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ORIGINAL ARTICLE

Endoplasmic reticulum stress regulates mechanical stress-induced ossification of posterior longitudinal ligament

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Abstract

Purpose The pathogenesis of ossification of posterior longitudinal ligament (OPLL) is not completely clear. Previous study has confirmed a single-pass type I endoplasmic reticulum (ER) membrane protein kinase (PERK), which is a major transducer of the ER stress, participates in the process of OPLL in vitro. This study aimed to demonstrate the role of ER stress in mechanical stress (MS)-induced OPLL.

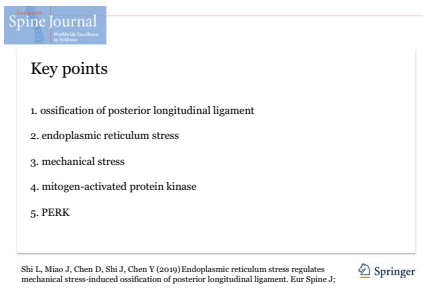
Methods The posterior longitudinal ligaments were collected intraoperatively. The expression of ER stress markers in ligament tissue samples was compared between OPLL and non-OPLL patients in vivo. Ligament fibroblasts were isolated and cultured. Loaded by MS, the expression of ER stress markers in fibroblasts deriving from non-ossified areas of the ligament tissues from OPLL patients was detected. The influence of inhibition of ER stress on MS-induced OPLL and activation of mitogen-activated protein kinase (MAPK) pathways by MS was also investigated.

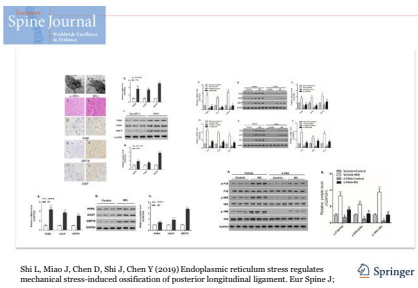
Results We confirmed the ER stress markers were highly expressed in non-ossified areas of the ligament tissues from OPLL patients but could barely be detected in the ligaments from non-OPLL patients in vivo. We also found ER stress could be activated by MS during the process of OPLL in vitro. Moreover, inhibition of ER stress could hinder MS-induced OPLL and activation of MAPK signaling pathways by MS in vitro.

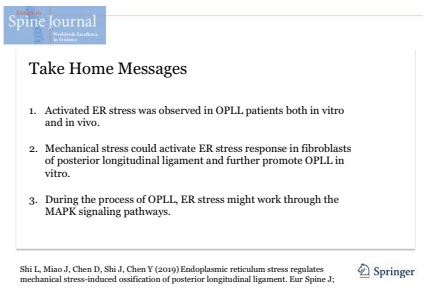
Conclusion Activated ER stress was observed in OPLL patients both in vitro and in vivo. Mechanical stress could activate ER stress response in posterior longitudinal ligament fibroblasts and further promote OPLL in vitro. In this process, ER stress might work through the MAPK signaling pathways.

Graphic abstract

These slides can be retrieved under Electronic Supplementary Material.







Keywords Ossification of posterior longitudinal ligament · Endoplasmic reticulum stress · Mechanical stress · Mitogen-activated protein kinase · PERK

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00586-019-06074-2) contains supplementary material, which is available to authorized users.

Extended author information available on the last page of the article

Introduction

Ossification of posterior longitudinal ligament (OPLL) is a pathological condition of ectopic bone formation in the spine ligament, which causes spinal cord compression and neurological symptoms. Although several studies have investigated the osteogenetic characteristic of the spinal ligament fibroblasts from OPLL patients, the pathogenesis is not completely clear.

Endoplasmic reticulum (ER) copes with the excessive accumulation of unfold proteins via signaling pathways, which is known as ER stress response. Recently, ER stress response has been proved to be very important in the skeletal development and osteoblast proliferation and differentiation [1–6]. Considering the similarities between the process of OPLL and osteoblast differentiation [7, 8], our previous study has confirmed a single-pass type I ER membrane protein kinase (PERK), which is a major transducer of the ER stress response, participates in the process of OPLL in vitro [9]. Thus, PERK-mediated ER stress response should play a role in OPLL.

It has been reported that the abnormal distribution of mechanical stress (MS) is highly correlated with the process of OPLL [10–12]. MS could induce osteogenic differentiation of posterior longitudinal ligament fibroblasts in vitro, which is expressed as up-regulation of osteogenic markers including osteocalcin (OCN), alkaline phosphatase (ALP) and type I collagen (COL I) [13–15]. At the same time, some studies have shown that MS could activate ER stress in particular cells [16–19]. Therefore, ER stress might also be activated by MS during the process of OPLL. Additionally, Yang et al. [20] reported that MS plays a regulatory role through ER stress in the process of ossification of periodontal ligament. Similarly, MS may also affect the process of OPLL through ER stress.

In mammals, there are three subfamilies of mitogen-activated protein kinase (MAPK), such as ERK1/2, p38 and JNK1/2. These mediate three parallel signaling transduction pathways. It has been confirmed that MS could promote osteogenic differentiation of posterior longitudinal ligament fibroblasts via ERK1/2 and p38 MAPK signaling pathways in vitro [21]. Meanwhile, previous studies have shown that ER stress plays an important role via MAPK signaling pathways in particular cells [22–24]. Thus, ER stress might influence the process of MS-induced OPLL through MAPK pathways.

In this study, we demonstrated the role of ER stress in MS-induced OPLL through a series of experiments.

Materials and methods

Patients

Sixteen patients presenting with OPLL and sixteen non-OPLL patient controls were selected (Table 1). All patients underwent cervical anterior decompression surgery. The diagnosis of OPLL or non-OPLL was confirmed by cervical radiographs, computer tomography, magnetic resonance

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/local	M/55	PLL	1	CDH	F/59	PLL
2	OPLL/local	F/60	PLL	2	Cervical fracture	M/49	PLL
3	OPLL/segmental	M/46	PLL	3	Cervical fracture	F/61	PLL
4	OPLL/local	F/53	PLL	4	CDH	F/54	PLL
5	OPLL/local	M/44	PLL	5	Cervical fracture	M/44	PLL
6	OPLL/continuous	F/47	PLL	6	CDH	M/62	PLL
7	OPLL/local	M/40	PLL	7	Cervical fracture	M/43	PLL
8	OPLL/segmental	F/60	PLL	8	CDH	F/48	PLL
9	OPLL/local	F/48	PLL	9	CDH	M/57	PLL
10	OPLL/local	M/42	PLL	10	CDH	M/42	PLL
11	OPLL/segmental	F/53	PLL	11	Cervical fracture	F/54	PLL
12	OPLL/mixed	M/37	PLL	12	Cervical fracture	M/46	PLL
13	OPLL/segmental	M/41	PLL	13	CDH	F/58	PLL
14	OPLL/local	M/56	PLL	14	Cervical fracture	F/39	PLL
15	OPLL/local	M/58	PLL	15	CDH	M/64	PLL
16	OPLL/mixed	F/51	PLL	16	CDH	M/49	PLL

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

imaging and clinical data. Informed consent was obtained from each patient, and this study was approved by the Ethics Committee of Second Military Medical University. In this study, ligament fibroblasts from each patient were cultured separately. Western blot and real-time polymerase chain reaction (RT-PCR) analysis were performed in triplicate for each sample.

Primary ligament fibroblasts culture

Posterior longitudinal ligament specimens were harvested during the anterior cervical decompression surgery [15]. The ligament tissues were carefully dissected from a non-ossified site to avoid any possible contamination with osteogenic cells. Tissues were minced into 0.5 mm^3 pieces and washed with PBS for several times. Afterward, the tissue fragments were placed into culture dishes and maintained with low-glucose DMEM culture mediate supplemented with 10% FBS. The cells derived from cultured explants were passaged with trypsin and further cultured under 5% CO_2, 95% air, and 37 °C humidified atmosphere. In the third passage, the cells grew in good condition, the morphology of the cells was stable, and the arrangement was orderly. Thus, we adopted the third passage of cells in following experiments.

Histochemistry and immunohistochemistry

For histochemistry and immunohistochemistry, posterior longitudinal ligaments from both OPLL and non-OPLL patients were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5-μm-thick sections and processed for hematoxylin and eosin (H and E) staining or immunohistochemistry. Immunohistochemistry was performed by using standard protocols with the following antibodies: PERK (1:500), CHOP (1:1000) and GRP78 (1:800). A computer-assisted genuine color image analysis system (Image-Pro Plus 6.0) was employed to detect the in vivo expression of PERK, CHOP and GRP78 in the OPLL and non-OPLL patients. Mean integrated optical densities were calculated.

Mechanical stretch apparatus

For mechanical stress loading, 3×10^5 cells were seeded into Flexcell plate (Flexcell Co.) for growing until 70% confluence [15]. Then cells were synchronized to ensure consistent cell growth, using DMEM with 1% FBS for 24 h. By using Flexcell 4000 Strain Unit (Flexcell Co.), the cells were then subjected to a cyclic tensile strain of 10% elongation at a frequency of 0.5 Hz, which is consistent with the low frequency and amplitude of the cervical physiological movement. The effect of mechanical stretch on fibroblasts for 24 h was observed.

The treatment with ER stress inhibitor

For 4-PBA administration, sodium phenylbutyrate (4-PBA, Sigma-Aldrich) was dissolved in double distilled water (ddH_2O) and prepared by titrating equimolar amounts of 4-PBA and sodium hydroxide to pH 7.4. Then 4-PBA (final concentration 2 mmol/L) was applied to cells for 24 h [25].

RNA isolation and quantitative PCR

RNA were extracted from tissues or cells using TRIzol (Invitrogen) according to manufacturer's protocol. 1 μg of isolated was applied for first-strand cDNA transcript (Invitrogen) and further used in quantitative PCR. SYBR Green qPCR system was adopted following manufacturer's instructions (Thermo). The amplification results were automatically analyzed using $2(^{-\Delta\Delta Ct})$ method with ABI Prism 7300 SDS software. The primer sequences are listed in Table 2.

Western blot analysis

Tissues or cells were lysed, and equal amount of 25 μg protein was loaded into each well of 10% or 15% SDS-PAGE gel. Separated proteins were transferred onto PVDF membrane followed by blocking for 1 h at room temperature in 5% non-fat milk in 1 × TBST buffer. The blots were then incubated with 1st antibody overnight at 4 °C (collagen I, abcam, Ab34710, 1:1000; ALP, abcam, Ab67228, 1:500; OCN, abcam, Ab76690, 1:2000; PERK, abcam, Ab65142, 1:500; CHOP, abcam, Ab11419, 1:1000; GRP78, abcam, Ab21685, 1:800; p-P38, Cell Signaling Tech, #9211, 1:1000; P38, Cell Signaling Tech, #9212, 1:1000; p-ERK1/2, Cell Signaling Tech, #9101, 1:1000; ERK1/2, Cell Signaling

Table 2 Primer sequences of PERK, CHOP, GRP78, ALP, OCN, COL I and GAPDH

Name	Primer sequences	
PERK	Forward	5′-CAGTTCACCAAAGGTGTATC-3′
	Reverse	5′-AGTCACTAACCCAAAGTCTC-3′
CHOP	Forward	5′-AACCAGGAAACGGAAACAG-3′
	Reverse	5′-TCACCATTCGGTCAATCAG-3′
GRP78	Forward	5′-CCCGTCCAGAAAGTGTTG-3′
	Reverse	5′-CAGCACCATACGCTACAG-3′
ALP	Forward	5′-AAGGAGGAAGCCTGGGAAG-3′
	Reverse	5′-TCAGTGGTGGAGCCAAGTC-3′
OCN	Forward	5′-GCAGCGAGGTAGTGAAGAGAC-3′
	Reverse	5′-GAAAGAAGGGTGCCTGGAGAG-3′
COL I	Forward	5′-GGGTCTGTGAGACGGATGTG-3′
	Reverse	5′-CAGCCTGCTGGCTTCTAGTG-3′
GAPDH	Forward	5′-CACCCACTCCTCCACCTTTG-3′
	Reverse	5′-CCACCACCCTGTTGCTGTAG-3′

Tech, #9102, 1:1000; p-JNK, Cell Signaling Tech, #9255, 1:2000; JNK, Cell Signaling Tech, #9252, 1:1000; GAPDH, Cell Signaling Tech, #5174, 1:2000). Finally, the membranes were washed with 1 × TBST for three times and incubated with ECL (Millipore) for image scanning. The density of each protein band was analyzed by ImageJ.

Statistical analysis

Data were presented as mean ± SD. Normal distribution was tested. Data between two groups were compared by independent *t* test. ANOVA and LSD *t* test were used for data among three or more groups. $p < 0.05$ was considered statistical significant.

Results

Endoplasmic reticulum stress response is activated in posterior longitudinal ligament tissue samples of OPLL patients in vivo

Previous study has reported PERK-mediated ER stress is activated in posterior longitudinal ligament fibroblasts of OPLL patients in vitro [9]. Here, we observed the proliferation, dilation and degranulation of the endoplasmic reticulum in non-ossified areas of the ligament tissue samples from OPLL patients by transmission electron microscopy (Fig. 1A, B). Subsequently, we performed H and E staining (Fig. 1C, D) and immunohistochemistry assays (Fig. 1E–J) on ligament tissue samples, which showed ER stress markers, such as PERK, 78 kDa glucose-regulated protein/immunoglobulin heavy chain-binding protein (GRP78), and CCAAT-enhancer-binding protein homologous protein (CHOP) were highly expressed in non-ossified areas of the ligament tissues from OPLL patients but could barely

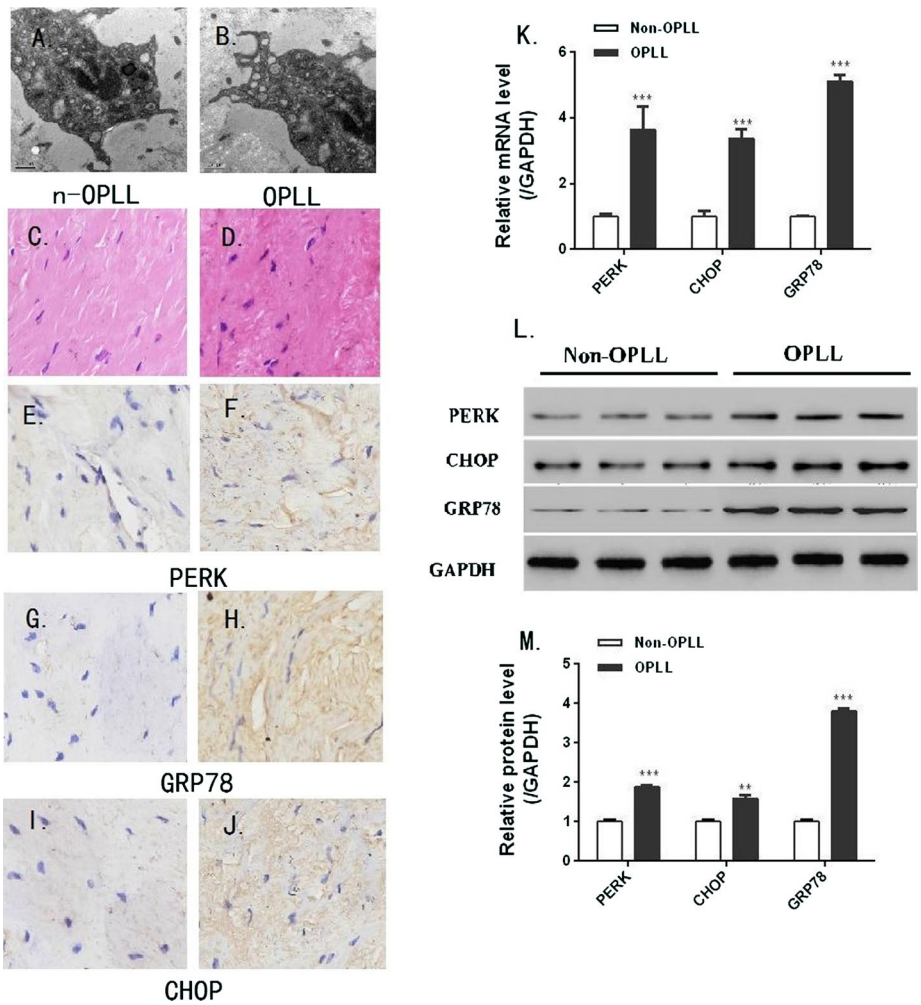


Fig. 1 **A** and **B** Morphology of endoplasmic reticulum in ligament tissue samples of OPLL patients by transmission electron microscopy. **H** and **E** staining (**C** and **D**) and immunohistochemistry assays (**E**–**J**) on posterior longitudinal ligament tissues. The mRNA (**K**) and protein (**L** and **M**) expression of ER stress markers in ligament tissue samples. The three OPLL and three non-OPLL bands presented in the western blot were technical replicates from the same sample. The representative figures were shown. The difference between two groups was significant (**$p < 0.01$, ***$p < 0.001$, $n = 16$)

be detected in the ligaments from non-OPLL patients. Then, we found the mRNA and protein expression of ER stress markers was much higher in ligament tissue samples from OPLL patients, via quantitative PCR (Fig. 1K) and western blot assays (Fig. 1L, M). These findings further verified ER stress response is activated in the process of OPLL in vivo.

Endoplasmic reticulum stress response is activated by MS during the process of OPLL in vitro

In previous study, we have confirmed mechanical stress could induce osteogenic differentiation of fibroblasts deriving from non-ossified areas of the ligament tissues from OPLL patients. However, this effect was insignificant in cells deriving from non-OPLL patients, which might attribute to genetic factors [15]. Thus, we employed fibroblasts deriving from non-ossified areas of the ligament tissues from OPLL patients in this study. Expectedly, ER stress was induced by MS as up-regulation of ER stress markers, such as PERK, GRP78 and CHOP (Fig. 2A–C). Quantitative PCR was used for mRNA expression analysis, and western blot was used for protein expression analysis, respectively.

Inhibition of endoplasmic reticulum stress response hinders MS-induced OPLL in vitro

Fibroblasts from OPLL patients were divided into 4 groups. We found the expression of ER stress markers was significantly down-regulated by 4-phenylbutyrate (4-PBA, Sigma-Aldrich, P21005, an ER stress inhibitor), with or without mechanical stress stimulation (Fig. 3A–C). Meanwhile, the expression of osteoblastic markers was significantly up-regulated by MS and osteogenic differentiation was reduced by 4-PBA, with or without MS stimulation. It suggested that MS-induced OPLL could be markedly hindered by inhibition of ER stress in vitro (Fig. 3D–F).

Inhibition of endoplasmic reticulum stress response hinders activation of MAPK signaling pathways by MS in vitro

It has been confirmed MS could activate MAPK and further promote osteogenic differentiation of posterior longitudinal ligament fibroblasts via MAPK signaling pathways in vitro [21]. Here, we found that the phosphorylation levels of ERK1/2, p38 MAPK and JNK1/2 were markedly reduced by 4-PBA, with or without MS stimulation. It suggested that inhibition of ER stress could block activation of MAPK signaling pathways by MS (Fig. 4A, B).

Discussion

Ossification of posterior longitudinal ligament is a disease characterized by heterotopic ossification of ligaments. The current studies have focused on its pathogenesis. Recently, ER stress response has been proved to be very important in the skeletal development and osteoblast proliferation and differentiation. PERK, a major transducer of the ER stress response, up-regulates the expression of OCN in osteoblasts through ER stress-related signal factor and core-binding factor a1 (Cbfa1). Additionally, BMP2 activates mTORC1 and regulates osteogenic differentiation via PERK-mediated ER stress [1]. On the other hand, it has been reported that lack of PERK weakens osteoblast proliferation. Expression of osteoblastic markers, such as ALP, OCN and COL I, was markedly down-regulated [26]. PERK-knockout mice suffer from severe osteoporosis [27]. In addition, the abnormal expression of PERK leads to Wolcott–Rallison syndrome, which is an autosomal recessive hereditary disease with typical symptoms such as early onset of type I diabetes mellitus, multiple skeletal dysplasia and growth retardation [28]. Moreover, it has been confirmed that PERK-mediated

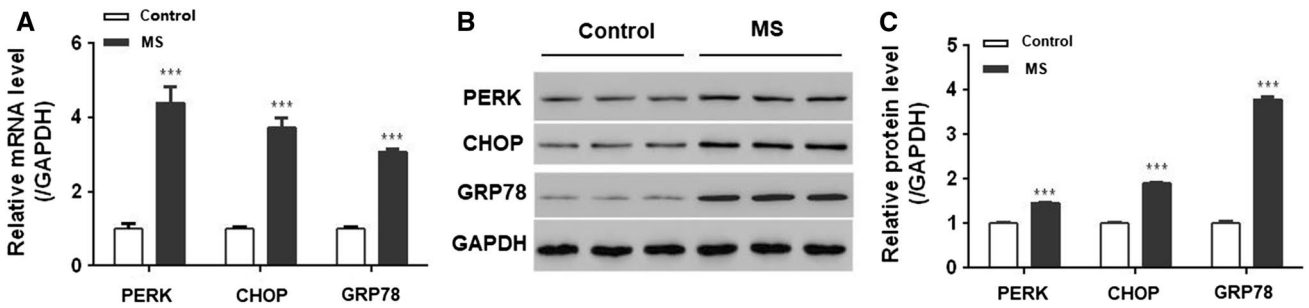


Fig. 2 The mRNA (**A**) and protein (**B** and **C**) expression of ER stress markers was analyzed after cells were loaded by mechanical stress for 24 h. The difference between two groups was significant (**$p < 0.01$, ***$p < 0.001$, $n = 16$)

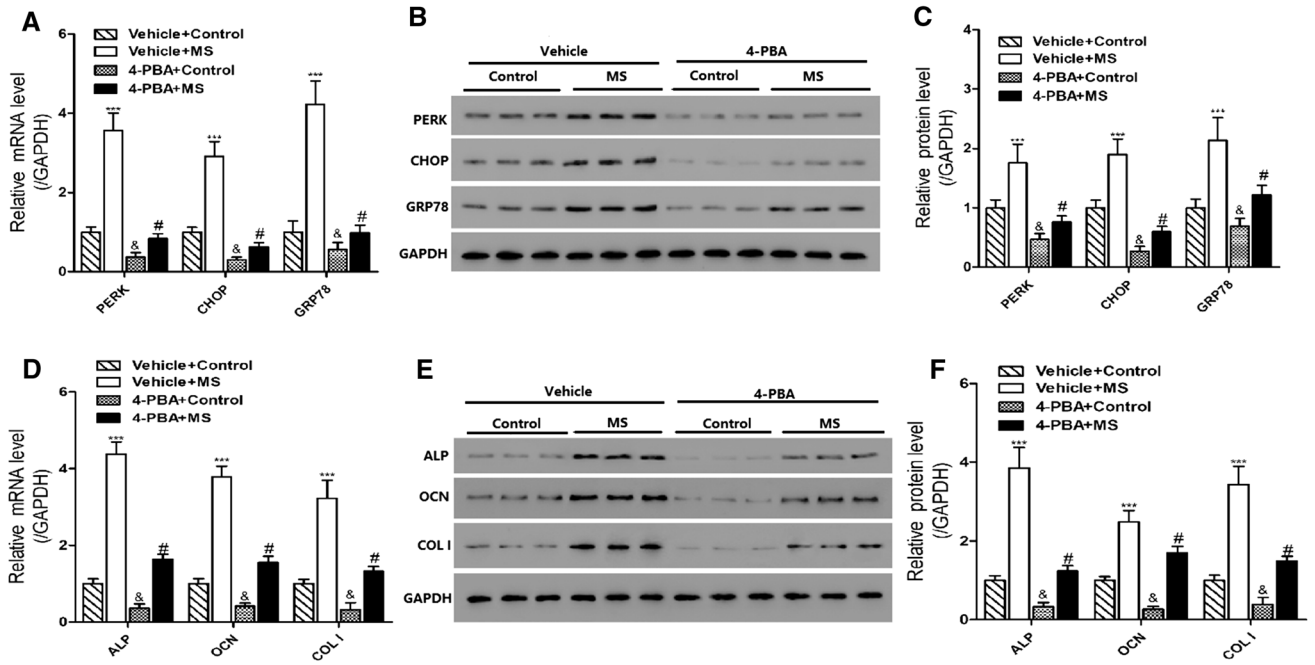


Fig. 3 (**A**–**C**) Expression of ER stress markers in mRNA and protein level. (**D**–**F**) Expression of osteoblastic markers in mRNA and protein level. The difference between vehicle +control group and vehicle + MS group was significant (***$p < 0.001$, $n = 16$). The difference between vehicle + control group and 4-PBA + control group was significant ($^{\&}p < 0.01$, $n = 16$). The difference between vehicle + MS group and 4-PBA + MS group was significant ($^{\#}p < 0.001$, $n = 16$)

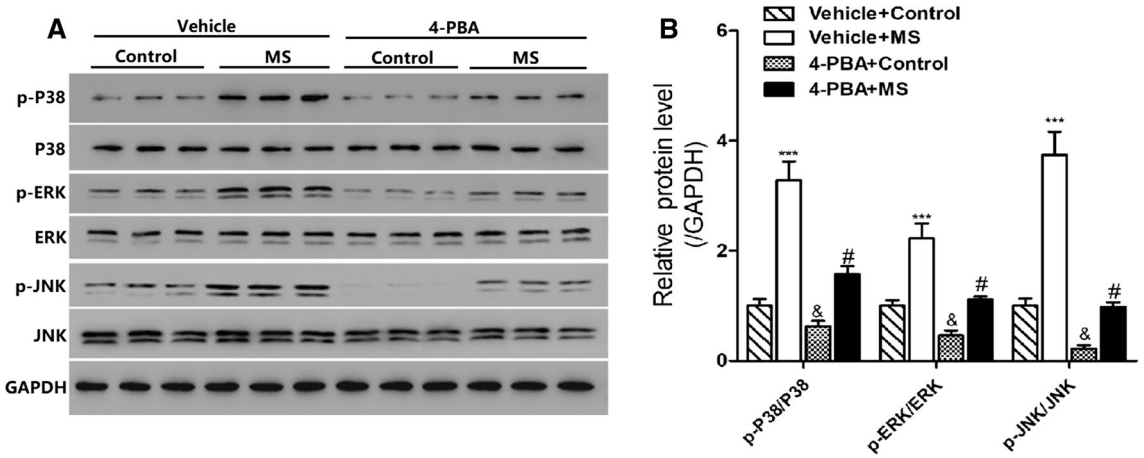


Fig. 4 **A** and **B** Phosphorylated protein and total protein expression of ERK1/2, p38 MAPK and JNK1/2. The difference between vehicle + control group and vehicle + MS group was significant (***$p < 0.001$, $n = 16$). The difference between vehicle + control group and 4-PBA + control group was significant ($^{\&}p < 0.01$, $n = 16$). The difference between vehicle + MS group and 4-PBA + MS group was significant ($^{\#}p < 0.001$, $n = 16$)

ER stress is involved in the process of OPLL in vitro [9]. In order to verify this in vivo, we firstly observed the proliferation, dilation and degranulation of the endoplasmic reticulum in non-ossified areas of the posterior longitudinal ligament tissue samples from OPLL patients by transmission electron microscopy. Then, the expression of ER stress markers, such as PERK, CHOP and GRP78 was detected by H and E staining and immunohistochemistry assays in ligament tissues. The expression of ER stress markers was further detected by quantitative PCR and western blot in ligament tissue sample. We found the expression of ER stress markers in OPLL patients was significantly higher than that in non-OPLL patients. These together indicate that ER stress response is activated in the process of OPLL in vivo.

Some studies have shown that ER stress response could be activated by stress stimulation in particular cells. In the study of cardiovascular disease, for example, ER stress of vascular smooth muscle and cardiomyocytes could be activated by stress stimulation and further induce apoptosis. At the same time, stress stimulation could activate ER stress of vascular endothelial cells and induce multiple kinds of anti-oxidation and NFκB-mediated inflammatory reaction [16–18]. In addition, studies on intervertebral disk have shown that mechnical stress activates ER stress in fibroannular cells and thus affects apoptosis [19]. On the other hand, many studies have reported that MS could lead to OPLL. MS is a very important pathogenic factor in the development of this disease. In previous studies, we have established a stress loading culture system for posterior longitudinal ligament fibroblasts in vitro. In this study, fibroblasts deriving from non-ossified areas of the ligament tissues from OPLL patients were stimulated by MS for 24 h. We found the mRNA and protein expression of ER stress markers was up-regulated, markedly. These suggest that ER stress response could be activated by MS during the process of OPLL in vitro.

Yang et al. [20] reported that cyclic mechanical stress could activate PERK-mediated ER stress and further promote OCN, BSP-mediated osteogenic differentiation of periodontal ligament cells in vitro. Thus, ER stress may also play a regulatory role in MS-induced OPLL. In order to confirm this inference, fibroblasts deriving from non-ossified areas of the ligament tissues from OPLL patients were treated by ER stress inhibitor, then stimulated by MS for 24 h. We found inhibition of ER stress could block OPLL induced by mechanical stress, markedly. In other words, MS could promote OPLL through ER stress response in vitro.

Previous study has illustrated that MS could promote OPLL via ERK1/2 and p38 MAPK signaling pathways in vitro [21]. At the same time, some studies have reported ER stress plays an important role via MAPK signaling pathways in particular cells. In human osteoblasts, for example, ER stress could activate apelin through the p38 MAPK pathway and further promote cell proliferation [22]. In addition, ER stress can regulate the carcinogenesis of HepG cells through ERK1/2 and JNK signaling pathways [23]. Moreover, ER stress mediates IL-6 release through p38 MAPK in the process of degenerative intervertebral disk disease [24]. Therefore, ER stress may also play a role via MAPK pathways in MS-induced OPLL. In current study, ligament cells from OPLL patients were treated by ER stress inhibitor and then stimulated by MS for 24 h. We observed that inhibition of ER stress hinders activation of MAPK signaling pathways by mechanical stress, significantly. Thus, MS could activate the MAPK signaling pathways through ER stress and further promote the process of OPLL in vitro.

In conclusion, activated ER stress was observed in OPLL patients both in vitro and in vivo. Mechanical stress could activate ER stress response in posterior longitudinal ligament fibroblasts and further promote OPLL in vitro. During this process, ER stress might work through the MAPK signaling pathways.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Ethics Committee of Second Military Medical University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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