Journal of Pharmacological Sciences 127 (2015) 467–473

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full paper

Decreased DNA methylation in the promoter region of the *WNT5A* and *GDNF* genes may promote the osteogenicity of mesenchymal stem cells from patients with ossified spinal ligaments

Noriyuki Chiba [a, b], Ken-Ichi Furukawa [a, *], Shohei Takayama [a], Toru Asari [b], Shunfu Chin [b], Yoshifumi Harada [b], Gentaro Kumagai [b], Kanichiro Wada [b], Toshihiro Tanaka [b], Atsushi Ono [b], Shigeru Motomura [a], Manabu Murakami [a], Yasuyuki Ishibashi [b]

[a] Department of Pharmacology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan
[b] Department of Orthopaedic Surgery, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan

ARTICLE INFO

Article history:
Received 2 December 2014
Received in revised form
23 March 2015
Accepted 25 March 2015
Available online 1 April 2015

Keywords:
Epigenetics
Mesenchymal stem cells
Microarray analysis
WNT5A
GDNF
DNA methylation

ABSTRACT

Mesenchymal stem cells (MSCs) isolated from spinal ligaments with ectopic ossification have a propensity toward the osteogenic lineage. To explore epigenetic control of the osteogenic features of MSCs, we treated MSCs obtained from the spinal ligaments of ossification of yellow ligament (OYL) patients and non-OYL patients with the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5AdC). We compared the non-OYL groups (untreated and treated with 5AdC) with the OYL groups (untreated and treated with 5AdC) by genome-wide microarray analysis. Next, we used methylated DNA immunoprecipitation combined with quantitative real-time PCR to assess gene methylation. Ninety-eight genes showed expression significantly increased by 5AdC treatment in MSCs from non-OYL patients but not from OYL patients. In contrast, only two genes, *GDNF* and *WNT5A*, showed significantly higher expression in OYL MSCs compared with non-OYL MSCs without 5AdC treatment. Both genes were hypermethylated in non-OYL MSCs but not in OYL MSCs. Small interfering RNA targeted to each gene decreased expression of the target gene and also several osteogenic genes. Both small interfering RNAs also suppressed the activity of alkaline phosphatase, a typical marker of osteogenesis. These results suggest that the osteogenic features of MSCs from OYL patients are promoted by unmethylated *WNT5A* and *GDNF* genes.

© 2015 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Ossifications of the posterior longitudinal ligament (OPLL) and/or yellow ligaments (OYL) are characterized by progressive ectopic bone formation in the spinal ligaments. In these patients, ectopic ossification sometimes occurs in other spinal ligaments. Consequently, OPLL and OYL patients have been regarded as a manifestation of diffuse idiopathic skeletal hyperostosis (DISH) (1,2). Based on genetic factors, these current findings may indicate that OPLL and OYL patients would have a tendency toward systemic ossification of numerous ligaments, similar to that observed in patients with DISH. Although the etiology of ectopic ossification of spinal ligaments has been analyzed extensively and linked to various epidemiological, genetic, metabolic, and mechanical factors (3), the pathogenesis of the disease is still unknown. The ossification of spinal ligaments can lead to narrowing of the spinal canal, which can eventually cause serious damage to the spinal cord, with patients suffering from various symptoms (4). Drug therapy is a symptomatic treatment and cannot prevent progress of ectopic bone formation. In severe cases, surgery is necessary to decompress the spinal cord. However, surgery puts the patient at risk of complications, such as injury to the spinal cord or nerve root and cerebrospinal fluid leakage (5). Furthermore, surgical treatment is not always effective for patients with myelopathy, because long-term compression results in irreversible degeneration of the spinal cord (6). Therefore, it is necessary to understand the mechanisms underlying the promotion of ossification and to find effective therapies that prevent ectopic bone formation (3).

Mesenchymal stem cells (MSCs) can be isolated from various human tissues and differentiated into osteoblastic, chondrogenic, myogenic, and adipogenic lineages (7). MSCs have been regarded as

* Corresponding author. Tel.: +81 172 39 5022; fax: +81 172 39 5023.
E-mail address: furukawa@cc.hirosaki-u.ac.jp (K.-I. Furukawa).
Peer review under responsibility of Japanese Pharmacological Society.

http://dx.doi.org/10.1016/j.jphs.2015.03.008
1347-8613/© 2015 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

a key player in several diseases and thus have been widely investigated for their potential role in treating human disease (8–10). We previously identified the presence of MSCs in human spinal ligaments and, using immunohistochemistry, demonstrated enrichment of MSCs in ossified areas of human spinal ligaments (11,12). Furthermore, the osteogenic differentiation potential of MSCs from patients with ossified spinal ligaments was increased; this propensity toward the osteogenic lineage might be a causal factor in the ossification of these ligaments (13). However, the mechanism by which MSCs obtain their osteogenicity remains unclear.

The behavior of MSCs, such as self-renewal, progression, and differentiation is regulated through posttranscriptional, translational, posttranslational, and epigenetic processes (14). Epigenetic regulation is based on heritable changes in the pattern of gene expression that occurs without alterations in the primary nucleotide sequence. Major epigenetic mechanisms include DNA methylation and histone deacetylation, both of which are relevant to gene expression. Recently, epigenetic regulation has emerged as an important factor in cell differentiation, and epigenetic deregulation has been associated with human diseases such as malignancies. Given its association with various disease states, epigenetic regulation has become an important focus of potential therapy (14).

Genetic factors are thought to be involved in ectopic ossification of spinal ligaments, because some patients have a positive family history (15). However, multiple etiologic factors, including some local factors, such as mechanical stress and cytokines, are also thought to be important in ossification of spinal ligaments (3). This highlights the significance of epigenetic changes in the characteristics of MSCs.

We hypothesized that the propensity toward the osteogenic lineage of MSCs from ossified spinal ligaments may be controlled by epigenetic mechanisms. To examine the validity of the hypothesis, we compared candidate genes that are suppressed by DNA methylation in MSCs between non-ossified spinal ligaments and ossified spinal ligaments, using genome-wide cDNA microarray analysis.

2. Materials and methods

2.1. Samples

The study was approved by the Medical Ethics Committee of Hirosaki University Graduate School of Medicine. All patients gave written informed consent to participate. Ligamentum flavum samples were obtained from eight donor (four non-OYL and four OYL) patients who underwent orthopedic surgery (patient information is given in Table 1). The diagnosis of OYL or non-OYL (other cervical disease not related to ectopic ossification, e.g., cervical spondylotic myelopathy) was confirmed before surgery by spinal surgeons using radiographs, computed tomography, and magnetic resonance imaging of the cervical spine.

2.2. Cell isolation and culture

For the isolation of MSCs, the ligamentum flavum was rinsed with phosphate-buffered saline to remove blood, debris, and surrounding tissue. Collected ligaments were minced into 0.5 mm^3 pieces and washed twice with phosphate-buffered saline, then digested using 3-mg/mL collagenase (Type 5; Sigma–Aldrich, St. Louis, MO) in α-modified Eagle's medium (α-MEM; Invitrogen, Carlsbad, CA) at 37 °C for 3 h. Digested tissue was filtered through a 70-μm nylon filter (BD Biosciences, San Jose, CA) to remove debris. Nucleated cells were resuspended after centrifugation, and then plated in non-coated plastic dishes (Nalge Nunc International, Rochester, NY) at a density of 5×10^5 cells/90-mm dish. Cells were maintained in complete culture medium (α-MEM + 10% fetal bovine serum (JRH Bioscience, Lenexa, KS), 100-U/ml penicillin G sodium and streptomycin sulfate (Invitrogen). Culture dishes were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 14 days as passage 0. Characterization of these cells was presented in our previous study (11). Cells identified as MSCs by flow cytometry, as described previously (13), were used.

Table 1
Clinical diagnosis, gender, and age of patients who donated tissue samples.

Non-OYL		OYL	
Age/sex	Diagnosis	Age/sex	Diagnosis
54/M	Syringomyelia	55/M	OYL
56/M	Fracture	56/M	OYL
76/F	Thoracic myelopathy	75/M	OYL
81/M	Thoracic myelopathy	75/F	OYL

M: male, F: female, OYL: ossification of yellow ligament of the spine.

2.3. Demethylation of genomic DNA

Genomic DNA was demethylated by treatment with the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5AdC) (Sigma, Tokyo, Japan) (16). For treatment with 5AdC, 2×10^5 cells/10-cm dish were seeded on day 0, and exposed to freshly prepared 30-μM 5AdC for 24 h on days 1 and 3. This dose suppressed cellular growth rates to approximately 70% of the growth rate of non-treated cells (data not shown). On day 5, total RNA was extracted from MSCs using RNeasy Mini kits (Qiagen, Valencia, CA) according to the manufacturer's instructions for mammalian cells, including on-column DNA digestion using an RNase-free DNase set (Qiagen) to avoid genomic DNA contamination. Genomic DNA was extracted from MSCs using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions for mammalian cells.

2.4. Gene expression analysis (microarray)

The concentration of total RNA extracted from MSCs treated with or without 5AdC was determined using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, NC) and the quality was assessed on an Agilent 2100 Bioanalyzer using an Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA). RNA integrity was calculated, and RNA was amplified and labeled using an Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Tokyo, Japan) according to the manufacturer's protocol, starting with 750 ng of total RNA. Amplified aRNA from the samples was labeled with Cy5. Universal Human Reference RNA (Stratagene, Santa Clara, CA) was labeled with Cy3 and used as reference RNA. Dye incorporation efficiency was measured using a NanoDrop Spectrophotometer. Labeled sample aRNA corresponding to 200-pmol Cy5 and reference aRNA corresponding to 130-pmol Cy3 were hybridized to in-house spotted arrays. Additionally, 20-pmol Cy5 sample aRNA and 20-pmol Cy3 reference aRNA were hybridized to Agilent SurePrint G3 Human GE K Microarrays (Agilent Technologies). Fragmentation, hybridization, and washing for both the spotted arrays and Agilent arrays were carried out using the Agilent Gene Expression Hybridization Kit and Gene Expression Wash Buffer Kit (Agilent Technologies) in a low ozone environment according to the manufacturer's protocol. Subsequently, the arrays were scanned using an Agilent G2565CA Microarray Scanner (Agilent Technologies). Microarray data have been deposited into the Gene Expression Omnibus.

2.5. Real-time PCR analysis

Real-time PCR was carried out with Power SYBR Green PCR Master Mix on an ABI Prism® 7000 Sequence Detection System

(Applied Biosystems, Foster City, CA). Specific primer pairs for each gene, as listed in Tables 2 and 3, were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The conditions for PCR were: 95 °C for 1 min, and 50 cycles of 95 °C for 15 s, 60 °C for 1 min. All samples were analyzed in parallel for glycerol 3-phosphate dehydrogenase (*G3PDH*) expression as an internal standard. Real-time PCR data were represented as Ct values, where Ct is defined as the cycle number at which the amount of amplified product exceeds the threshold level. We used the comparative Ct method to compare the RNA expression in samples to that of the control in each experiment. The primers were constructed so that the dynamic range of the targets and the *G3PDH* reference was similar over a wide range of dilutions (1:1 to 1:10,000). Reactions were performed in triplicate for each cell preparation. The results were expressed as the mean ± SEM ($n = 4$) and the graphs show the relative expression levels compared with the control (non-OYL MSCs), unless otherwise stated.

2.6. Methylated DNA immunoprecipitation (MeDIP)

Gene methylation status was quantitatively assessed by MeDIP combined with real-time PCR using a primer set for the transcription regulatory site of the genes of interest (17). MeDIP was carried out using a MethylAffinity™ Methylated DNA Enrichment Kit according the manufacturer's instructions (GeneCopoeia, Inc., Rockville, MD). Briefly, genomic DNA (1 μg) was fragmented by sonication to 250–500-bp fragments and then mixed with GCM™ beads followed by overnight incubation at 4 °C on a rotisserie shaker. The tubes were then centrifuged at 1000 ×*g* for 1 min to pellet the beads. The supernatants were transferred to new tubes and used to determine the fraction of unmethylated DNA. The proportion of DNA in which the promoter regions were unmethylated was assessed by real-time PCR using primers for CpG islands in the promoter regions (Table 3). *G3PDH* was used as an internal standard, because its expression was not changed by 5AdC treatment (data not shown).

2.7. RNA interference

The expression of *WNT5A* and *GDNF* was specifically inhibited using OriGene 27 siRNA Knockdown Duplexes (OriGene, Rockville, MD) according to the manufacturer's protocol. Briefly, MSCs were transfected with 3 nM small interfering RNA (siRNA) for each gene or universal scrambled negative control siRNA duplex (SR30004; OriGene) using siTran 1.0 Transfection Reagent (OriGene). Cells at 90% confluency were incubated in siRNA with siTrans1.0 + siRNA at 37 °C in a 5% CO_2 incubator for 24 h. The following siRNAs were used: *WNT5A* (SR305106A) (5′–3′): GGAUGUAGAAUGAUUAA AGGUUGGT, *GDNF* (SR301776B) (5′–3′): GGAGUGCACUUGUUUGG AAGUUCTG.

Table 2
Sequences of real-time PCR primers used for gene expression.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
G3PDH	TGCACCACCAACTGCTAGC	GGCATGGACTGTGGTCATGAG
WNT5A	TCACAGGTTCTCAGCCCAAG	AGGGCTCAGTGTGAAGAGGA
GDNF	CCAGAGGAAAAGGTCGGAGA	CATAGCCCAGACCCAAGTCA
RUNX2	GCCTTCAAGGTGGTAGCCC	CGTTACCCGCCATGACAGTA
BMP2	AGATGAACACAGCTGGTCACAGA	GGAAGGATGCCCTTTTCCA
ALP	ACGAGCTGAACAGGAACAACGT	CACCAGCAAGAAGAAGCCTTTG

G3PDH: glyceraldehyde-3-phosphate dehydrogenase, *WNT5A*: wingless-type MMTV integration site family, member 5A, *GDNF*: glial cell-derived neurotrophic factor, *RUNX2*: runt-related transcription factor 2, *BMP2*: bone morphogenetic protein 2, *ALP*: alkaline phosphatase.

Table 3
Sequences of PCR primers for CpG islands.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
G3PDH	CAGAGCCTCGAGGAGAAGTTC	GGAACGACTGAGATGGGGAA
WNT5A	TGGAAAAACGGGGAAAAGAGAG	CTTATTGCTGTGCGGTGAGC
GDNF	GGCTCTTTCGTTCTCGGTATTTG	GAGACAAACTGAGAAGCCGAG

G3PDH: glyceraldehyde-3-phosphate dehydrogenase, *WNT5A*: wingless-type MMTV integration site family member 5A, *GDNF*: glial cell-derived neurotrophic factor.

2.8. Alkaline phosphatase (ALP) assay

MSCs from OYL patients were seeded into a 6-well plate and grown for 2 days until 90% confluent, then transfected with siRNA for *GDNF*, *WNT5A*, or universal scrambled negative control siRNA before further culture for 24 h. The proteins were collected from the cells using a lysis buffer (600 μl) containing 0.05% Triton X-100. After two freeze/thaw cycles, ALP activity in the lysates was measured using a LabAssay™ ALP kit (Wako Pure Chemical Industries, Osaka, Japan) and standardized to whole protein content in the lysate according to the manufacturer's instructions. The experiment was performed once with duplicate wells for each sample ($n = 4$). ALP activity was expressed as IU/μg of protein.

2.9. Statistical analysis

Data from eight samples (untreated and treated with 5AdC) in each group (non-OYL and OYL) were used for statistical analyses. Calculations were performed with SPSS ver.12.0 J (SPSS, Inc., Chicago, IL). A comparison was made of all non-OYL groups (untreated and treated with 5AdC) to all OYL groups (untreated and treated with 5AdC). All quantitative values are expressed as mean ± SEM. Experimental data from the microarray analysis between the 5AdC-treated group and the non-treated group were compared using the unequal variance unpaired *t*-test (Welch test) with Benjamini–Hochberg correction for multiple comparison. Gene expression levels of each mRNA were compared between the non-OYL group and the OYL group. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of 5AdC on gene expression in OYL and non-OYL samples

The strategy to identify genes responsible for the osteogenic feature of MSCs from patients with ossification of spinal ligaments was as follows (see also Fig. 1). In our experimental conditions, three groups of genes were observed. Group 1 comprises genes whose expression was significantly increased by 5AdC in non-OYL samples. These genes are normally suppressed by hypermethylation. Group 2 comprises genes whose expression did not change significantly upon 5AdC treatment in OYL samples. These genes were released from restriction by hypermethylation. Group 3 comprises genes whose expression in OYL samples was significantly higher than that in non-OYL samples under normal conditions (without 5AdC treatment). Candidate genes will reside in the intersection among Groups 1–3. In the non-OYL group, 1269 genes were down-regulated and 818 up-regulated by 5AdC compared with control (considering $p < 0.05$ as the cut-off). In the OYL group, 1182 genes were down-regulated and 472 up-regulated by 5AdC treatment compared with control (considering $p < 0.05$ as the cut-off). 5AdC induced a significant increase in the expression of 98 genes in MSCs from non-OYL patients, but did not change gene expression in MSCs from OYL patients (i.e., the intersection between Groups 1 and 2). Only two genes, glial cell-derived

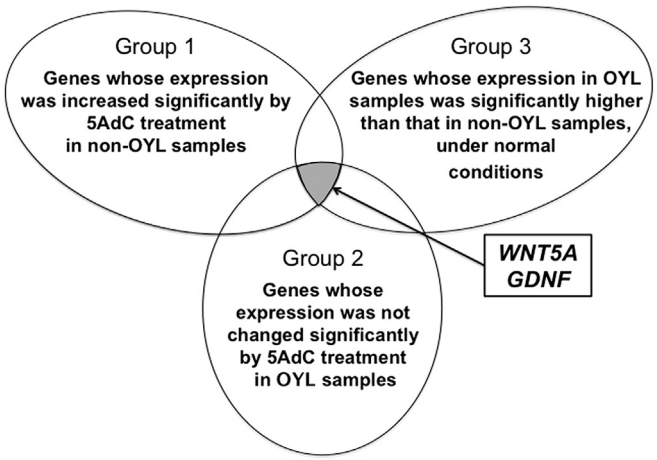

Fig. 1. Strategy for the identification of susceptibility genes for ectopic ossification of spinal ligaments that are regulated by DNA methylation. To identify genes responsible for the osteogenic features of MSCs from patients with ossification of spinal ligaments, we focused on three groups. The candidate genes will reside in the intersection among Groups 1–3.

neurotrophic factor (*GDNF*) and wingless-type MMTV integration site family member 5A (*WNT5A*), showed significantly higher expression (>2×, considering $p < 0.05$ as the cut-off) in OYL MSCs compared with non-OYL MSCs in normal culture conditions without 5AdC (*i.e.*, the intersection among Groups 1–3).

3.2. Real-time reverse transcriptase PCR analysis

To confirm the microarray findings, we measured the mRNA expression levels of *WNT5A* and *GDNF* by quantitative real-time reverse transcriptase PCR. We demonstrated significantly higher expression of these two genes in MSCs from OYL samples compared with those from non-OYL samples. This was consistent with the microarray data (Fig. 2).

3.3. Methylated DNA immunoprecipitation (MeDIP)

To assess the DNA methylation status of the promoter region of the two genes, we carried out MeDIP combined with quantitative real-time PCR. In the non-OYL group, the amount of unmethylated CpG island in the promoter region of *WNT5A* and *GDNF* DNA was low, but it increased significantly after 5AdC treatment. In contrast, there was no significant change in the amount of unmethylated *WNT5A* or *GDNF* DNA in the OYL group (Fig. 3). This suggests that the expression of these genes was inhibited by DNA methylation of the promoter region in non-OYL MSCs but not in OYL MSCs under normal culture conditions.

3.4. Effect of siRNA-mediated gene silencing of GDNF and WNT5A on osteogenic gene expression in OYL MSCs

siRNAs targeting *GDNF* or *WNT5A* significantly decreased the expression of each target gene in MSCs from OYL patients (Fig. 4). Furthermore, siRNA treatment resulted in significant down-regulation of osteogenic genes such as *RUNX2*, *BMP2*, and *ALP*. Negative control siRNA did not affect the expression of these genes (see Fig. 4).

3.5. Effect of siRNA-mediated gene silencing of GDNF and WNT5A on enzymatic ALP activity

Next, we investigated the effect of siRNA silencing of *GDNF* and *WNT5A* on the functional activity of ALP, a marker of osteogenesis. ALP leads to mineralization of the tissue (18). Both siRNAs significantly decreased ALP activity in cell lysate prepared from OYL MSCs (Fig. 5).

4. Discussion

By both microarray and real-time RT-PCR, we showed that *WNT5A* and *GDNF* showed significantly higher expression in OYL MSCs than in non-OYL MSCs under normal culture conditions without 5AdC treatment. In non-OYL MSCs, both genes were hypermethylated, while in OYL MSCs, these genes were unmethylated. Knockdown with siRNAs decreased not only the expression of the target gene but also that of several other osteogenic genes. Furthermore, siRNA treatment suppressed the enzymatic activity of ALP, a marker of osteogenesis. These data suggest that the expression of *WNT5A* and *GDNF* was restricted by DNA methylation of the promoter region in non-OYL MSCs, and this epigenetic regulation of both genes led to a tendency for ossification of the spinal ligaments in OYL patients.

The *WNT* gene encodes a secreted signaling protein that has been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis (19). During skeletal development, WNT signaling is implicated in proximal–distal outgrowth and dorsoventral limb

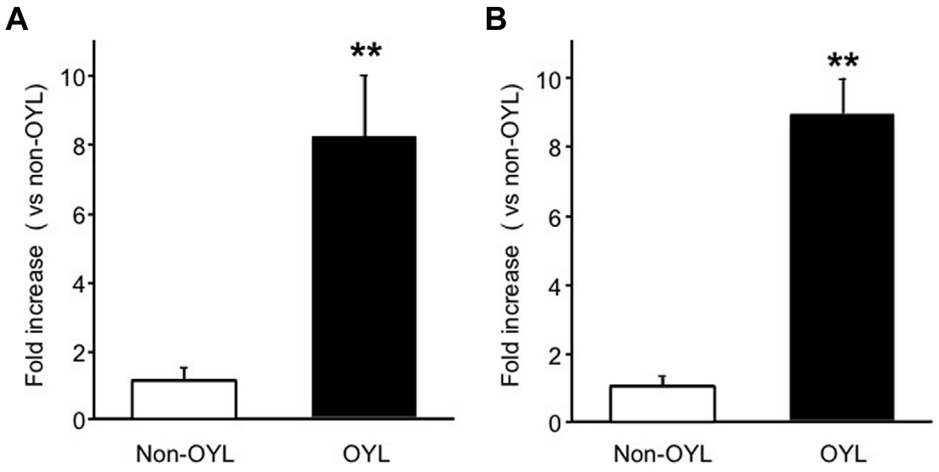

Fig. 2. Confirmation of expression level of (A) *WNT5A* and (B) *GDNF* by real-time RT-PCR. mRNA expression levels of *WNT5A* and *GDNF* in non-OYL and OYL MSCs without 5AdC treatment, analyzed by real-time RT-PCR. Specific primer pairs for each gene are listed in Table 2. Gene expression levels were normalized to those of *G3PDH*. $^{**}p < 0.01$ ($n = 4$).

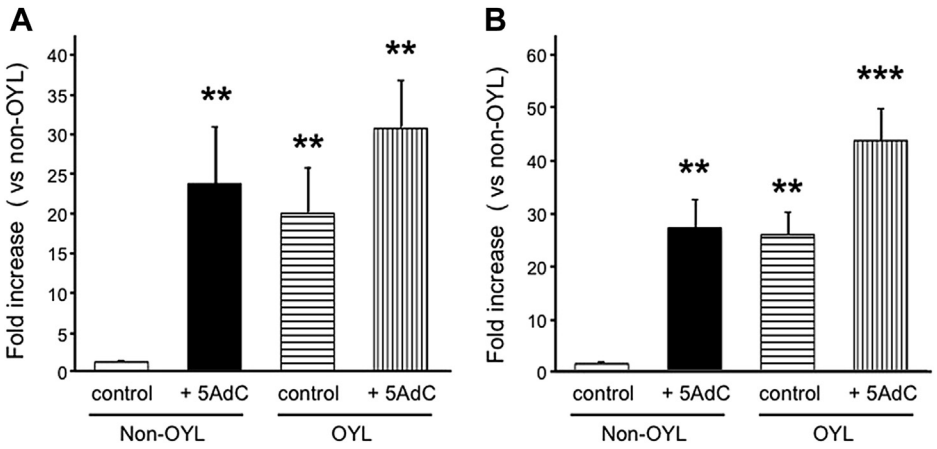

Fig. 3. Effect of 5AdC on the amount of unmethylated DNA in the promoter region of (A) *WNT5A* and (B) *GDNF*. Non-OYL and OYL MSCs were treated with or without 5AdC and their genomic DNA extracted. Fragmented DNAs were mixed with beads coated with anti-methyl cytidine antibody (GCM™ beads) and the methylated DNA fraction was sedimented with the beads by centrifugation. The supernatants were used to determine the amount of unmethylated DNA in the *WNT5A* and *GDNF* promoter region by real-time PCR. $^{**}p < 0.01$; $^{***}p < 0.001$ ($n = 4$).

patterning. Later, it plays a crucial role in MSC lineage commitment and progression (20). As previously reported, the WNT signaling pathway has emerged as a key regulator, including of skeletal patterning (21). WNT signaling may regulate the maintenance and differentiation of stem cells (22) and its inappropriate activation is associated with a high frequency of tumors in specific tissues including the intestine, liver, skin, and mammary gland (23). WNT signaling has also been shown to be important in regulating bone formation in mammals (24). WNT5A regulates osteoblastogenesis and adipogenesis through the up-regulation of WNT/β-catenin signaling (25). Osteoblast-lineage cells from WNT5A-deficient mice exhibited impaired osteoblast differentiation. On the basis of the above findings, it is probable that epigenetic regulation of the WNT signaling pathway, *i.e.*, higher expression of these genes, leads to the tendency for ossification of the spinal ligaments in OYL patients.

GDNF was first discovered in glial cells (26), and its expression has been found in a variety of tissues (27). GDNF is the most potent survival factor identified in motor neurons (28): heterozygous GDNF-knockout mice lack 22% of their lumbar motor neurons (29). In contrast, GDNF is produced by osteoblasts as well as bone marrow stromal cells (30). GDNF is able to influence the proliferation of calvarial osteoblasts and may be involved in the regulation of osteoblast recruitment in bone growth and remodeling (31). On the basis of the above findings, it is probable that the epigenetic regulation of GDNF signaling is related to the tendency for ossification of the spinal ligaments in OYL patients. Furthermore, GDNF/WNT11 signaling plays an important role in morphogenesis of the kidney (32). GDNF is mislocalized in WNT5A-deficient kidneys (33). Thus, there may be an as-yet-unknown crosstalk between GDNF and WNT5A in the repair processes of spinal ligaments. This warrants further study.

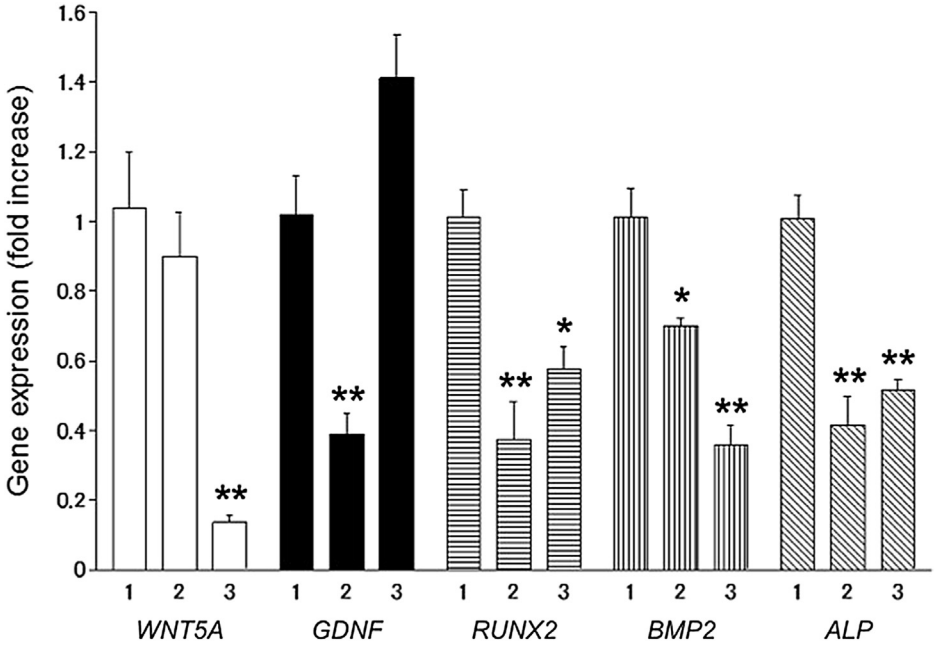

Fig. 4. Inhibition of gene expression by siRNAs targeting *WNT5A* and *GDNF*. MSCs from OYL patients ($n = 4$) were transfected with siRNA and cultured for 24 h. Total RNA was harvested and used for real-time RT-PCR. 1: negative control siRNA, 2: siRNA targeting *GDNF*, 3: siRNA targeting *WNT5A*. Gene expression levels were normalized to *G3PDH* and expressed as fold increase compared with control (MSCs without siRNA transfection). $^{*}p < 0.05$; $^{**}p < 0.01$ ($n = 4$).

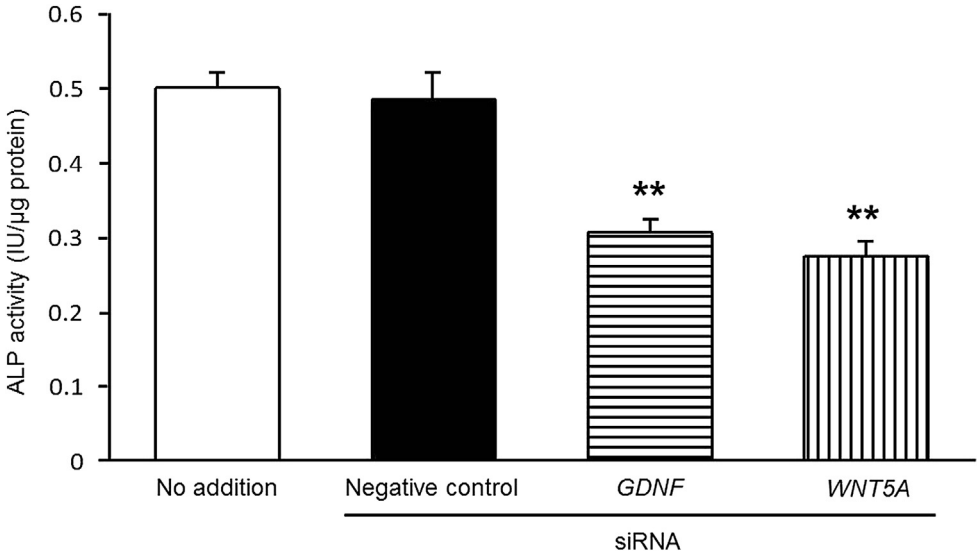

Fig. 5. Alkaline phosphatase (ALP) activity in MSCs treated with siRNAs targeting *WNT5A* and *GDNF*. Cell lysates prepared from siRNA-transfected OYL MSCs were assayed for ALP enzymatic activity. $^{**}p < 0.01$ ($n = 4$).

This study has several limitations. First, microarray analysis revealed that many genes demonstrated altered expression in MSCs from non-OYL or OYL patients treated with 5AdC. A proportion of such changes may directly reflect demethylation of the target gene; however the rest might be indirect effects resulting from interactions among the genes. Therefore, further studies should examine the effect of other genes on methylation status. Second, we used an indirect method to measure DNA methylation status — the sensitivity of mRNA expression to 5AdC. It may be more valid to assess this directly using microarray analysis of the methylation status of promoter regions and CpG islands. Third, epigenetic changes include not only DNA methylation but also histone modification (acetylation, methylation, phosphorylation, ubiquitination, sumoylation); frequently, more than one mechanism is tightly linked to the regulation of gene expression. Therefore, it is necessary to examine the influence of histone modification. Fourth, we did not attempt direct inhibition of the ossification of OYL MSCs, because this requires multiple siRNA transfections over several weeks, resulting in cytotoxicity. In future studies, we will continuously inhibit the two genes using methods such as gene editing with CRISPR/Cas9.

In summary, by genome-wide microarray analysis we revealed that 5AdC induced a significant increase in the expression of 98 genes in MSCs from non-OYL patients, which were not changed in MSCs from OYL patients. Only two such genes, *GDNF* and *WNT5A*, showed significantly higher expression in OYL MSCs compared with in non-OYL MSCs under normal culture conditions without 5AdC treatment. Then, using MeDIP combined with real-time PCR, we quantitatively assessed the methylation status of these two genes. They were hypermethylated in non-OYL MSCs but not in OYL MSCs. These results suggest that the osteogenic features of MSCs from OYL patients are promoted by unmethylated *WNT5A* and *GDNF* genes.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by Japanese Pharmacological Society.

Acknowledgments

This project was funded by an AOSpine Japan research grant (2013); a Grant-in-Aid from the Investigation Committee on Ossification of the Spinal Ligaments; the Public Health Bureau of the Japanese Ministry of Labour, Health, and Welfare; and by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (no. 24590310). We thank Drs. Kazumasa Ueyama, Naoki Echigoya, Akio Sannohe, Takuya Numasawa, Toru Yokoyama, Kazunari Takeuchi, Shuichi Aburakawa, Yoshihito Yamasaki, Taisuke Nitobe, Kenji Kowatari and Takashi Tomita for their contributions to sample collection. Japanese Pharmacological Society acknowledge to Dr. Taito Itabashi for his valuable comments.

References

(1) Resnick D, Shaul SR, Robins JM. Diffuse idiopathic skeletal hyperostosis (DISH): Forestier's disease with extraspinal manifestations. Radiology. 1975;115:513–524.
(2) Utsinger PD. Diffuse idiopathic skeletal hyperostosis. Clin Rheum Dis. 1985;11:325–351.
(3) Furukawa K-I. Pharmacological aspect of ectopic ossification in spinal ligament tissues. Pharmacol Ther. 2008;118:352–358.
(4) Matsunaga S, Kukita M, Hayashi K, Shinkura R, Koriyama C, Sakou T, et al. Pathogenesis of myelopathy in patients with ossification of the posterior longitudinal ligament. J Neurosurg. 2002;96:168–172.
(5) Choi S, Lee SH, Lee JY, Choi WG, Choi WC, Choi G, et al. Factors affecting prognosis of patients who underwent corpectomy and fusion for treatment of cervical ossification of the posterior longitudinal ligament: analysis of 47 patients. J Spinal Disord Tech. 2005;18:309–314.
(6) Koyanagi I, Iwasaki Y, Hida K, Imamura H, Abe H. Magnetic resonance imaging findings in ossification of the posterior longitudinal ligament of the cervical spine. J Neurosurg. 1998;88:247–254.
(7) Luu HH, Song WX, Luo X, Manning D, Luo J, Deng ZL, et al. Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. J Orthop Res. 2007;25:665–677.
(8) Min KJ, So KA, Ouh YT, Hong JH, Lee JK. The effects of DNA methylation and epigenetic factors on the expression of CD133 in ovarian cancers. J Ovarian Res. 2012;5:28.
(9) Yan H, Yu N, Tong J. Effects of 5-Aza-2′-deoxycytidine on the methylation state and function of the WWOX gene in the HO-8910 ovarian cancer cell line. Oncol Lett. 2013;6:845–849.
(10) Wang Z, Li L, Su X, Gao Z, Srivastava G, Murray PG, et al. Epigenetic silencing of the 3p22 tumor suppressor DLEC1 by promoter CpG methylation in non-Hodgkin and Hodgkin lymphomas. J Transl Med. 2012;10:209.

(11) Asari T, Furukawa K-I, Tanaka S, Kudo H, Mizukami H, Ono A, et al. Mesenchymal stem cell isolation and characterization from human spinal ligaments. Biochem Biophys Res Commun. 2012;417:1193–1199.
(12) Chin S, Furukawa K-I, Ono A, Asari T, Harada Y, Wada K, et al. Immunohistochemical localization of mesenchymal stem cells in ossified human spinal ligaments. Biochem Biophys Res Commun. 2013;436:698–704.
(13) Harada Y, Furukawa K-I, Asari T, Chin S, Ono A, Tanaka T, et al. Osteogenic lineage commitment of mesenchymal stem cells from patients with ossification of the posterior longitudinal ligament. Biochem Biophys Res Commun. 2014;443:1014–1020.
(14) Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature. 2004;429:457–463.
(15) Nakajima M, Takahashi A, Tsuji T, Karasugi T, Baba H, Uchida K, et al. A genome-wide association study identifies susceptibility loci for ossification of the posterior longitudinal ligament of the spine. Nat Genet. 2014;46:1012–1017.
(16) Yamashita S, Takahashi S, McDonell N, Watanabe N, Niwa T, Hosoya K, et al. Methylation silencing of transforming growth factor-beta receptor type II in rat prostate cancers. Cancer Res. 2008;68:2112–2121.
(17) Mohn F, Weber M, Schubeler D, Roloff TC. Methylated DNA immuno precipitation (MeDIP). Methods Mol Biol. 2009;507:55–64.
(18) Narisawa S, Yadav MC, Millán JL. In vivo overexpression of tissue-nonspecific alkaline phosphatase increases skeletal mineralization and affects the phosphorylation status of osteopontin. J Bone Miner Res. 2013;28:1587–1598.
(19) Rijsewijk F, Schuermann M, Wagenaar E, Parren P, Weigel D, Nusse R. The Drosophila homology of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. Cell. 1987;50:649–657.
(20) Hu H, Hilton MJ, Tu X, Yu K, Ornitz DM, Long F. Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development. 2005;132:49–60.
(21) Cabrera CV, Alonso MC, Johnston P, Phillips RG, Lawrence PA. Phenocopies induced with antisense RNA identify the wingless gene. Cell. 1987;50:659–663.
(22) Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. Nature. 2001;411:349–354.
(23) Noort van M, Clevers H. TCF transcription factors, mediators of Wnt-signaling in development and cancer. Dev Biol. 2002;244:1–8.
(24) Long F. Building strong bones: molecular regulation of the osteoblast lineage. Nat Rev Mol Cell Biol. 2011;13:27–38.
(25) Okamoto M, Udagawa N, Uehara S, Maeda K, Yamashita T, Nakamichi Y, et al. Noncanonical Wnt5a enhances Wnt/β-catenin signaling during osteoblastogenesis. Sci Rep. 2014;4:4493.
(26) Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science. 1993;260:1130–1132.
(27) Suter-Crazzolara C, Unsicker K. GDNF is expressed in two forms in many tissues outside the CNS. Neuroreport. 1994;20:2486–2488.
(28) Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, et al. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science. 1994;266:1062–1064.
(29) Moore MW, Klein RD, Fariñas I, Sauer H, Armanini M, Phillips H, et al. Renal and neuronal abnormalities in mice lacking GDNF. Nature. 1996;382:76–79.
(30) Jiang J, Lv Z, Gu Y, Li J, Xu L, Xu W, et al. Adult rat mesenchymal stem cells differentiate into neuronal-like phenotype and express a variety of neuroregulatory molecules in vitro. Neurosci Res. 2010;66:344–346.
(31) Gale Z, Cooper PR, Scheven BA. Glial cell line-derived neurotrophic factor influences proliferation of osteoblastic cells. Cytokine. 2012;57:276–281.
(32) Majumdar A, Vainio S, Kispert A, McMahon J, McMahon AP. Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. Development. 2003;130:3175–3185.
(33) Yun K, Ajima R, Sharma N, Costantini F, Mackem S, Lewandoski M, et al. Noncanonical Wnt5a/Ror2 signaling regulates kidney morphogenesis by controlling intermediate mesoderm extension. Hum Mol Genet. 2014;23:6807–6814.