Upregulated expression of PERK in spinal ligament fibroblasts from the patients with ossification of the posterior longitudinal ligament

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Abstract

Purpose Molecular mechanism of ossification of the posterior longitudinal ligament (OPLL) remains unclear. This study was to investigate different expressions of PERK between the spinal ligament fibroblasts from OPLL patients and non-OPLL patients, and demonstrate knock-down of PERK protein expression by RNA interference inhibiting expression of osteocalcin (OCN), alkaline phosphatase (ALP), and type I collagen (COL I) in the cells from OPLL patients.

Methods Spinal ligament cells were cultured using tissue fragment cell culture and identified by immunocytochemistry and immunofluorescence. The mRNA expression of osteoblast-specific genes of OCN, ALP and COL I was detected in the cells from OPLL and non-OPLL patients by semiquantitative reverse transcription-polymerase chain reaction. The protein expression of PERK was detected by Western blotting. And then, after 72 h, when RNA interference against PERK was performed on the cells from OPLL patients, expression of the osteoblast-specific genes was compared again between the transfection group and non-transfection group.

Results Spinal ligament fibroblasts were observed 7–10 days after cell culture. Immunocytochemistry and

immunofluorescence exhibited positive results of vimentin staining. The mRNA expressions of OCN, ALP and COL I and protein expression of PERK in the cells from OPLL patients were significantly greater than those from non-OPLL patients. In addition, knockdown of PERK protein expression inhibited the mRNA expressions of OCN, ALP and COL I remarkably in the transfection group compared with the non-transfection group, at 72 h after RNA interference targeting PERK was performed on the cells from OPLL patients.

Conclusions The cultured fibroblasts from OPLL patients exhibited osteogenic characteristics, and PERK-mediated ER stress might be involved in development of OPLL.

Keywords Ossification of the posterior longitudinal ligament · Fibroblast · Osteogenic differentiation · PERK · Endoplasmic reticulum stress

Introduction

Ossification of the posterior longitudinal ligament (OPLL) is a pathological condition causing ectopic bone formation in the spine ligament [3, 7, 14]. It is known that the presence of OPLL does not always indicate cervical myelopathy, and clinical symptoms may occur only when the ossified ligament develops to a certain degree [17]. Most researchers have agreed that the endochondral ossification process plays a major role in OPLL; however, during the development of hyperostotic OPLL, the intramembranous ossification process might also play some role. These two processes might differ in terms of the speed of bone formation [6, 31]. Although several studies have investigated the osteogenetic characteristic of the spinal ligament

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fibroblasts from OPLL patients, the intracellular signaling pathways still remain unclear [4, 8, 9, 12, 24, 28].

PERK, a single-pass type I endoplasmic reticulum membrane protein kinase, is a major transducer of the endoplasmic reticulum (ER) stress response. It contains a luminal regulatory domain and cytoplasmic eIF2 α kinase domain that is conserved among the four-member family of kinase that regulate translation initiation in mammals [5, 22]. PERK is negatively regulated by the ER chaperones 78 kDa glucose-regulated protein/Immunoglobulin heavy chain-binding protein (GRP78/BIP) and 94 kDa glucose-regulated protein (GRP94). The high concentration of calcium within the ER lumen promotes binding of PERK to these ER chaperones and inhibits PERK's dimerization and activation [12, 13]. However, PERK is activated during ER stress when the folding capacity of the ER is compromised by Ca²⁺ depletion or when the protein load exceeds the capacity of the ER chaperones to regulate proper folding [26].

Recently, such PERK-mediated ER stress response has been proved to be very important in the neonatal skeletal development and osteoblast proliferation and differentiation [21, 25]. Because the mechanism of development of OPLL is known to be similar to that of normal skeletal development, expression of PERK in the spinal ligament fibroblasts derived from OPLL patients is particularly likely to be upregulated and plays an important role in the signal transmission during development of OPLL.

To explore this possibility, expression of PERK between the spinal ligament cells from OPLL patients and non-OPLL patients was evaluated via Western blotting in this study. In addition, we performed RNA interference, targeting PERK in the cells from OPLL patients, and then detected the messenger RNA (mRNA) expression changes of osteoblast-specific genes including osteocalcin (OCN), alkaline phosphatase (ALP) and type I collagen (COL I) in the transfection group compared with those in the non-transfection group. In the literature, the expression of OCN demonstrates osteogenesis, ALP offers phosphonic acid for the deposition of hydroxyapatite crystals, and COL I is essential for the appropriate bone formation and its strength. These three marker genes may present different stages for bone formation [3, 27].

Materials and methods

Patients

This study was approved by the Ethics Committee of Second Military Medical University, and informed consent was obtained from each patient. Between January 2012 and June 2012, 16 consecutive patients presenting with OPLL

who underwent anterior cervical decompression surgery were enrolled in this study. The diagnosis of OPLL was confirmed by X-ray photographs, computer tomography (CT), magnetic resonance imaging (MRI) of the cervical spine. X-ray provided a general view of the cervical spine, and significant OPLL could be viewed on the lateral image. Three-dimensional (3D) reconstructions were obtained by use of the CT console at 0.5-mm intervals from the 0.75-mm scan slice data. Bone window CT images were used to construct 3D CT images, which provided enough information about OPLL, including thickness, extent, type, and even OPLL in the early stage. MRI provided more information about compression of the spinal cord. The patients included nine males and seven females, with a mean age of 47.3 ± 6.8 years. At the same period, another 16 patients with non-OPLL (10 of cervical trauma and 6 of cervical disc herniation) were selected for the control group. Controls were collected on age-matched and sex-matched controls (9 males and 7 females, mean age: 49.2 ± 7.2 years). Demographics of these patients were provided in Table 1. No patients were administrated on Didronel or other related medications.

In vitro culture of primary ligament fibroblasts using tissue fragment cell culture

During the anterior cervical decompression surgery, 32 posterior longitudinal ligament specimens from 16 OPLL patients and 16 non-OPLL patients were harvested. The ligaments were extirpated carefully from a non-ossified site to avoid any possible contamination with osteogenic cells. After mincing into approximately 0.5 mm³ pieces and washed twice with phosphate-buffered saline (PBS), the ligament fragments were plated in 90-mm culture dishes and maintained in low-glucose Dulbecco's modified Eagle's media (DMEM) supplemented with 10 % FBS. The explants were incubated in a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C. The cells derived from the explants were harvested from the dishes with 0.05 % trypsin for further passages. Inverted phase contrast microscopy was used to observe cell morphology.

Cell identification

Hematoxylin–eosin (HE) staining

Cell morphology was observed via conventional HE staining [29]. Third passage cells were seeded on a microscopic glass in 6-well plates at a density of 10³ cells/well. After cultures reached confluence, the cells were stained according to conventional HE staining protocol and then photographed using an inverted phase contrast microscope.

Table 1 Tissue samples used in this study, including the clinical diagnosis, patient gender, age, and origin of each

OPLL				Non-OPLL			
Code	Diagnosis/type	Sex/age (years)	Tissue	Code	Diagnosis	Sex/age (years)	Tissue
1	OPLL/segmental	M/45	PLL	1	Cervical fracture	M/52	PLL
2	OPLL/local	M/60	PLL	2	Cervical fracture	F/56	PLL
3	OPLL/local	M/36	PLL	3	Cervical fracture	M/65	PLL
4	OPLL/local	F/56	PLL	4	CDH	M/44	PLL
5	OPLL/segmental	F/44	PLL	5	Cervical fracture	F/44	PLL
6	OPLL/continuous	M/44	PLL	6	Cervical fracture	M/46	PLL
7	OPLL/segmental	M/42	PLL	7	Cervical fracture	M/44	PLL
8	OPLL/local	F/54	PLL	8	CDH	F/48	PLL
9	OPLL/segmental	M/48	PLL	9	Cervical fracture	M/54	PLL
10	OPLL/local	M/44	PLL	10	CDH	F/42	PLL
11	OPLL/local	F/46	PLL	11	Cervical fracture	F/44	PLL
12	OPLL/local	F/37	PLL	12	Cervical fracture	M/46	PLL
13	OPLL/continuous	M/44	PLL	13	CDH	M/56	PLL
14	OPLL/mixed	F/56	PLL	14	Cervical fracture	F/39	PLL
15	OPLL/local	M/50	PLL	15	CDH	M/60	PLL
16	OPLL/segmental	F/52	PLL	16	CDH	F/47	PLL

OPLL ossification of the posterior longitudinal ligament, CDH cervical disc herniation, PLL posterior longitudinal ligament, M male, F female

Immunocytochemistry and immunofluorescence

Cells were characterized via immunocytochemistry/immunofluorescence with vimentin, which was a type of intermediate filament that mainly resides in fibroblasts, and had been a routine identification marker of fibroblasts [20]. Third passage cells were seeded on microscopic glasses in 6-well plates at a density of 10^3 cells/well. After cultures reached confluence, the cells were washed thrice with cool PBS for 10 min, and then fixed with 4 % paraformaldehyde for 20 min, followed by a PBS wash. Cells were then permeabilized with 0.1 % Triton X-100 (0.01 mol/L) PBS for 5 min and washed with PBS. Cells were blocked for 1 h using 5 % goat serum at room temperature and then incubated with mouse anti-vimentin (1:100, Sigma Aldrich Co., St. Louis, MO, USA) at 4 °C overnight. The cells were washed with PBS the next day and then incubated with horse anti-mouse antibody conjugated with fluorescein isothiocyanate for 1 h at room temperature. After a wash with PBS, cell nuclei were stained with 4',6-diamidino-2-phenylindole for 10 min and washed again in PBS. Fixed cells were then mounted using glycerine and photographed under fluorescent microscopy.

RNA preparation and complementary DNA synthesis

Expression of three osteoblast-specific genes, including OCN, ALP and COL I, was detected through semiquantitative RT-PCR [16]. The third passage cells of OPLL and

non-OPLL groups were seeded in 6-well plates at a density of 3×10^5 cells/well. After cultures reached confluence, the cells were incubated in DMEM supplemented with 1 % fetal bovine serum for 24 h. After that, the cells were washed 2–4 times with PBS before RNA isolation. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, CA) according to the manufacturer's protocol. After separation, sedimentation, reduction, and washing, the RNA concentration was detected. Each 1 µg of total RNA was reversely transcribed into the first strand of complementary DNA (cDNA) using a RevertAid first-strand cDNA synthesis kit (Fermentas, MD) according to the manufacturer's instruction. The production was stored at −20 °C until use, for amplification by a polymerase chain reaction (PCR).

PCR analysis

Polymerase chain reaction amplification was carried out in a volume of 20 µL containing 10 µL $2 \times$ PCR master mix, 8 µL nuclease-free water, 1 µL cDNA and 1 µL primer. Specific oligonucleotide primers (designed by Sangon Biotech Co. Ltd, Shanghai, China) are shown in Table 2. The cycling conditions were 94 °C for 5 min as an initial denaturation step, followed by 35 cycles of denaturation at 94 °C for 30 s. Renaturation and extension were both performed for 1 min each. The renaturation temperatures for OCN, ALP, COL I, PERK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 56.7, 56.7, 54.9,

Table 2 Primer sequences of OCN, ALP, COL I and GAPDH

Index	Primer sequences
OCN	
Forward primer	5'-AGGGCAGCGAGGTAGTGA-3'
Reverse primer	5'-CCTGAAAGCCGATGTGGT-3'
ALP	
Forward primer	5'-GTGGACTATGCTCACAACAA-3'
Reverse prime	5'-GGAGAAATACGTTTCGCTAGA-3'
COL I	
Forward primer	5'-CGAAGACATCCCACCAATC-3'
Reverse prime	5'-ATCACGTCATCGCACAACA-3'
GAPDH	
Forward primer	5'-CGCGGGCTCCAGAACATCAT-3'
Reverse prime	5'-CCAGCCCCAGCGTCAAAGGTG-3'

55.8 and 59.1 °C, respectively. A final extension step at 72 °C for 10 min was performed. Real time-PCR (RT-PCR) products were electrophoresed on a 1.0 % agarose gel with 0.5 mg/mL ethidium bromide. Bands were detected by UV illumination of ethidium bromide-stained gels. Band intensities were quantitatively analyzed by Quantity One Software (BioRad, CA, USA) for each gene, and were normalized to the corresponding GAPDH values. The ratio of each gene per GAPDH was considered as the value of mRNA expression. Each specimen was repeated three times.

Western blotting

Expression of PERK protein was detected by Western blotting [2]. For Western blot analysis, third passage OPLL and non-OPLL cells were placed at a density of 3×10^5 cells/well. Equal amount of protein (20 µg/well) from each sample was separated on a 10 % sodium dodecyl sulfate–polyacrylamide gel. Separated proteins were transferred from the gel to a nitrocellulose membrane followed by a blocking for 1 h at room temperature, in 5 % nonfat dried milk in Tris-buffered saline Tween-20 buffer (Ximei Chen Co. Ltd, Shanghai, China). Blots were then probed with anti-PERK (rabbit, Sigma Aldrich Co.) at 1:500 dilutions at 4 °C overnight. The membranes were then washed and incubated with a goat anti-rabbit IgG linked to horseradish peroxidase (1:1,000) for 2 h at room temperature. Bound antibody was then revealed using 3,3'-diaminobenzidine as a substrate (DAB, 0.5 mg/mL). Finally, the membranes were dried and then scanned using an Epson Perfection Photo Scanner (Epson Corporation, CA, USA). Protein intensities were quantitatively analyzed by Quantity One software (BioRad). GAPDH was used as the internal control. The value of PERK protein expression was the ratio of

Table 3 PERK-specific siRNA sequence and negative control sequence

Primer sequences	
SiRNA	
Sense strand	5'-CCTTGTGCCTGTCAATGA-3'
Antisense strand	5'-GCAGCAGCCATGGCGGT-3'
Negative control	
Forward primer	5'-GTGGACTATGCTCACAACAA-3'
Reverse primer	5'-GGAGAAATACGTTTCGCTAGA-3'

PERK per GAPDH. Each specimen was repeated three times.

Transfection of short interfering RNA (siRNA)

To determine whether PERK plays a role in the signal transmission and the effect on OCN, ALP, and COL I expression in the cells from OPLL patients, RNA interference targeting PERK was performed. Third passage cells cultured from OPLL patients were placed in 6-well plates at a density of 3×10^5 cells/well and allowed to reach confluence. Lipofectamine 2000 reagent (10 µL) (Invitrogen) was added to 500 µL Opti-Modified Eagle Media (Opti-MEM, Invitrogen) for 5 min. Then, the mixture was divided into two equal parts: one part was put into 250 µL Opti-MEM containing 5 µL PERK siRNA, and the other part was put into 250 µL Opti-MEM containing 5 µL negative control siRNA, and the things in each part were made to totally mix together for 20 min. In this process, the cells were washed twice with serum-free DMEM. After 20 min, the DMEM was removed and 500 µL Lipofectamine-siRNA-Opti-MEM mixture and 300 µL serum-free DMEM were added to each well. After 8 h of transfection, 800 µL of 10 % fetal bovine serum-DMEM was added to each well. The siRNAs were synthesized in duplex (GenePharma Co., Shanghai, China). The sense and antisense strands of the PERK siRNA are shown in Table 3. After 72 h of transfection, we performed Western blotting to clarify whether the transfection of siRNA downregulated PERK protein expression and the knockdown efficiency.

Statistical analysis

Data are expressed as mean $\pm$ SD. Data analysis between OPLL and non-OPLL groups was conducted using independent sample *t* test. The difference between groups of non-transfection and siRNA transfection with OPLL cultures was analyzed using a paired *t* test. All analyses were performed with the Statistical Package for Social Sciences (SPSS 6.1.1; Norusis/SPSS Inc, Chicago, IL, USA)

Fig. 1 Identification of the cultured cells: **a** hematoxylin–eosin staining showed the cells were multi-angle shaped and also had relatively long cell antennas; however, it is interesting that the cells from OPLL patients had larger cell size and nucleus; **b** immunocytochemistry and immunofluorescence demonstrated vimentin (green fluorescence, fluorescein isothiocyanate-labeled) in the cytoplasm

software, and were two-tailed with a level of significance set at 0.05.

Results

HE staining and immunocytochemistry/immunofluorescence

Slender cells could be seen around the isolated tissue fragments (from OPLL and non-OPLL patients) at the 7–10th day after tissue fragment culture. The cells were gradually rearranged into fascicles on approximately the 10th day after they were observed around the tissue fragment. After the cells reached confluence, they were harvested with 0.05 % trypsin to stimulate uniformity to facilitate cell karyokinesis.

Fig. 2 Comparison of mRNA expression of osteocalcin (OCN), alkaline phosphatase (ALP), and type I collagen (COL I) between the cells from ossification of the posterior longitudinal ligament (OPLL) and non-OPLL patients through semiquantitative real time-polymerase chain reaction, demonstrating a significantly upregulation in the cells from OPLL patients, with levels of expression 73, 80, and 62 %, respectively (** $p < 0.01$)

Fig. 3 Western blotting result of PERK protein expression in the cells from OPLL and non-OPLL patients, showing a significantly upregulation in the cells from OPLL patients, with level of expression 167 % (** $p < 0.01$). PERK indicates endoplasmic reticulum membrane protein kinase; *OPLL* ossification of the posterior longitudinal ligament

As shown in Fig. 1a, cells derived from the cervical posterior longitudinal ligament (OPLL and non-OPLL patients) showed much fibroblast-like morphological characters. They were multi-angle shaped and also had relatively long cell antennas; however, it is interesting that the cells from OPLL patients had larger cell size and

Fig. 4 Western blotting result showing a knockdown efficiency of PERK in the transfection group compared with the negative control group, with level of expression 77 % (** $p < 0.01$). PERK indicates endoplasmic reticulum membrane protein kinase

nucleus, which may represent some kind of pathological change. On the other hand, given that vimentin is a type of intermediate filament that mainly resides in fibroblast, here we took vimentin as a fibroblast marker, and performed the immunofluorescence assay (Fig. 1b). The results showed that the cells derived from the cervical posterior longitudinal ligament (OPLL and non-OPLL patients) were fibroblast-like cells, with green fluorescence in cytoplasm.

Different expression of OCN, ALP, COL I and PERK between the cells from OPLL and non-OPLL patients

The expression of OCN, ALP, and COL I was significantly upregulated in the cells from OPLL patients, with levels of expression 73 % ($p < 0.01$), 80 % ($p < 0.01$), and 62 % ($p < 0.01$) greater than those from non-OPLL patients, respectively (Fig. 2). The protein expression of PERK in the cells from OPLL patients was significantly upregulated, with levels of expression 167 % ($p < 0.01$) greater than those from non-OPLL patients (Fig. 3).

Knockdown efficiency of siRNA targeting PERK

After 72 h of transfection in the cells from OPLL patients, the result of Western blotting showed a downregulation of PERK protein expression by 77 % ($p < 0.01$) in the transfection group compared with that in the non-transfection group (negative control, Fig. 4). However, in the cells from non-OPLL patients, only a slight downregulation of PERK protein expression by 18 % ($p > 0.05$) was

Fig. 5 The mRNA expression of osteoblast-specific genes after 72 h of transfection, showing there were a significant upregulated expression of OCN, ALP and COL I by approximately 48, 68 and 63 % (** $p < 0.01$). OCN osteocalcin, ALP alkaline phosphatase, COL I type I collagen, NC negative control

found in the transfection group compared with that in the non-transfection group after 72 h of transfection. We supposed it was because the expression of PERK was not activated in the cells from non-OPLL patients by ER stress.

Influence of siRNA targeting PERK on expression of OCN, ALP and COL I

After 72 h of transfection, mRNA expression of OCN, ALP, and COL I was detected via RT-PCR and then was compared between the transfection group and the negative group. The results showed there were a significant down-regulated expression of OCN, ALP and COL I by approximately 48 % ($p < 0.01$), 68 % ($p < 0.01$) and 63 % ($p < 0.01$), respectively, after 72 h of transfection in the cells from OPLL patients (Fig. 5).

Discussion

Ectopic bone formation in the cervical spinal ligament is the typical characteristic of OPLL. Fibroblasts cells can differentiate into osteoblasts or osteocytes [4, 8, 9, 12, 24, 28]. This differentiation should be accompanied by an upregulation of several marker genes related to bone formation, such as OCN, ALP and COL I. The OCN, deposited in the bone matrix, is a vitamin K-dependent non-collagenous protein secreted by mature osteoblasts. The carboxyglutamic acid of OCN binds to Ca^{2+} with high specificity. The expression of OCN demonstrates osteogenesis. ALP is secreted by osteoblasts in the process of

osteogenic differentiation. Under osteogenic stimulus, ALP can hydrolyze the organophosphate and offer phosphonic acid for the deposition of hydroxyapatite crystals on collagen fibers and subsequently make osteogenesis development. Cells from ALP-knockout rats exhibited only proliferation with no calcification [27]. COL I is a major bone matrix protein secreted by osteoblasts and is essential for the appropriate bone formation and its strength. Mutations in the genes, which encode type I collagen are linked to misfolding or decrease of the molecule, and consequently cause osteogenesis imperfecta. There has been evidence suggesting that OCN and ALP activity are high in OPLL cells compared with non-OPLL cells [3]. In this study, we confirmed that the fibroblasts derived from OPLL patients in contrast to those derived from non-OPLL patients exhibited a greater degree of mRNA expression of OCN and ALP, as well as a significant upregulation of COL I.

PERK has been reported to play an important role in the differentiation of osteoblasts. Analysis of gene expression demonstrated the expression of mature osteoblast markers OCN, ALP and COL I was reduced in PERK^{-/-} osteoblasts in vivo and in vitro. Correlated with reduced differentiation, PERK^{-/-} osteoblasts also exhibited reduced proliferation both in mice and in primary calvarial osteoblast cultures [21]. PERK^{-/-} mice were reported to exhibit severe osteopenia, and the phenotypes observed in bone tissue were very similar to those ATF4^{-/-} mice, and ATF4 is an essential transcription factor for osteoblast terminal differentiation and bone formation [25]. Loss of function mutations of PERK in human caused Wolcott-Rallison syndrome (WRS), an autosomal recessive disorder that is characterized by the early onset of type I diabetes, growth retardation and multiple skeletal dysphasia including epiphyseal dysplasia, bone fractures and later osteoporosis [1]. However, there has not been any report about the different expression of PERK in the fibroblasts from OPLL patients. Thus, we hypothesized that the expression of PERK should be upregulated in the fibroblasts derived from OPLL patients. Indeed, we found that upregulated expression of PERK protein in the fibroblasts from OPLL patients, and OCN, ALP as well as COL I was significantly downregulated after siRNA interfering targeting PERK in the fibroblasts compared with those in the non-transfection group. Our results suggested that the effect of siRNA interfering targeting PERK on stopping the cells developing osteoblastic differentiation was consistent with a previous study on Cx43 [29], and thus a similar PERK-mediated signaling pathway might be also involved in development of OPLL. In addition, PERK is a major ER stress transducer and is activated in response to the ER stress. Therefore, fibroblasts from the OPLL patients may be in an ER stress condition.

There has been suggestion about that genes coding for collagens, cytokines as well as growth factors are involved in the pathophysiology of OPLL. Among them, the role of BMP-2 has definitely been demonstrated in development of OPLL [10, 11, 23], and ER stress response mediated by PERK-eIF2 α -ATF4 pathway was proved to be involved in osteoblast differentiation induced by BMP-2 [21]. In this study, treatment of wild-type primary osteoblasts with BMP-2, which is required for osteoblast differentiation, induced ER stress, leading to an increase in ATF4 protein expression levels. Additionally, it is well known that mechanical stress to the cervical spine may be an important factor in progression of OPLL [15]. Attempts have been made to simulate the mechanical stress in vitro, mostly with a motor-driven uniaxial sinusoidal cyclic stretch [18]. The system induced the transcription of messenger ribonucleic acids of several growth factors, including BMP-2, BMP-4, prostaglandin I₂ synthase, and osteoblast-specific transcription factor in the cells from OPLL patients [9, 19, 24, 30]. At the same time, several studies have suggested that ER stress response can be activated by mechanical stress in some cellular pathological processes, such as apoptosis in rat annulus fibrosus cells or human connective tissue cells [32]. Thus, based on above biological information, we hypothesized that PERK-mediated ER stress might be downstream mechanism in development of OPLL induced by some primary genetic or mechanical factors. Further studies are needed to clarify how ER stress response is activated by these primary factors in development of OPLL.

In conclusion, we demonstrate that the fibroblasts derived from OPLL patients exhibited osteoblast-like properties. PERK was significantly upregulated in the cells from OPLL patients in contrast to those from non-OPLL patients. The mRNA expressions of OCN, ALP and COL I in the cells from OPLL patients were significantly reduced after 72 h of transfection of specific siRNA targeting PERK. On the basis of these observations, we proposed that the PERK-mediated ER stress might be involved in development of OPLL.

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Conflict of interest None.

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