

Original Article

Expression and role of osteopontin in ossification of ligamentum flavum

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Abstract: Ossification of ligamentum flavum is an ectopic ossification in the spinal ligament, which may lead to severe myelopathy. However, the exact pathogenesis has not been elucidated. Osteopontin (OPN), overexpressed in the ossified sites of different ossification diseases, is an essential protein mediating cell attachment during bone remodeling. The purpose of this study is to clarify the role and mechanism of OPN expression in ligamentum flavum cell (LFC) isolated from patient with normal ligamentum flavum (NLF) and patient with ossification of ligamentum flavum (OLF). The expression of alkaline phosphatase (ALP) is assessed by reverse transcriptase-PCR and Western blotting, while activation of mitogen-activated protein kinases (MAPK) ERK1/2, p38 and JNK are detected by Western blotting. The results show that OPN expression is higher in ossified human ligamentum flavum, and OPN treatment promotes the cell proliferation both in NLF-LFC and OLF-LFC. In OLF group, the expression level of ALP is dose-dependently increased by OPN treatment, which had no significant change in NLF group. MAPK pathway contributes importantly to OPN-induced mineralization, as revealed by the activation of p38, but not JNK or ERK1/2. By using SB203580, a p38 inhibitor, to pharmacologically inhibit this activation, which causes significantly lower ALP expression. Taken together, this study shows a possibility that OPN plays an important role in ossification of ligamentum flavum.

Keywords: Osteopontin, ligamentum flavum, MAPK, ossification

Introduction

Ossification of ligamentum flavum (OLF) is an ectopic ossification disease that may lead to spinal cord compression, but identifying pathogenesis still needs further effort. However, the incidence of OLF is strongly correlated with ethnicity [1], stress [2], and inflammation [3]. As the molecular mechanism of OLF is complicated, researchers have done considerable numbers of studies that shows that BMP-2 (bone morphogenetic protein 2), TGF-Beta1 (Transforming growth factor beta 1) [4], Runx-2 (Runt-related transcription factor 2) [5], IGF-I (Insulin-like growth factor-I) [6] and Leptin [7] are believed to be involved in the progress of OLF. It is difficult to identify the specific roles of the factors above in the pathogenesis of OLF.

Ossification of ligamentum flavum progresses by cell-mediated endochondral ossification [8], and bone remodeling, which plays an important

role in endochondral ossification and relies on the attachment of osteoblasts and osteoclasts to bone matrix. Osteopontin (OPN), which mediates attachment of cells to bone matrix [9], is upregulated during the ossification process of ligamentum flavum [10]. Therefore, OPN is considered as a critical factor in the regulation of OLF.

OPN is generally regarded as an indicator of ossification in many studies, but the osteogenic effect of osteopontin in OLF has not been reported. Our study is targeted to investigate the expression and role of OPN in ossification of ligamentum flavum.

Materials and methods

Agents

Following primary antibodies were used: Anti-Vimentin (Abcam Inc., Cambridge, MA, USA),

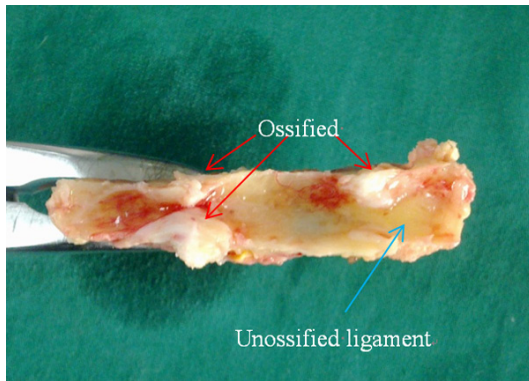


Figure 1. A sample of OLF patient dissected during surgery, consists of ossified area (red arrow) and unossified area (blue arrow). We cultured cells derived from the unossified areas.

Anti-COL1 (Abcam Inc, Cambridge, MA, USA), Anti-Osteopontin (Abcam), Anti-Integrin (Abcam), Anti-CD44 (Abcam), Anti-ALP (Santa Cruz biotechnology, CA, USA), Anti-osteocalcin (Santa Cruz). U0126 and SB203580 were from Merck & Co., Inc. (Darmstadt, Germany).

Specimens

All the specimens were obtained from Shanghai Changzheng Hospital, and the specimens were divided into 2 groups as OLF (ossification of ligamentum flavum) and NLF (normal ligamentum flavum). Each group had 8 cases, and the average age in OLF is 50.13 ± 7.57 , of which in NLF group was 48.88 ± 9.67 . All patients had been informed and consent was obtained. First, ligaments obtained from surgery were irrigated with normal saline, and surrounding tissue was removed with microsurgery scissors. Then, we put ligaments into sterile flasks full of ice-cold normal saline and transported them to lab in an ice box.

Histology

OPN, CD44, Integrin in OLF unossified tissue from the ossified segment were measured by immunohistochemistry.

Cell culture

The LF cells were obtained by tissue explant method. The ligaments were extirpated carefully from the unossified areas (**Figure 1**) to avoid contamination with osteogenic cells. The ligaments were minced into 1-mm^3 pieces, and the fragments were washed and explanted

onto 100-mm diameter dish in 8 mL of Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, New York, U.S.) and supplemented with 10% fetal bovine serum qualified, 1% penicillin/streptomycin (hycline) at 37°C in humidified atmosphere of 95% air/5% CO_2 . The cells derived from explants were removed from the dish with 0.02% EDTA, 0.05% trypsin for passage.

Immunocytochemistry

The ligament cells were placed at a density of $5 \times 10^4/\text{well}$ in six-well plates. After an one-day culturing, cells were irradiated 3 times with phosphate-buffered saline (PBS) for a period of 5 minutes, followed by fixation with 4% paraformaldehyde for 15 min, permeabilized with 0.3% TritonX-100 for 30 min, blocked for non-specific binding by incubation in 3% BSA for 30 min at 20°C . Then, they were respectively covered with Anti-OPN antibody, Anti-CD44 antibody, Anti-Integrin antibody overnight at 4°C . They were then incubated with a HRP secondary antibody, and finally with an avidin-biotin peroxidase complex for 20 min at room temperature. Color was developed when using diaminobenzidine (DAB) reaction. Semi-quantitative analysis method was used for classification of results: negative (-) means no staining is observed; positive (+) means staining could be observed; and strongly positive (++) means cells are deeply stained.

Immunofluorescence staining

The ligament cells were placed at a density of $5 \times 10^4/\text{well}$ in six-well plates. After an one-day culturing, cells were irradiated 3 times with phosphate-buffered saline (PBS) for a period of 5 minutes, followed by fixation with 4% paraformaldehyde for 15 min and 0.3% permeabilized TritonX-100 for 30 min. Then, they were blocked for non-specific binding by incubation in 3% BSA for 30 min at 20°C and were respectively covered with Anti-OPN antibody, Anti-CD44 antibody, Anti-Integrin antibody overnight at 4°C . They were later incubated with an HRP-fluorescence secondary antibody for 40 min and observed under fluorescence microscope.

CCK-8 assay

Cell proliferation was measured by CCK-8 assay. Namely, cells were seeded into 96-well plates overnight and exposed to the OPN at dif-

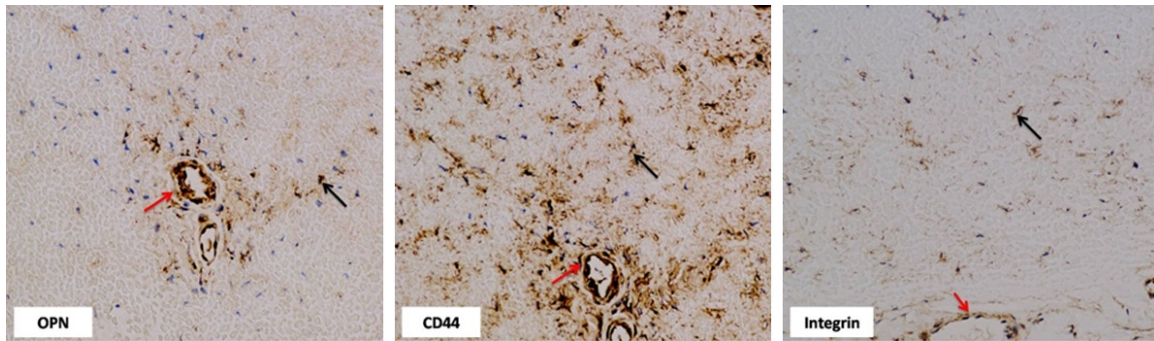


Figure 2. OPN, CD44, integrin in OLF ligaments were deep stained (black arrow), especially around the vessels.

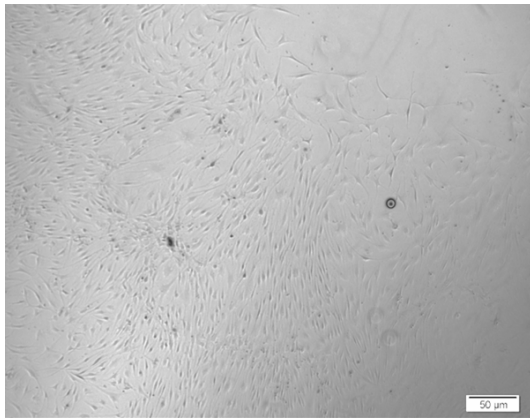


Figure 3. OLF-LFC (4 × 10): The shape of LFCs was mainly fibroblast-like, seldom polygonal or oval. Small nodules (black arrow) could be observed.

ferent concentrations for multiple times. After the medium was replaced, the cells were incubated with 10 uL CCK-8 solution for 4 h at 37°C. After removal of CCK-8, the absorbance was measured at 450 nm using a microplate reader.

RT-PCR

Total RNA was extracted from the cell monolayers using TRIzol reagents (Invitrogen). mRNA expression of various genes was examined by reverse transcription (RT)-PCR.

For PCR amplification, specific oligonucleotide primers were designed as following-Actin sense: 5'-CAC CCA GCA CAA TGA AGA TCA AGA T-3', and anti-sense: 5'-CCA GTT TTT AAA TCC TGA GTC AAG C-3', length 317; OPN sense: 5'-GAG GAA AAG CAG ACC CTT CCA-3', and anti-sense: 5'-ACG GCT GTC CCA ATC AGA AG-3', length 456; CD44 sense: 5'-AGT GAA CAG AAC TGC ACC AGC-3', and anti-sense: 5'-TCC TCC AGC

CAA TCA GTG ATC-3', length 181; Integrin sense: 5'-CTG TG C TAT CCC TGT ACG CCT CTG-3', and anti-sense: 5'-GTG ATC TCC TTC TGC ATC CTG TCG-3', length 200; OCN sense: 5'-AGG GCA GCG AGG TAG TGA AG-3', and anti-sense: 5'-TCC TGA AAG CCG ATG TGG TC-3', length 151. RT-PCR System total of 25 μl includes NA sample solution 1 μl, upstream and downstream of each primer, 0.5 μl (pmol/μL), dNTP1.0 μl (10 mmol/L), Taq enzyme 0.5 μl (2 U/μl), and 10 × Buffer (containing MgCl₂) 2.5 μL. PCR conditions consist of initial denaturing at 95°C for 2 min, 30 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 50 s, and a final extension at 72°C for 7 min. The PCR products were separated in a 2.5% agarose gel and visualized by staining with FastStart Universal SYBR Green Master (Rox). The SYBR Green fluorescence was converted into a TIFF image by a 7900HT machine. Relative expression is estimated by using β-actin as an internal control.

Western blot

LFCs were washed with cold PBS and lysed with RIPA lysis buffer. Cell lysates were separated by 10% SDS-PAGE for protein analyses and subsequently electrotransferred onto polyvinylidene difluoride membranes. The membrane blots were probed with a primary antibody overnight at 4°C and incubated with appropriate horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature, then visualized by enhanced chemiluminescent kit (Thermo, USA). Signals were visualized on The ChemiDoc MP (Bio-Rad, USA).

Statistical method

Ridit analysis was used for the difference of immunohistochemistry staining intensity. Other

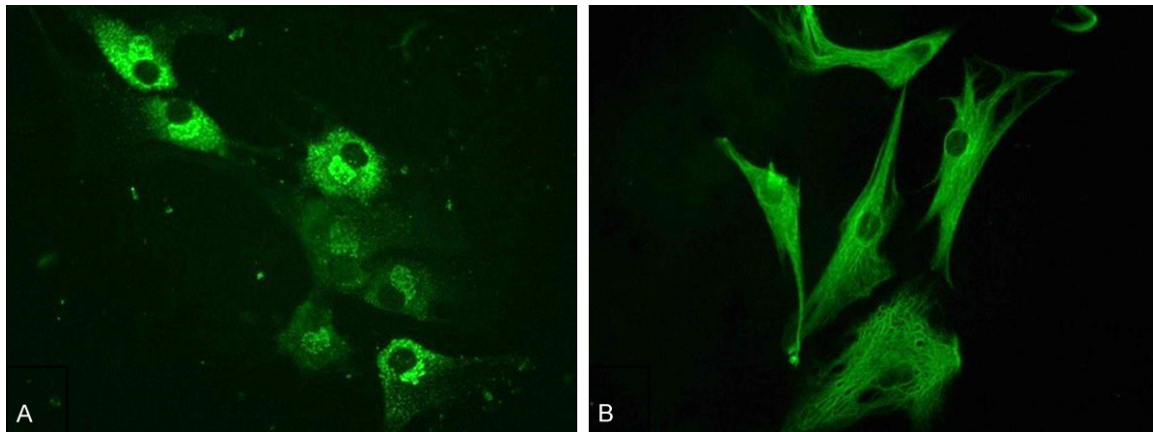


Figure 4. A. Positive immunofluorescence staining of COL-I. B. Positive immunofluorescence staining of Vimentin.

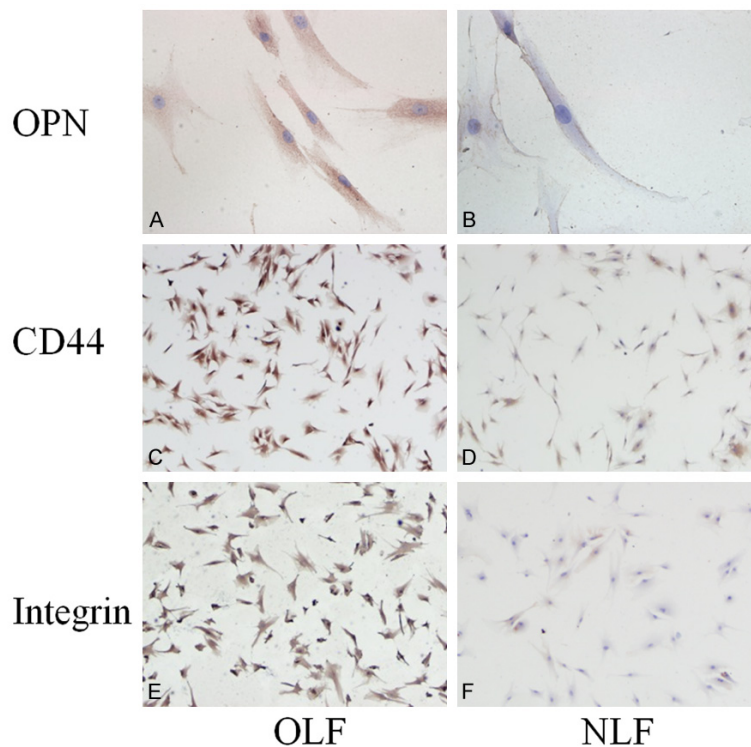


Figure 5. A. In OLF-LFC, nuclei were blue stained and the cytoplasm was deep brown stained for OPN. B. In NLF-LFC, nuclei and cytoplasm was blue stained and the cytoplasm was deep brown stained for OPN. C. In OLF-LFC, nuclei were blue stained and the cytoplasm was deep brown stained for CD44. D. In NLF-LFC, nuclei was blue stained and the cytoplasm was light yellow stained for CD44. E. In OLF-LFC, nuclei were blue stained and the cytoplasm was deep brown stained for Integrin. F. In NLF-LFC, nuclei was blue stained and the cytoplasm was light yellow stained for Integrin.

data were expressed as the mean \pm SEM. ANOVA with the Dunnett type test was used for multiple comparisons against control. $P < 0.05$ is considered significant. The OD values of

CCK-8 were analyzed with SNK test. $P < 0.05$ is considered significant.

Results

Expression of OPN, CD44, Integrin were found in OLF ligament

The study showed increasing OPN expression in the ossification areas [10]. Thus, we used immunohistochemistry to investigate the expression of OPN, CD44, and Integrin in OLF ligaments. The result presented that all proteins were distinctively stained (**Figure 2**).

Cell culture

A total of 16 cases of LFC were harvested: eight cases each in OLF group and NLF group. Cells were crawled out in 12-21 days, 14.5 ± 3 d in OLF group while 12.4 ± 1.8 d in NLF group. And there was no significant difference between two groups ($t=1.696$, $P=0.11 > 0.05$). The shape of LFCs was mainly fibroblast-like, seldom polygonal or oval. OLF-LFCs had a few more polygonal and oval cells than NLF-LFCs. A few small nodules were scattered in OLF-LFC (**Figure 3A**).

Table 1. Expression of OPN

Group	Cases	-	+	++	R
NLF	8	7	1	0	0.289
OLF	8	1	1	6	0.711

$u=-5.109$, $P < 0.001$

In NLF group, OPN staining had 7 negative cases, 1 positive case, and 0 strong positive case, $R=0.289$; in OLF group, OPN staining had 1 negative case, 1 positive case, and 6 strong positive case, $R=0.711$. The difference between the two groups is significant ($u=-5.109$, $P < 0.001$).

Table 2. Expression of CD44

Group	Cases	-	+	++	R
NLF	8	3	4	1	0.271
OLF	8	0	2	6	0.7

$u=-4.755$, $P < 0.001$

In NLF group, CD44 staining had 3 negative cases, 4 positive cases, and 1 strong positive case, $R=0.271$; in OLF group, CD44 staining had 0 negative cases, 2 positive cases, and 6 strong positive case, $R=0.7$. The difference between the two groups is significant ($u=-4.755$, $P < 0.001$).

Table 3. Expression of integrin

Group	Cases	-	+	++	R
NLF	8	2	5	1	0.305
OLF	8	0	1	7	0.695

$u=-4.204$, $P < 0.005$

In NLF group, Integrin staining had 2 negative cases, 5 positive cases, and 1 strong positive case, $R=0.305$; in OLF group, Integrin staining had 0 negative cases, 1 positive cases, and 7 strong positive case, $R=0.695$. The difference between the two groups is significant ($u=-4.204$, $P < 0.005$).

In order to determine whether the isolated cells were ligamentum flavum cells, we stained type I collagen (COL-I) and Vimentin with immunofluorescence staining (**Figure 4**). As the ligamentum flavum is derived from embryonic mesoderm [11], and retains the feature of mesenchymal cell. COL-I is the primary extracellular matrix of fibroblast, and Vimentin is main component of cytoskeleton in mesenchymal cells. With both positive stained of two proteins and the fibroblast-like shape, we preliminarily considered the cells as LFC.

The expression of OPN, CD44, and Integrin in OLF-LFC is higher than that in NLF-LFC

Due to the foregoing experiments, we found increased expression of OPN and its receptors in ossification tissue. In order to detect whether

this increased expression was from LFCs, we performed immunocytochemistry (ICC) staining and PCR on LFCs.

By ICC staining results, we found the staining of OPN, CD44, integrin in OLF-LFC were mainly dark brown. While in NLF-LFC, OPN stain stayed blue, CD44 and integrin stain were light yellow (**Figure 5**). By RT-PCR, mRNA expression intensity of OPN, CD44 and Integrin in each sample was estimated. The intensity of all three indexes in OLF-LFC was higher than that in NLF-LFC (**Tables 1-3**). The difference of expression intensity between two groups was significant according to ridit analysis. (OPN: $u=-5.109$, $P < 0.001$; CD44: $u=-4.755$, $P < 0.001$; Integrin: $u=-4.204$, $P < 0.005$).

The relative expression of each mRNA has been presented in **Figure 6**. Expression of OPN, CD44 and Integrin in OLF group significantly increased in comparison with that in NLF group (OPN: $t=7.644$, $P < 0.05$, CD44: $t=9.216$, $P < 0.05$, Integrin: $t=12.397$, $P < 0.05$).

Effect of OPN on OLF-LFCs and NLF-LFCs

ALP and OCN expression was determined by PCR, WB, and other methods. Meanwhile, in OLF-LFCs, OPN increased ALP expression in a concentration-dependent manner (**Figures 8 and 9**). In order to investigate the role of OPN in the process of OLF, we designed a proliferation experiment with CCK-8 assay. Both OLF-LFCs and NLF-LFCs were exposed to rhOPN at the concentration of 1 ng/ml, 10 ng/ml and 100 ng/ml for different time as 24 h, 48 h, 72 h, and CCK-8 (Dojindo, Japan) was performed as described under "Experimental Procedures". **Figure 7** shows that OPN stimulated the proliferation of both OLF-LFCs and NLF-LFCs. Meanwhile, the RT-PCR results shows that OPN could increase the expression of ALP in OLF-LFCs, not in NLF-LFCs (**Figure 8**). But the OPN treatment had no significant effect on OCN expression (**Figure 8**). In agreement with RT-PCR experiments, Western blotting demonstrated that the protein expression of ALP was elevated in response to OPN stimulation in OLF-LFCs, but not in NLF-LFCs. However, OPN had no significant effect on OCN expression. (**Figures 8 and 9**).

Molecular events involved in OPN-stimulated osteogenesis in OLF-LFCs

The local function of OPN is activated by binding to the receptors [9]. The activation of the

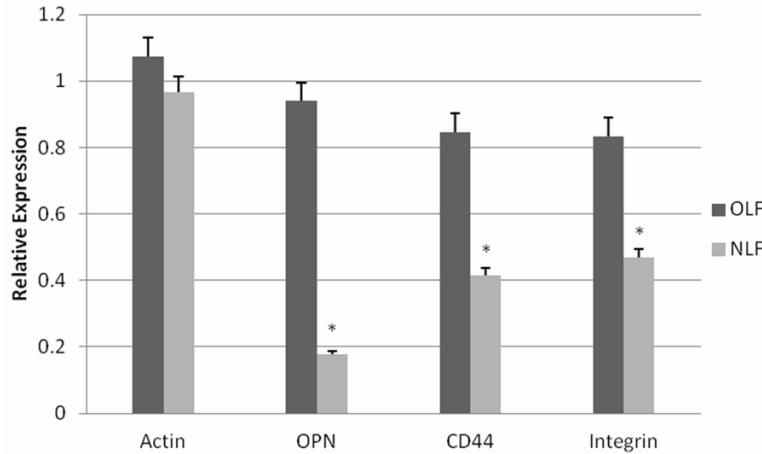


Figure 6. Expression of OPN, CD44 and Integrin in OLF group was significantly increased in comparison with that in NLF group. *Significantly different from OLF group (OPN: $t=7.644$, $P < 0.05$, CD44: $t=9.216$, $P < 0.05$, Integrin: $t=12.397$, $P < 0.05$).

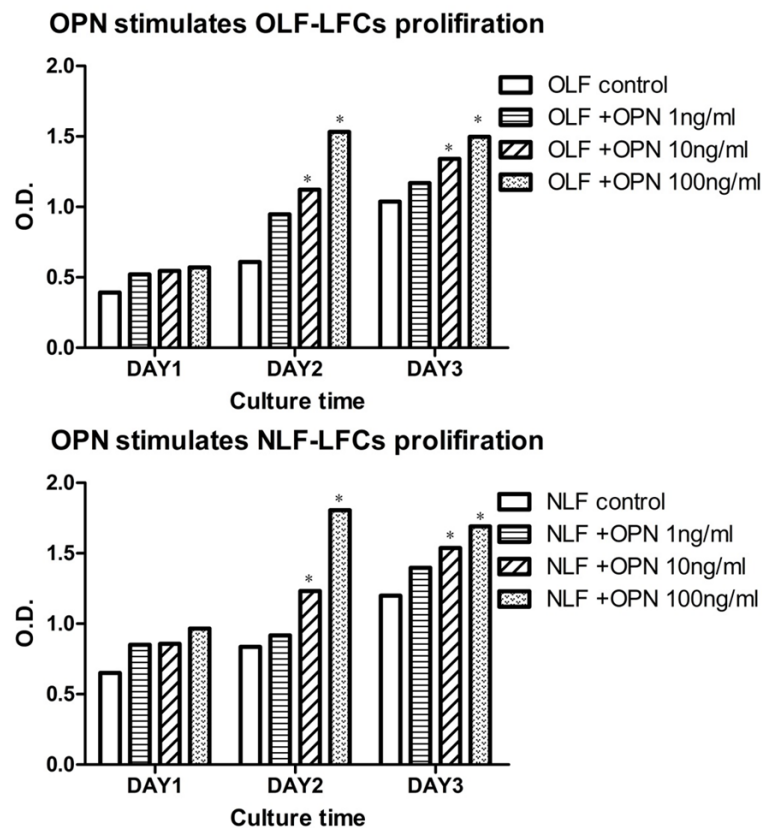


Figure 7. OPN treatment significantly stimulated proliferation of both OLF-LFCs and NLF-LFCs, and the effect was both dose- and time-dependent. ($P > 0.05$).

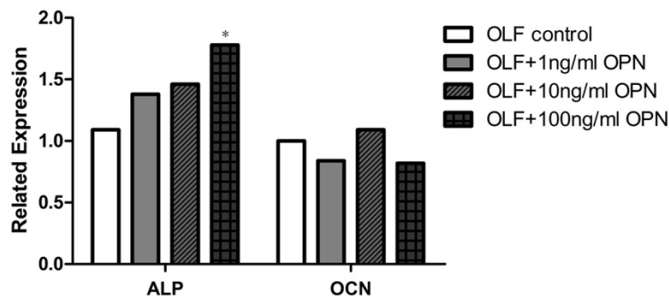
receptors may on the phosphorylation of ERK1/2, JNK, and p38 [12]. We evaluated the expression and phosphorylation of ERK1/2,

JNK, p38 in the OLF-LFCs stimulated with OPN. Results (**Figure 10**) showed high expression of ERK and p38, and it had a time-dependent increase. However, the expression and phosphorylation of JNK turned out low. To investigate whether activation of ERK1/2 and p38 signaling pathways are indeed linked to osteogenic effect of OPN in OLF-LFC, we used selective inhibitors to block phosphorylation of ERK1/2 and p38 and detected the expression of ALP by RT-PCR under the treatment of OPN. Treatment of OLF-LFCs with ERK1/2 phosphorylation-selective inhibitor U0126 and p38 phosphorylation-selective inhibitor SP-204580 selectively inhibit the phosphorylation of ERK1/2 and p38. Measurement of ALP expression indicated that blocking phosphorylation of ERK1/2 only had marginal effects on ALP expression, whereas blocking p38 phosphorylation resulted in a significant reduction of OPN-induced ALP expression but not OCN (**Figure 11**), suggesting that p38 signaling pathway is the major mediator for the osteogenic effect of OPN in OLF-LFCs.

Discussion

The expression of OPN is found in bone cells, osteoblasts, osteoclasts, chondrocytes, fibroblasts, smooth muscle cells, etc. And these cells may also be major sources of OPN in OLF process [13]. Sodek et al [14] found that OPN protein in extract of porcine long bone and calvaria increased with mineral density under X-ray photography. In our study, the expression of OPN, CD44 and Integrin was found around the vessels by histological analy-

ALP and OCN expression after OPN treatment on OLF-LFCs



ALP and OCN expression after OPN treatment on NLF-LFCs

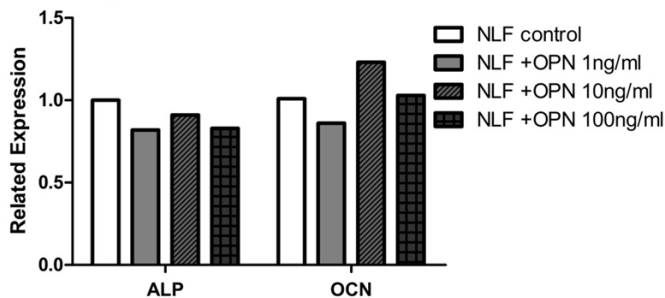


Figure 8. Cells were induced by 100 ng/ml OPN for 48 h, followed by measurement of ALP and OCN mRNA expression with RT-PCR. Panels show that OPN could increase the expression of ALP in OLF-LFCs but not in NLF-LFCs, but the OPN treatment had no significant effect on OCN expression both in OLF-LFCs and NLF-LFCs. The asterisks indicate statistical significance ($P < 0.05$).

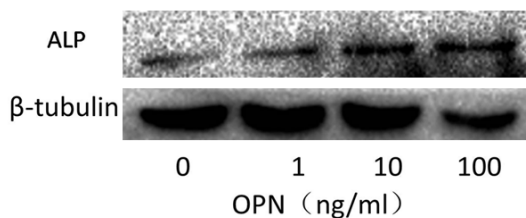


Figure 9. The ALP protein in OLF-LFCs was estimated by Western-Blot test, after cells were induced by 100 ng/ml OPN for 48 h. ALP protein had a dose-dependent increased.

sis in the unossified ligaments from OLF patients (**Figure 2**). To confirm the source of OPN, OLF-LFCs were stained by immunocytochemistry and immunofluorescence assays, which showed that OPN, CD44, integrin were strongly stained while in NLF-LFCs the proteins were only mild stained. Results confirmed that the secretion of OPN is increased in ossifying area, which means OPN plays an important role in bone remodeling.

Since the expression of OPN and its receptors were increased with the process of ossification,

the exact function of OPN appears intriguing.

One of the functions of OPN may be proliferation-promoting. Proliferation of LFC is a basic progress in ligamentum flavum hypertrophy, which is necessary in OLF. We found that the LFC proliferation was significantly promoted with OPN treatment, and the increase was dose- and time-dependent.

After OPN treatment, we detected the ALP expression with RT-PCR and Western-blot in OLF-LFCs, which was significantly increased. ALP is a homodimeric glycoprotein and is considered as an indicator of bone formation, which can anchor to osteoblast cells membrane by glycosyl-phosphatidylinositol (GPI). During bone formation, ALP's functions are various. On the one hand, ALP can hydrolyze

organophosphate into inorganic phosphorus, which provides phosphate for hydroxyapatite (HA). On the other hand, ALP can hydrolyze inhibitors of crystal growth such as pyrophosphate and ATP. Genge et al [15] also reported that the ALP expression is an early indicator of the reaction cell osteogenic differentiation with high sensitivity and specificity.

Our experiment proved that OPN could promote the progress of OLF by increasing the expression of ALP, but in the normal ligamentum flavum, the OPN treatment was not effective. MAPK plays an important role in the regulation of cell osteogenic differentiation [16-18]. By binding to CD44 and integrin receptors, OPN can initiate several intracellular signaling pathways, including MAPK signaling pathway. Our study supported OPN-induced activation of MAPK in OLF-LFCs. First, OPN treatment induced the phosphorylation of ERK1/2 and p38, but had not effect on JNK. Second, SB203580, a p38 specific inhibitor, blocked OPN-induced expression of ALP in OLF-LFCs. These discoveries illustrated that OPN induced p38 MAPK in OLF-LFCs. Previous studies had

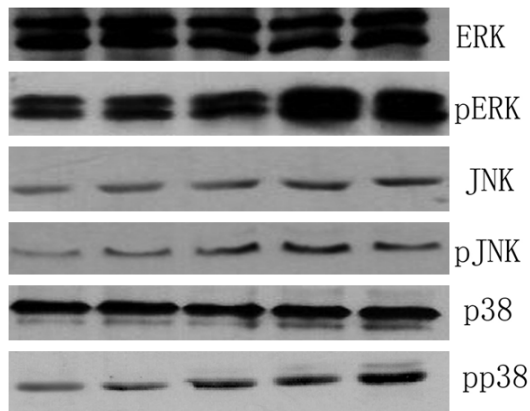


Figure 10. OLF-LFCs were treated with 100 ng/ml rhOPN for the time indicated: 0 min, 15 min, 30 min, 60 min and 2 h. The phosphorylation of ERK1/2, P38 and JNK was examined by Western-Blot. The pictures showed that the expression of pERK1/2 and pp38 were time-dependent increased, while the OPN treatment did not affect the expression and phosphorylation of pJNK in OLF-LFCs. Thus, OPN treatment could activate the phosphorylation of ERK1/2 and p38 but not JNK.

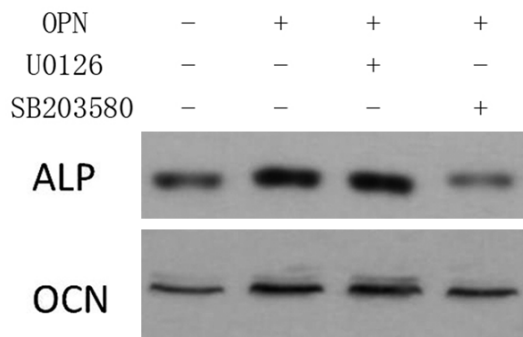


Figure 11. Effects of U0126 (ERK inhibitor) and SB203580 (p38 inhibitor) on rhOPN-induced osteogenic differentiation of OLF-LFCs. LFCs were cultured for 10 days in the presence or absence of rhOPN (100 ng/ml) combined with or without U0126 or SB203580 at 20 μ M. The expression of ALP and OCN protein were measured.

shown that OPN induced the phosphorylation of ERK1/2 and p38 in HEC-1A cells [19] and activates p38 in human umbilical vascular smooth muscle cells [20], which is consistent with our result.

The activation of MAPK signaling is involved in osteogenic differentiation of LFCs induced by tensile stress and other factors, which are considered as the causes of OLF. However, whether MAPK pathway effects on OPN-induced LFCs, differentiation needs further studies. In our

experiments, the p38 inhibitor (SB203580) inhibited significantly OPN-induced increase in ALP expression, which suggests that OPN-induced osteogenic differentiation of LFCs is mediated by the activation of p38.

In conclusion, we found that OPN may cause hypertrophy of ligamentum flavum by promoting cell proliferation. Simultaneously, inhibiting the phosphorylation of p38 could decrease the expression of ALP in OLF-LFCs, which means OPN has an essential role in the OLF process.

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Disclosure of conflict of interest

None.

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