (Fig. 3). The expression of *Cbfa1* mRNA and *osteocalcin* mRNA was enhanced by BMP-2 in USAC cell culture, and these productions were accelerated APase activity for differentiation to osteoblasts (Fig. 2A, 4B).

There are few reports about the relations with chondrocyte or osteoblasts and the neurocyte. Setoguchi et al.[21] demonstrated that gene modification to inhibit BMP signaling by *Noggin* expression promoted differentiation of neural precursor cells into neurons and oligodendrocytes. They also reported that functional recovery of the recipient mice with spinal cord injury was observed when *Noggin*-expressing neural precursor cells were transplanted. BMPs, also act as growth stimulation factors for neuron[22, 23]. Sonic hedgehog from midline structures collaborates with Netrin-1 to guide commissural axons. Failure to activate the *Hhs* pathway can also explain absence of floor plate *Netrin-1* expression in midbrain and hindbrain. *Sonic hedgehog*, acting via *Smoothened*, is a midline-derived chemoattractant for commissural axons and can be act as an axonal chemoattractant. USAC cells increased *Indian hedgehog* (*Ihh*) mRNA expression immediately after the treatment with BMP-2. It was indicated that differentiation passed through a signal of *Ihh* by BMP-2 for chondrogenesis or osteogenesis of USAC cells. Moreover, *Ihh* may act as a substitution for neurogenesis, even if Netrin-1 is repressed by BMP-2.

Togari et al.[24] found that neuropeptides were present in osteoblasts and osteoclasts[14, 15, 16] demonstrated that the expressions of neuropeptides were accelerated in bone metabolism, suggesting that the functions of neuropeptides involve not only the growth of neurons, but also the differentiation of osteoblasts. They also suggested the extension of axons of peripheral sensory and sympathetic neurons to osteoblastic and osteoclastic cells and the possible neural regulation of bone metabolism in these osteogenic cells. As for the results of USAC cells, *Netrin-1* was strongly expressed at the early stage of culture, but the expression became weak at the later stage of culture (Fig. 2 and 3). Examination using diffusion chambers indicated that Netrin-1 was produced by the small cell layer and prehypertrophic cell layer of the tissue in BMP-2 treated diffusion chambers, just before the appearance of metachromasia (Fig. 1A-J). These results indicate that Netrin-1 is expressed in immature cells and then its expression is decreased in the maturation process for chondrocytes or osteoblasts of USAC cells by BMP-2 or noggin (Fig. 5).

Netrin family is highly conserved, and the members have also been identified in several invertebrate species, *Netrin-1, -2, -3, -4* and UNC-6[1, 2]. *Netrin-1* has been cloned from several vertebrates, including humans[25, 26]. In addition, *Netrin-3* and *Netrin-4* have been identified[27, 28], but their functions have not been understood clearly. The C domain of Netrin has been shown to bind heparin with high affinity[29]. They showed that heparan sulfate-bound Netrin remains functional and can bind BMPs at the plasma membrane. Our results imply that interactions between

Netrin-1 and matrix components were regulated by BMP-2 activity.

In this study suggested that Netrin-1 may act as chondrogenic and osteogenic regulator on USAC cells. Addtionaly, decrease of Netrin-1 function will be delay the velocity of neurodegenerative growth in cartilage and bone.

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