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Osteogenic lineage commitment of mesenchymal stem cells from patients with ossification of the posterior longitudinal ligament

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ABSTRACT

Ectopic bone formation is thought to be responsible for ossification of the posterior longitudinal ligament of the spine (OPLL). Mesenchymal stem cells (MSCs) were isolated from spinal ligaments and shown to play a key role in the process of ectopic ossification. The purpose of this study was to explore the capacity of these MSCs to undergo lineage commitment and to assess the gene expression changes between these committed and uncommitted MSCs between OPLL and non-OPLL patients. Spinal ligament-derived cells were obtained from OPLL patients or patients with cervical spondylotic myelopathy (non-ossified) for comparison ($n = 8$ in each group). MSCs from the two patient cohorts were evaluated for changes in colony forming ability; osteogenic, adipogenic and chondrogenic differentiation potential; and changes in gene expression following induction with lineage-specific conditions. We show that the osteogenic differentiation potential was significantly higher in MSCs from OPLL patients than in those from non-OPLL patients. In addition, alkaline phosphatase activity and several osteogenic-related genes expressions (bone morphogenetic protein 2, runt-related transcription factor 2 and alkaline phosphatase) were significantly higher in the OPLL group than in the non-OPLL group. However, single cell cloning efficiency, adipogenic and chondrogenic differentiation, and the expression of adipogenic and chondrogenic-related genes were equivalent between MSCs harvested from OPLL and non-OPLL patient samples. These findings suggest an increase in the osteogenic differentiation potential of MSCs from OPLL patients and that this propensity toward the osteogenic lineage may be a causal factor in the ossification in these ligaments.

1. Introduction

The posterior longitudinal ligament of the spine lies adjacent to the posterior aspect of the vertebral bodies. Ossification of this ligament is characterized by ectopic bone formation, and is frequently linked with similar ossification in other surrounding spinal ligaments. These ossified lesions often enlarge with time and compress the spinal cord and its roots, leading to neurological deficiencies and a high risk of spinal cord injury [1,2]. Patients with severe symptoms are considered definitive candidates for surgical treatment such as spinal decompression [3]. However, it is worth noting that there are no therapies for preventing the formation and progression of ossified lesions.

Given that some patients have a familial history of OPLL, it is quite possible that genetic factors are partly responsible for this pathogenic condition. The genetic factors pertaining to OPLL are described as multifactorial genetic inheritance [4] and the genes playing a key role in its onset remain unclear. In OPLL patients, ectopic ossification occurs throughout the spine, frequently involving ossification of other spinal ligaments. Consequently, OPLL has been regarded as one of the manifestations of diffuse idiopathic skeletal hyperostosis (DISH) [5,6]. Based on genetic factors, these current findings may indicate that OPLL patients would have a tendency toward systemic ossification of numerous ligaments, similar to that observed in patients with DISH.

Mesenchymal stem cells (MSCs) have been isolated from various tissues and are thought to play essential roles in supplying damaged tissues with a source of progenitor cells for repair. However, several studies have discussed the undesirable effects of MSCs in pathogenic conditions such as fibrodysplasia ossificans progressiva (FOP) [7], ectopic ossification following burn injury [8] and aortic valve calcification [9]. We have focused our attention on the roles of MSCs in the process of ectopic ossification in spinal ligamentous tissues. As a first step, we isolated MSCs from human spinal ligaments and performed immunophenotypic analysis of

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cell surface markers [10]. Furthermore, we previously demonstrated the localization of MSCs in ossified human spinal ligaments [11]. Chondrocytes near the ossification front in ossified spinal ligaments are positive for MSC marker expression, and there is an increase in the prevalence of MSCs in the collagenous matrix near the ossified spinal ligament. Therefore, we suspect that MSCs play a key role in the ectopic ossification process of spinal ligaments. This study was aimed to explore the capacity of MSCs to undergo lineage commitment and to determine the gene expression changes that occur in committed and uncommitted MSCs and how this differs between OPLL and non-OPLL patients.

2. Materials and methods

2.1. Samples

The study was approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine. All patients gave written informed consent to participate. Ligamentum flava at the C3 level were collected aseptically during surgery of the cervical spine from eight patients with OPLL (OPLL group) and eight patients with cervical spondylotic myelopathy as a control (non-OPLL group). The ligamentum flava in OPLL patients showed no evidence of ossification at the time of harvesting. The clinical diagnosis, patient gender and age of the tissue samples used in this study are shown in Table 1. There was no significant difference in patient ages between OPLL (mean, 66.9 ± 6.9 years) and non-OPLL (mean, 65.9 ± 6.6 years).

2.2. Cell isolation and culture

MSCs were isolated as described in the literature [10]. Briefly, collected samples were washed with phosphate-buffered saline (PBS), minced and digested with 3 mg/ml collagenase (Type5; Sigma–Aldrich, St. Louis, MO, USA) in α-modified Eagle's medium (α-MEM; Invitrogen, Carlsbad, CA, USA) at 37 °C for 3 h. Nucleated cells were plated in plastic dishes (Nalge Nunc International, Rochester, NY, USA) at density of 5×10^5 cells/90-mm dishes. Cells were maintained in complete culture medium (α-MEM + 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA), 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.

2.3. Flow cytometric analysis and cell sorting

For flow cytometric analysis of cell surface antigens and cell sorting, MSCs were stained for the expression of CD34 and CD105 using a mouse anti-human CD34 antibody coupled with PE and a mouse anti-human CD105 antibody coupled with PerCP-Cy5.5, respectively (BD Biosciences, San Jose, CA, USA). Passage 1 cells (1×10^6 cells) were harvested and washed with PBS containing 2% FBS. Cells were suspended in 50 μl PBS containing specific antibodies and incubated for 45 min on ice. The cells were washed in PBS, filtered using a 35-μm strainer (BD Falcon), resuspended in 1 ml of PBS and analyzed using a FACSAria™ II instrument (BD Biosciences). For cell sorting, gates were defined as negative for CD34 ($CD34^-$) and positive for CD105 ($CD105^+$) according to the isotype control fluorescence intensity. Data were analyzed using BD FACSDiva™ software v6.1.3 (BD Biosciences).

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

non-OPLL group		OPLL group	
Sex/age	Diagnosis	Sex/age	Diagnosis
F/56	CSM	M/53	C-OPLL, T-OLF
M/60	CSM	M/62	C-OPLL, T-OPLL, T-OLF
M/60	CSM	M/65	C-OPLL
M/60	CSM	F/67	C-OPLL, T-OPLL, T-OLF
M/71	CSM	M/71	C-OPLL, T-OLF
M/72	CSM	M/71	C-OPLL, T-OPLL, T-OLF
F/73	CSM	F/71	C-OPLL, T-OPLL
M/75	CSM	M/75	C-OPLL

M: male, F: female, CSM: cervical spondylotic myelopathy, C: cervical spine, T: thoracic spine, OPLL: ossification of the posterior longitudinal ligament of the spine, OLF: ossification of the ligamentum flavum of the spine.

2.4. Single cell cloning efficiency

Sorted cells at passage 1 were seeded in complete culture medium into two 96-well culture plates (Nalge Nunc International) per sample at a density of one cell per well by FACS. After 14 days of culture, wells were stained with 0.5% crystal violet (Wako Pure Chemical Industries, Osaka, Japan). Wells that reached 30 or more cells per well were counted as single-cell clones [12].

2.5. In vitro differentiation and histological analysis

2.5.1. Osteogenic differentiation

Sorted MSCs at passage 1 were seeded at densities of 100 cells/cm^2 for cytohistological staining and cultured at subconfluent levels. Osteogenic induction medium was then added for 21 days. Osteogenic induction medium consisted of complete culture medium supplemented with 1 nM dexamethasone (ICN Biomedicals Inc., Costa Mesa, CA, USA), 10 mM β-glycerol phosphate and 50 μg/ml ascorbic acid (Wako Pure Chemical Industries), as previously reported [13]. To evaluate the mineralized matrix, cells were fixed with 4% formaldehyde and stained with 1% Alizarin Red S (Sigma–Aldrich) for 10 min. The calcium-bound dye was extracted using 100 mM hexadecylpyridinium chloride monohydrate (Wako Pure Chemical Industries) and quantified at an optical density (OD) of 570 nm. The experiment was performed once with triplicate wells for each sample. ALP activity was measured at days 0, 7 and 14 using a LabAssay™ ALP kit (Wako Pure Chemical Industries) 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.

2.5.2. Adipogenic differentiation

Cells from passage 2 were seeded at densities of 10,000 cells/cm^2. Upon reaching the appropriate subconfluent level, cells were incubated in adipogenic induction medium (complete culture medium supplemented with 100 nM dexamethasone, 0.5 mM isobutylmethylxanthine and 100 μM indomethacin (Sigma–Aldrich)) for 21 days, as previously reported [14]. To evaluate lipid vesicle formation, cells were fixed with 4% formaldehyde and stained with 0.3% Oil Red O for 20 min. The dye was extracted using 60% isopropanol and the OD was measured at 490 nm. The experiment was performed once with duplicate wells for each sample.

2.5.3. Chondrogenic differentiation

5×10^5 cells at passage 2 were centrifuged for 10 min at 450×*g* in a 15-mL polypropylene tube. The pellet was treated with chondrogenic induction medium for 21 days. Chondrogenic induction medium consisted of complete culture medium supplemented with 500 ng/ml bone morphogenetic protein (BMP) 2, 10 ng/ml transforming growth factor (TGF)-β3 (R&D Systems, Minneapolis, MN, USA) and 100 nM dexamethasone, as previously reported [10]. The pellets were evaluated in terms of diameter and pellet wet weight. The diameter was measured using ImageJ software (Rasband, WS, rsb.info.nih.gov/ij). The pellets were fixed in 10%

Table 2
List of primers used for real-time PCR.

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
GAPDH	TGCACCACCAACTGCTAGC	GGCATGGACTGTGGTCATGAG
BMP2	AGATGAACACAGCTGGTCACAGA	GGAAGGATGCCCTTTTCCA
Runx2	GCCTTCAAGGTGGTAGCCC	CGTTACCCGCCATGACAGTA
ALP	ACGAGCTGAACAGGAACAACGT	CACCAGCAAGAAGAAGCCTTTG
Osteocalcin	TGAGAGCCCTCACACTCCTC	ACCTTTGCTGGACTCTGCAC
Osteopontin	CGCAGACCTGACATCCAGT	GGCTGTCCCAATCAGAAGG
PPARγ2	TGAATGTGAAGCCCATTGAA	CTGCAGTAGCTGCACGTGTT
LPL	ATGTGGCCCGGTTTATCA	CTGTATCCCAAGAGATGGACATT
Sox9	GTACCCGCACTTGCACAAC	TCGCTCTCGTTCAGAAGTCTC
COL2A1	CCGGGCAGAGGGCAATAGCAGGTT	CATTGATGGGGAGGCGTGAG
COL10A1	CATGTTTGGGTAGGCCTGTATAAGA	ACTCCCTGAAGCCTGATCCA

G3PDH: glyceraldehyde-3-phosphate dehydrogenase, BMP2: bone morphogenetic protein 2, Runx2: runt-related transcription factor 2, ALP: alkaline phosphatase, PPARγ2: peroxisome proliferator-activated receptor gamma 2, LPL: lipoprotein lipase, Sox9: sex-determining region Y-type high mobility group box 9, COL2A1: collagen type 2 alpha 1, COL10A1: collagen type 10 alpha 1.

formaldehyde, dehydrated through serial ethanol dilutions and embedded in paraffin. Blocks were cut into 5-μm sections and stained with Alcian Blue (Sigma–Aldrich). Images were captured on a light microscope (BX41; Olympus, Tokyo, Japan) and ImageJ software (Rasband) was used to quantify differences in Alcian Blue staining between samples. To standardize measurements, the ImageJ automatic thresholding routine was used to avoid bias introduced by an observer manually setting the imaging threshold. The Alcian Blue stained area of the pellet was quantified with the percentage of the positive area to the total area [15]. The experiment was performed once with duplicate pellets for each sample.

2.6. RNA isolation and Real-Time PCR analysis

The differences between the mRNA levels of osteogenic-, adipogenic- and chondrogenic-related genes in stem cell differentiation were analyzed using real-time PCR. Total RNA was extracted on days 0, 7, 14 and 21 of each induction periods using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Real-time PCR was conducted using Power SYBR Green PCR Master Mix on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Specific primer pairs for each gene (Table 2) were designed using Primer Express software (Applied Biosystems). The expression level of each gene was normalized with that of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Graphs show the relative expression levels compared with the control on day 0.

2.7. Statistical analysis

Data from eight samples in each group were used for statistical analyses, with the exception of chondrogenic-related gene expressions ($n = 5$ for each group). Data input and calculations were performed with SPSS ver.12.0 J (SPSS Inc., Chicago, IL, USA). All quantitative values are expressed as mean ± SEM. Experimental data between non-OPLL group and OPLL group were compared using Mann–Whitney *U*-test. Gene expression levels of each mRNA were compared between non-OPLL group and OPLL group on each day. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation and flow cytometric analysis of MSCs from spinal ligament

MSCs obtained from spinal ligaments were plastic-adherent and demonstrated characteristic spindle-shaped and fibroblast-like morphology in both OPLL and non-OPLL groups (Fig. 1A). Flow cytometric analysis was used to examine the proportions of $CD34^-/CD105^+$ cells, which is indicative of MSCs, in both groups (Fig. 1B). The percentages of $CD34^-/CD105^+$ passage-1 cells in non-OPLL and OPLL groups were 90.7 ± 4.5% and 89.6 ± 4.9%, respectively (Fig. 1C).

3.2. Single cell cloning efficiency

Passage 1 cells were seeded at a statistical density of one cell per well in 192 wells for each sample. After 14 days, colonies were stained with crystal violet. Single cell cloning efficiency was 48.3 ± 3.9 clones in non-OPLL group and 52.0 ± 8.9 clones in OPLL group per 192 wells (Fig. 1D).

3.3. In vitro differentiation and histological analyses

3.3.1. Osteogenesis

After 21 days in basal growth media, none of the MSC cultures showed evidence of spontaneous mineralization (Fig. 2A). However, under osteogenic induction medium, mineralization was observed in both OPLL and non-OPLL groups, with calcium deposition visualized by Alizarin Red S staining. To quantify the mineralized content, bound Alizarin Red S dye was dissolved and measured at 570 nm. The OD values were 1.9 ± 0.9 in the non-OPLL group and 13.1 ± 4.0 in the OPLL group (Fig. 2B), demonstrating a significant difference between the two groups ($P = 0.0117$). Furthermore, ALP activity on day 7 under the osteogenic conditions and day 14 under basal media and osteogenic induction media were significantly higher in the OPLL group as compared with those values in the non-OPLL group (Fig. 2C). This finding indicates that the osteogenic potential of MSCs derived from the ligaments of patients with OPLL is greater than that of MSCs derived from the ligaments of non-OPLL patients.

3.3.2. Adipogenesis

In basal growth conditions, MSCs from both OPLL and non-OPLL patients formed a monolayer, with no apparent spontaneous lipid vacuole formation (Fig. 3A). Following 21 days of adipogenic induction, light microscopy of Oil Red O-stained sections revealed the formation of lipid vacuoles in both groups. To quantify the staining intensity, bound Oil Red O dye was extracted and measured at 490 nm. The OD values were 0.30 ± 0.06 in the non-OPLL group and 0.27 ± 0.07 in the OPLL group (Fig. 3B).

3.3.3. Chondrogenesis

After 21 days of chondrogenic induction, MSCs from both groups formed semitranslucent pellets of an increased size owing

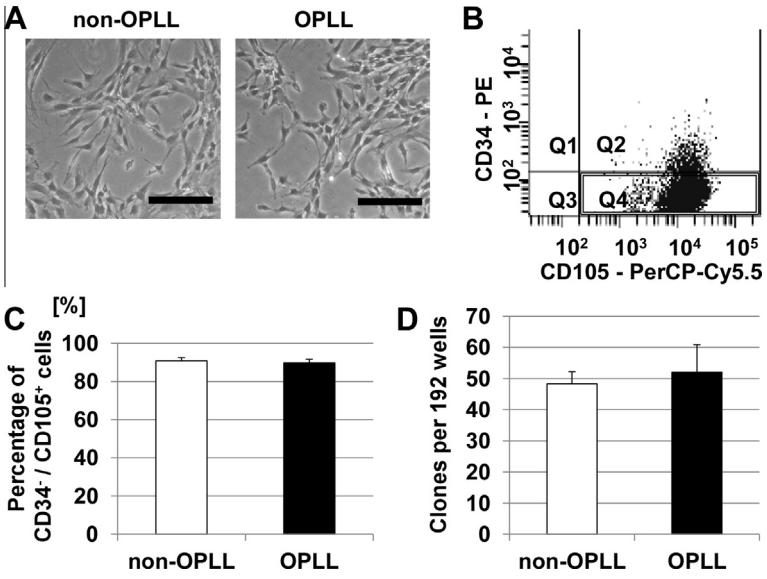

Fig. 1. (A) Phase contrast microscopic view of mesenchymal stem cells (MSCs) at passage 0. (B) Flow cytometric analysis of cell surface antigens. Cells inside the box (Q4) were negative for CD34 ($CD34^-$) and positive for CD105 ($CD105^+$). (C) Percentages of $CD34^-$ and $CD105^+$ cells. (D) Single cell cloning efficiency. Scale bars: 100 μm.

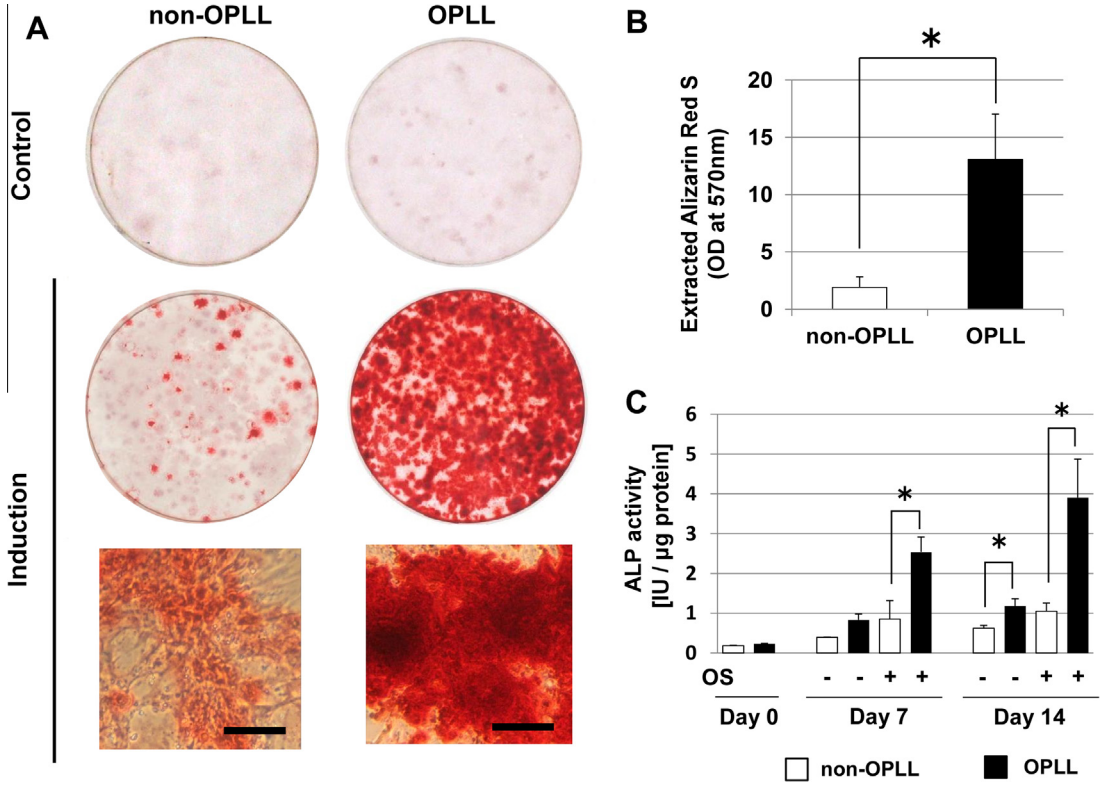

Fig. 2. Osteogenic differentiation potential. (A) Alizarin Red S staining was used to assess calcium deposition after 21 days in osteogenic induction medium. (B) Bound Alizarin Red S was dissolved and measured at 570 nm to quantify mineral content. (C) Alkaline phosphatase (ALP) activity was assessed at days 0, 7 and 14 under osteogenic induction media (OS+) and basal media (OS−). The *asterisk* indicates a significant difference; $P < 0.05$. Scale bars: 100 μm.

to production of extracellular matrix (Fig. 3C). Comparing the non-OPLL and OPLL groups, the diameters of pellets were 0.97 ± 0.07 mm and 0.99 ± 0.06 mm (Fig. 3E), and the weights were 0.49 ± 0.09 mg and 0.43 ± 0.05 mg (Fig. 3F), respectively. Pellets were then sectioned and stained with Alcian Blue to assess the degree of chondrogenesis between the groups. Alcian blue staining of pellets formed in basal medium was weak or negative, while clearly positive after 21 days in chondrogenic differentiation medium (Fig. 3D). Percentages of Alcian Blue staining per pellet were 42.7 ± 3.7% in the non-OPLL group and 38.3 ± 6.2% in the OPLL group (Fig. 3G).

3.4. Gene expression

The expression of osteogenic, adipogenic and chondrogenic-related genes during the course of lineage-specific differentiation

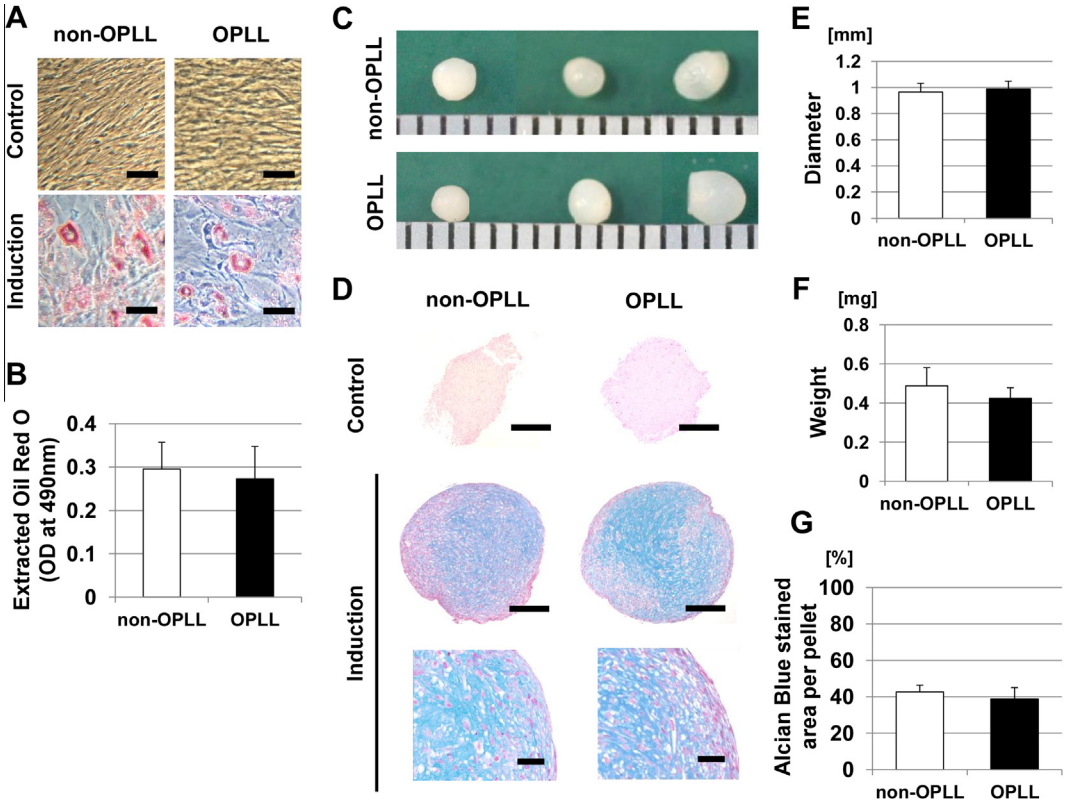

Fig. 3. Adipogenic and chondrogenic differentiation potential. (A) Oil Red O staining was used to assess lipid vacuole formation after 21 days in adipogenic induction medium. (B) Bound Oil Red O dye was extracted and measured the OD at 490 nm. Scale bars: 50 μm. (C) Representative pellet formation after 21 days culture in chondrogenic induction medium measured against a 0.5-mm scaled ruler. (D) Histological sections stained with Alcian blue. Scale bars: 200 μm. Higher magnifications of sections (Bottoms). Scale bars: 50 μm. (E) Diameter and (F) weight of the pellets were equivalent in cells from the two groups. (G) Percentages of Alcian Blue-positive areas per pellet.

was investigated. The mRNA levels of BMP2, Runx2, ALP, osteocalcin and osteopontin, known osteogenic markers, were all enhanced by osteogenic induction in both groups, as compared with expression in basal media (Fig. 4A–E; fold-change increase over control levels). In particular, BMP2 and ALP were higher in OPLL group than in the non-OPLL group at day 7, and Runx2 was higher on days 14 and 21 in OPLL group than in the non-OPLL group significantly. The mRNA levels of adipogenic markers PPARγ2 and LPL were also elevated under adipogenic conditions in both groups compared with control conditions (Fig. 4F and G). However, there were no significant differences in the expression of these two genes between the OPLL and non-OPLL groups under adipogenic conditions. Similarly, while the mRNA levels of the chondrogenic markers Sox9, COL2A1 and COL10A1 were elevated by chondrogenic induction, no significant difference was observed between OPLL and non-OPLL groups (Fig. 4H–J).

4. Discussion

In this study, we sought to assess the capacity of MSCs residing in the spinal ligament tissue to undergo lineage commitment. Using various assays, we clearly showed that MSCs harvested from the ligaments of patients with OPLL have an increased osteogenic differentiation potential as compared with MSCs from patients without evidence of ligament ossification. However, single cell cloning efficiency, adipogenic and chondrogenic differentiation potentials, and the expression levels of adipogenic- and chondrogenic-related genes were equivalent between the two groups. These results suggest that MSCs in the ligaments of patients with OPLL have a propensity toward becoming osteogenic cells.

As previously reported, one disadvantage of conventional MSC isolation techniques is the unavoidable contamination from hematopoietic and heterogeneous cells, including various progenitor and fibroblastic cells [16–18]. Indeed, the expression of CD34, a known hematopoietic cell marker, was small but not negligible because of this unavoidable contamination using the conventional method. $CD105^+$ cells have been reported to have a more homogeneous population of colony forming unit-fibroblasts and the capacity to form bone *in vivo* [19] as well as the potential to differentiate into cells of a chondrogenic lineage [20]. For these reasons, we selected $CD34^-/CD105^+$ cells from cells isolated by conventional MSC preparation for our experiments. From this point, cell sorting created a homogeneous population with which to rigorously evaluate the characteristics of MSCs.

Ectopic ossification is the process by which bone forms in soft tissues in response to injury, inflammation, or genetic diseases or through excess signaling, such as that which occurs when exogenous growth factors are supplemented at a wound site. However, despite numerous studies, the biological mechanism responsible for the development of ectopic ossification has not been fully elucidated. Perhaps the most extreme cases of ectopic ossification can be found in patients with FOP [21], which is induced by a combination of genetic mutations and acute inflammatory responses. Reportedly, aberrant stem cell differentiation can contribute to ectopic ossification in the onset of FOP as a result of activin-like kinase-2 mutations and the conversion of endothelial cells to cells of a mesenchymal-like phenotype [7]. In this study, the origin and localization of isolated MSCs in spinal ligament tissues was not determined, but one possibility is that MSCs were induced by through this mechanism of enhanced endothelial-mesenchymal conversion.

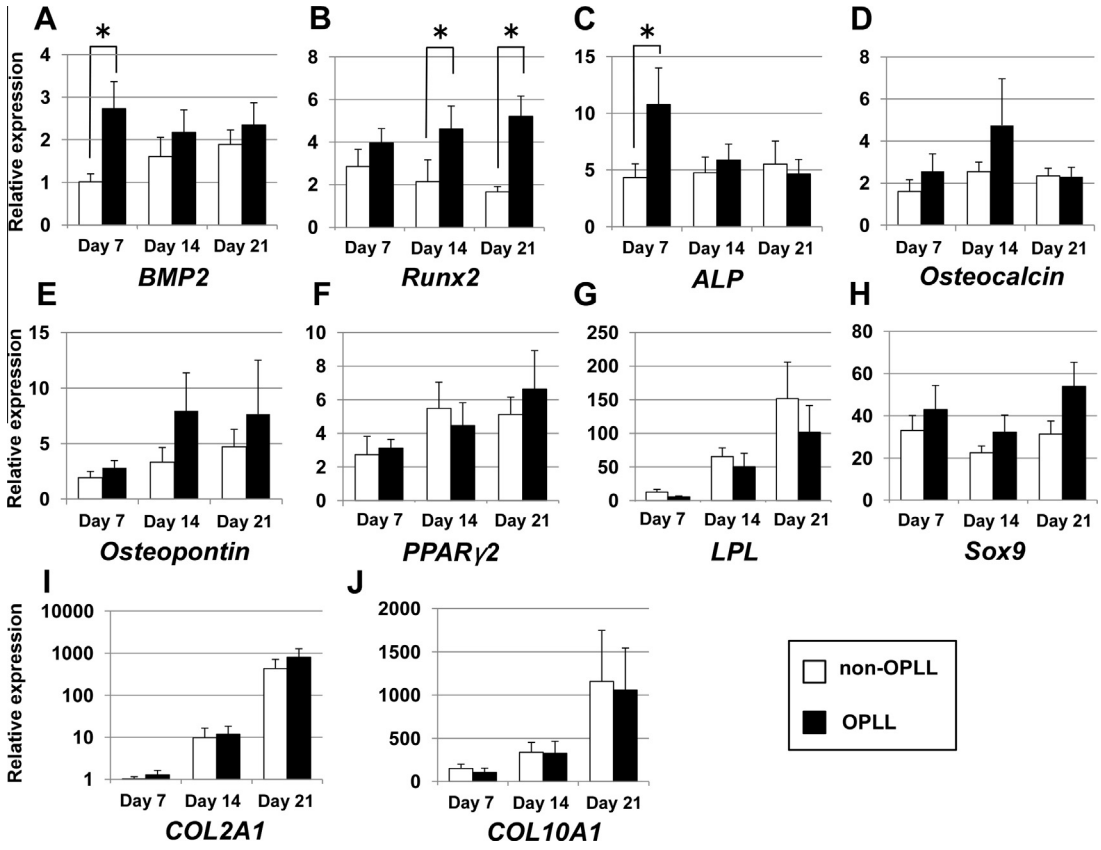

Fig. 4. Expression of osteogenic, adipogenic and chondrogenic genes. (A) BMP2, (B) Runx2, (C) ALP, (D) osteocalcin, and (E) osteopontin are osteogenic markers. (F) PPARγ2 and (G) LPL are adipogenic markers. (H) Sox9, (I) COL2A1 and (J) COL10A1 are chondrogenic markers. The *asterisk* indicates a significant difference between two groups at a particular each day; $P < 0.05$.

Putative osteoprogenitor cells have been identified by others in certain tissues such as traumatized muscle and aortic valves [9,22]. However, the process for the spontaneous phenotypic differentiation of MSCs into osteoprogenitor cells or osteoblasts has not been clearly elucidated. Some authors suggest that miRNA and epigenetic alterations regulate osteogenic differentiation of progenitor cells in muscle sites and *in vitro* [23,24]. We, too, hypothesize that genetic and epigenetics aspects are major factors controlling lineage commitment of MSCs.

One caveat of this study is that samples were harvested from non-ossified ligamentum flavum. However, as previously mentioned, OPLL patients have a tendency toward systemic ossification of numerous ligaments and soft tissues, thus it is likely that even MSCs obtained from non-ossified ligaments would exhibit the features of this pathological condition. There are some other limitations that should be noted. First, because of ethical reasons, control samples were not from healthy patients but from patients with cervical spondylotic myelopathy without evidence of ligament ossification. Second, this is an *in vitro* study. Some reports have suggested that *in vitro* mineral deposition does not correlate with *in vivo* bone formation [25,26]. Thus, this investigation should be followed up using an *in vivo* model to study the mechanism of osteogenic differentiation of MSCs that leads to ectopic ossification in a physiological system. Third, we used only two markers instead of more to sort the cell, as discussed above. And finally, the current study included a relatively limited number of tissue samples ($n = 8$) and is thus not adequately powered to perform all of the necessary statistical analyses.

In conclusion, this study indicates an increase in the osteogenic differentiation potential of MSCs from OPLL patients and the findings suggest that this propensity toward increased osteogenesis of these MSCs may be a causal factor for ossification in the ligaments of these patients.

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References

[1] S. Matsunaga, M. Kukita, K. Hayashi, et al., Pathogenesis of myelopathy in patients with ossification of the posterior longitudinal ligament, J. Neurosurg. 96 (2002) 168–172.

[2] K. Furukawa, Pharmacological aspect of ectopic ossification in spinal ligament tissues, Pharmacol. Ther. 118 (2008) 352–358.

[3] M. Mochizuki, A. Aiba, M. Hashimoto, et al., Cervical myelopathy in patients with ossification of the posterior longitudinal ligament, J. Neurosurg. Spine 10 (2009) 122–128.
[4] W.R. Stetler, F. La Marca, P. Park, The genetics of ossification of the posterior longitudinal ligament, Neurosurg. Focus 30 (2011) E7.
[5] D. Resnick, S.R. Shaul, J.M. Robins, Diffuse idiopathic skeletal hyperostosis (DISH): Forestier's disease with extraspinal manifestations, Radiology 115 (1975) 513–524.
[6] P.D. Utsinger, Diffuse idiopathic skeletal hyperostosis, Clin. Rheum. Dis. 11 (1985) 325–351.
[7] D. Medici, E.M. Shore, V.Y. Lounev, et al., Conversion of vascular endothelial cells into multipotent stem-like cells, Nat. Med. 16 (2010) 1400–1406.
[8] E.R. Nelson, V.W. Wong, P.H. Krebsbach, et al., Heterotopic ossification following burn injury: the role of stem cells, J. Burn Care Res. 33 (2012) 463–470.
[9] J.H. Chen, C.Y. Yip, E.D. Sone, et al., Identification and characterization of aortic valve mesenchymal progenitor cells with robust osteogenic calcification potential, Am. J. Pathol. 174 (2009) 1109–1119.
[10] T. Asari, K. Furukawa, S. Tanaka, et al., Mesenchymal stem cell isolation and characterization from human spinal ligaments, Biochem. Biophys. Res. Commun. 417 (2012) 1193–1199.
[11] S. Chin, K. Furukawa, A. Ono, et al., Immunohistochemical localization of mesenchymal stem cells in ossified human spinal ligaments, Biochem. Biophys. Res. Commun. 436 (2013) 698–704.
[12] V. Dexheimer, S. Mueller, F. Braatz, et al., Reduced reactivation from dormancy but maintained lineage choice of human mesenchymal stem cells with donor age, PLoS One 6 (2011) e22980.
[13] Y. Sakaguchi, I. Sekiya, K. Yagishita, et al., Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source, Arthritis Rheum. 52 (2005) 2521–2529.
[14] I. Sekiya, B.L. Larson, J.T. Vuoristo, et al., Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs), J. Bone Miner. Res. 19 (2004) 256–264.
[15] S.J. Bruce, N.C. Butterfield, V. Metzis, et al., Inactivation of Patched1 in the mouse limb has novel inhibitory effects on the chondrogenic program, J. Biol. Chem. 285 (2010) 27967–27981.
[16] S. Morikawa, Y. Mabuchi, Y. Kubota, et al., Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow, J. Exp. Med. 206 (2009) 2483–2496.
[17] Y. Jiang, B.N. Jahagirdar, R.L. Reinhardt, et al., Pluripotency of mesenchymal stem cells derived from adult marrow, Nature 418 (2002) 41–49.
[18] L. da Silva Meirelles, P.C. Chagastelles, N.B. Nardi, Mesenchymal stem cells reside in virtually all post-natal organs and tissues, J. Cell Sci. 119 (2006) 2204–2213.
[19] H. Aslan, Y. Zilberman, L. Kandel, et al., Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105+ cells, Stem Cells 24 (2006) 1728–1737.
[20] M.K. Majumdar, V. Banks, D.P. Peluso, et al., Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells, J. Cell. Physiol. 185 (2000) 98–106.
[21] E.M. Shore, F.S. Kaplan, Insights from a rare genetic disorder of extra-skeletal bone formation, fibrodysplasia ossificans progressiva (FOP), Bone 43 (2008) 427–433.
[22] W.M. Jackson, A.B. Aragon, J.D. Bulken-Hoover, et al., Putative heterotopic ossification progenitor cells derived from traumatized muscle, J. Orthop. Res. 27 (2009) 1645–1651.
[23] T. Oishi, A. Uezumi, A. Kanaji, et al., Osteogenic differentiation capacity of human skeletal muscle-derived progenitor cells, PLoS One 8 (2013) e56641.
[24] Z. Li, C. Liu, Z. Xie, et al., Epigenetic dysregulation in mesenchymal stem cell aging and spontaneous differentiation, PLoS One 6 (2011) e20526.
[25] K.H. Larsen, C.M. Frederiksen, J.S. Burns, et al., Identifying a molecular phenotype for bone marrow stromal cells with in vivo bone-forming capacity, J. Bone Miner. Res. 25 (2010) 796–808.
[26] P. Janicki, S. Boeuf, E. Steck, et al., Prediction of in vivo bone forming potency of bone marrow-derived human mesenchymal stem cells, Eur. Cell Mater. 21 (2011) 488–507.