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Original Article

The endoplasmic reticulum stress response participates in connexin 43-mediated ossification of the posterior longitudinal ligament

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Abstract: Ossification of the posterior longitudinal ligament (OPLL) manifests as ectopic bone formation in spinal ligament tissue. As revealed by *in vitro* studies, fibroblasts from patients with OPLL or healthy ligament fibroblasts undergo mechanical stress (MS). We previously demonstrated that a cell-cell junction protein, connexin 43 (Cx43), is significantly up-regulated in OPLL cells and previous data indicated that some proteins related to the endoplasmic reticulum (ER) stress response are elevated during the development of OPLL. The present study utilized gain- and loss-of-function tools to delineate the contribution of the ER stress response within ligament fibroblasts under OPLL-inducing stimuli and the crosstalk between Cx43 signaling and the ER stress response. The ER stress process was augmented by the induction of Cx43 expression in OPLL cells or cells under MS. Cx43 over-expression also promoted ER stress and ossification in OPLL cells. Moreover, the activation of ER stress was accompanied with increased oxidative stress, which was inhibited by Cx43 gene silencing. Cx43 knockdown also improved ER stress-related ossification in OPLL cells. The blockage of ER stress using a chemical compound or small interfering RNA was sufficient to overcome MS-induced ossification in OPLL cells. These findings were further validated in patients with OPLL, as the mRNA levels of Cx43 and PKR-like endoplasmic reticulum kinase (a single-pass type I ER membrane protein kinase), a major transducer of ER stress, were significantly increased compared with non-OPLL subjects. In conclusion, this study demonstrates that ER stress participates in Cx43-related OPLL.

Keywords: Endoplasmic reticulum stress, Cx43, mechanical stress, ossification of the posterior longitudinal ligament, reactive oxygen species

Introduction

Ossification of the posterior longitudinal ligament (OPLL) is a condition in which the posterior longitudinal ligament structure becomes less flexible due to ectopic bone formation in the cervical spinal ligament [1-3]. Severe OPLL may cause compression of the spinal cord, pain, difficulties with walking, and other neurological symptoms. The current treatments largely rely on pain medication, anti-inflammatory drugs, or surgery according to the severity of the symptoms. However, the causes of OPLL are not understood fully. Several studies have found that the incidence of cervical OPLL is highly correlated with an abnormal distribution of mechanical strain [4-6]. *In vitro* tests from our group and others have confirmed that mechanical stress (MS) loading leads to the osteogenic differentiation of ligament fibroblasts and the release of inflammation cytokines and vasoactive peptides [7, 8]. Several transcription factors have been proposed to be involved in this process [9-12].

We have demonstrated that a gap-junction protein, connexin 43 (Cx43), plays multiple important roles in mediating the osteogenesis of ligament fibroblasts. Cx43 expression levels are induced both in OPLL tissue *in vivo* and in cultured OPLL fibroblasts *in vitro* [7, 13-15]. Similarly, mechanical strain induces the expression of Cx43 [7]. Inhibition of Cx43 expression suppresses the osteogenic process, partially through the inactivation of the extracellular signal-related protein kinase (ERK)1/2 and p38

Table 1. Demographic information of the study subjects

Item	OPLL group (n=52)	Non-OPLL group (n=16)
Sex (Male n, %)	21, 40.4%	6, 37.5%
Age (Mean, min-max), years	48.6, 33.0-74.0	51.8, 46.0-64.0
Type (n, %)	Local, 20, 38.5%	Cervical fracture, 11, 68.7%
	Mixed, 5, 9.6%	CDH, 5, 31.3%
	Segmental, 27, 51.9%	

OPLL, ossification of the posterior longitudinal ligament; CDH, cervical disc herniation.

mitogen-activated protein kinase (MAPK) pathways and the amelioration of nuclear factor (NF)-κB inflammation signaling [14, 15]. However, the cellular signaling pathways underlying osteogenesis mediated by Cx43 remain unclear and need further investigation.

The osteogenesis of ligament cells involves the synthesis of extracellular matrix proteins, such as osteogenesis marker collagen I (coded by gene COLA1). Additionally, a few signaling pathways were activated from the endoplasmic reticulum (ER) to prevent the excessive accumulation of unfolded proteins [16] and these processes are known as the ER stress response, which is known be important in osteoblast proliferation and differentiation [17, 18]. We recently demonstrated that PKR-like endoplasmic reticulum kinase (PERK), a major ER stress transducer and single-pass type I endoplasmic reticulum membrane protein kinase is involved in the development of OPLL *in vitro* [19]. Old astrocyte specifically induced substance, another novel type of ER stress response transducer, has been reported to share similar features with PERK in terms of OPLL development [20]. Nevertheless, why the ER stress response occurs during OPLL and its major contribution to this process remains largely unknown.

In this study, we applied gain- and loss-of-function tools to delineate the contribution of Cx43 and the ER stress response within ligament fibroblasts under stimuli inducing OPLL and the crosstalk between Cx43 signaling and the ER stress response.

Materials and methods

Patient information

Sixteen control individuals and 52 patients with OPLL who underwent anterior cervical decompression surgery were enrolled in this study. OPLL was diagnosed by a series of clinical examinations including X-ray photographs, computed tomography, and magnetic resonance imaging of the cervical spine. Informed consent was obtained from each subject, and this study was approved by the ethics committee of our hospital. Detailed information of the study subjects is listed in **Table 1**.

Primary ligament fibroblast cell culture

Posterior longitudinal ligament specimens from all study subjects were harvested during anterior cervical decompression surgery [7]. The ligament tissues were dissected carefully from a non-ossified site to avoid any possible contamination with osteogenic cells. The tissues were minced into 0.5-mm^3 pieces and washed several times with phosphate-buffered saline (PBS). Afterwards, the tissue fragments were placed into culture dishes and maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells derived from the cultured explants were digested with trypsin and further cultured at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. A compound named 12/15-Lipoxygenase inhibitor (5,8, 11-eicosatriynoic acid, Sigma-Aldrich; E3140) was used to induce ER stress with a dose of 20 nM while 4-phenylbutyrate (4-PBA; Sigma-Aldrich, St. Louis, MO; P21005) was used as ER stress inhibitor with a dose of 10 nM.

Flow cytometry measurement of reactive oxygen species

Following the indicated treatments, the cells were stained with a 10 μM dichloro-dihydro-fluorescein diacetate working solution for 20 min at 37°C. After incubation, the cells were washed three times with PBS and resuspended for reactive oxygen species (ROS) detection at an excitation wavelength of 480 nm and emission wavelength of 525 nm on a flow cytometer. Cells treated with Rosup, an active oxygen-positive control reagent, were used as a positive control. The X-axis in the figures indicates ROS signal strength as assessed by flow cytometry. The experiments were repeated three times independently.

Table 2. All siRNA sequences used in silencing Cx43 and PERK

Name	siRNA sequence
Cx43-1	5'-CCTGGCTCATGTGTTCTAT-3'
Cx43-2	5'-GCGTTAAGGATCGGGTTAA-3'
Cx43-3	5'-CCGCAATTACAACAAGCAA-3'
PERK-1	5'-GCAATGAGAAGTGGAATTT-3'
PERK-2	5'-GCAGTCATCAGTCAGAATT-3'
PERK-3	5'-CCGGGCATTGTTAGATATT-3'

Table 3. The primer pairs used in quantitative real-time PCR

Name	Sequence
Cx43-Forward	5'-TATTTCAATGGCTGCTCCTC-3'
Cx43-Reverse	5'-ATGGCTAGTGGCTGTAATTC-3'
PERK-Forward	5'-CAGTTCACCAAAGGTGTATC-3'
PERK-Reverse	5'-AGTCACTAACCCAAAGTCTC-3'
CHOP-Forward	5'-AACCAGGAAACGGAAACAG-3'
CHOP-Reverse	5'-TCACCATTCGGTCAATCAG-3'
GRP-78-Forward	5'-CCCGTCCAGAAAGTGTTG-3'
GRP-78-Reverse	5'-CAGCACCATACGCTACAG-3'
ALP-Forward	5'-AAGGAGGAAGCCTGGGAAG-3'
ALP-Reverse	5'-TCAGTGGTGGAGCCAAGTC-3'
OCN-Forward	5'-GCAGCGAGGTAGTGAAGAGAC-3'
OCN-Reverse	5'-GAAAGAAGGGTGCCTGGAGAG-3'
COL I-Forward	5'-GGGTCTGTGAGACGGATGTG-3'
COL I-Reverse	5'-CAGCCTGCTGGCTTCTAGTG-3'
GAPDH-Forward	5'-CACCCACTCCTCCACCTTTG-3'
GAPDH-Reverse	5'-CCACCACCCTGTTGCTGTAG-3'

Construction of gene silencing/over-expression systems

Standard procedures for plasmid construction, lentivirus production, and concentration determination were performed following a previous protocol [21]. Briefly, small interfering RNAs (siRNAs) targeting either Cx43 or PERK were designed (See **Table 2** for sequences), synthesized, and cloned into the pLKO.1 puro plasmid (Addgene, Watertown, MA). 293T cells were cultured until 90% confluence and transfected with modified pLKO.1, psPAX2, and pMD2G using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 72 h after transfection, the cells were harvested for virus collection.

Full-length Cx43 (NM_000165.4) was amplified and cloned into the pcDNA 3.1 (+) expression plasmid. The cloned sequence was verified by Sanger sequencing. The Cx43-expression plasmid was transfected into primary fibroblasts using Lipofectamine 2000 for 48-72 h.

Western blot analysis

Third passage OPLL and non-OPLL cells were lysed, and an equal amount of protein (25 μg) was loaded into each well of a 10% or 15% sodium dodecyl sulfate-polyacrylamide gel. Separated proteins were transferred onto a polyvinylidene fluoride membrane, followed by blocking for 1 h at room temperature in 5% non-fat milk in 1× Tris-buffered saline Tween 20 (TBST) buffer. The blots were incubated with the following primary antibodies overnight at 4°C: anti-Cx43 (Abcam, Cambridge, UK; Ab11370, 1:6000), anti-COL I (Abcam; Ab34710, 1:1000), anti-alkaline phosphatase (ALP; Abcam; Ab67-228, 1:500), anti-osteocalcin (OCN; Abcam, Ab-76690, 1:2000), anti-PERK (Abcam; Ab65142, 1:500), anti-CCAAT-enhancer-binding protein homologous protein (CHOP; Abcam; Ab11419, 1:1000), anti-78 kDa glucose-regulated protein/immunoglobulin heavy chain-binding protein (GRP78; Abcam; Ab21685, 1:800), anti-phosphorylated (p)-P38 (Cell Signaling Technology, Danvers, MA; #9211, 1:1000), anti-P38 (Cell Signaling Technology; #9212, 1:1000), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology; #5174, 1:2000). Finally, the membranes were washed three times with 1× TBST and incubated with an enhanced chemiluminescence kit (Millipore, Burlington, MA) for image scanning. The density of each protein band was analyzed by Image J software (National Institutes of Health, Bethesda, MD).

RNA isolation and quantitative real-time PCR

RNA from OPLL and non-OPLL cells was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. Isolated RNA (1 μg) was utilized to generate first-strand cDNA transcripts (Invitrogen) and further used in quantitative real-time PCR. A 25-μL SYBR qPCR system was adopted following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). The amplification results were analyzed automatically using the $2^{(-\Delta\Delta Ct)}$ method with ABI Prism 7300 SDS software (Applied Biosystems, Foster City, CA). The primer sequences of all targeted genes measured in this study are listed in **Table 3**.

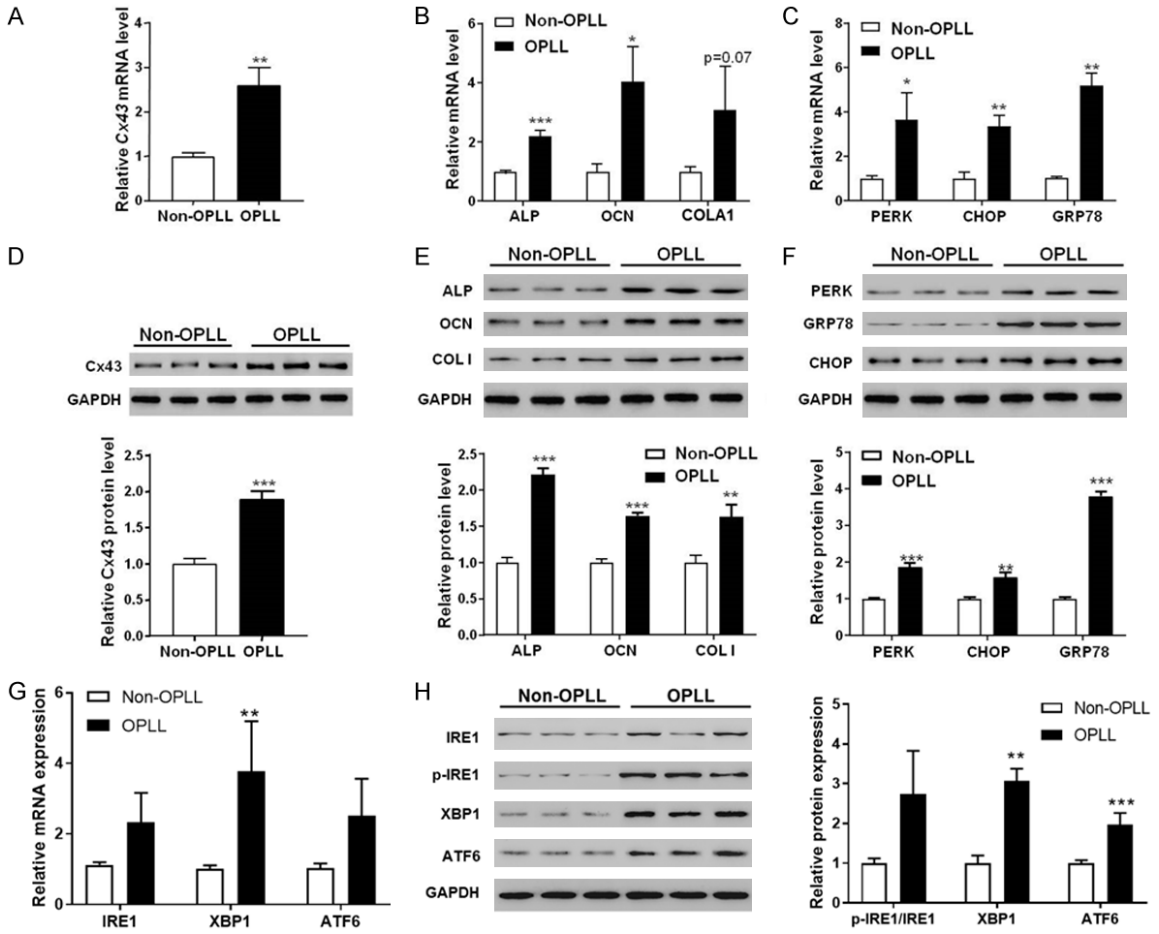

Figure 1. Cx43 expression, ossification, and ER stress were enhanced in OPLL cells. (A-C) mRNA expression levels of CX43 (A), ossification markers (ALP, OCN, and COLA1) (B) and ER stress markers (PERK, CHOP, GRP78) (C) as compared in non-OPLL and OPLL cells. (D-F) Protein expression levels of Cx43 (D), ossification markers (E) and ER stress markers (F) in non-OPLL and OPLL cells. (G, H) mRNA (G) and protein (H) levels of additional ER stress markers XBT-1, ATF6, and IRE1 were examined in non-OPLL and OPLL cells. n=3 in each group. Differences between two samples were conducted using an unpaired Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001; non-OPLL group vs. OPLL group.

Mechanical stretch apparatus

For MS loading, 3.0×10^5 cells were seeded in a Flexcell plate (Flexcell Co., Burlington, NC) for growth until 70% confluence [7]. The cells were then synchronized using DMEM with 1% FBS for 24 h. Subsequently, the cells were subjected to a cyclic tensile strain of 10% elongation at a frequency of 0.5 Hzusing a Flexcell 4000 Strain Unit (Flexcell Co.) [7].

Statistical analysis

Data are presented as the mean ± standard deviation. Differences between two samples were conducted using an unpaired Student's *t*-test. For comparisons of more than two groups, one-way analysis of variance followed by a post hoc test was performed. *P*-values <0.05 were considered statistically significant.

Results

The ER stress response is activated in OPLL cells

Spinal ligament fibroblasts derived from non-OPLL and OPLL patients were isolated and cultivated. Cx43 mRNA and protein levels were induced in the OPLL group (**Figure 1A** and **1D**). In parallel, the OPLL group demonstrated enhanced ossification as reflected by the induction of osteogenic markers, namely, ALP, OCN, and COL I, at the mRNA (**Figure 1B**) and protein

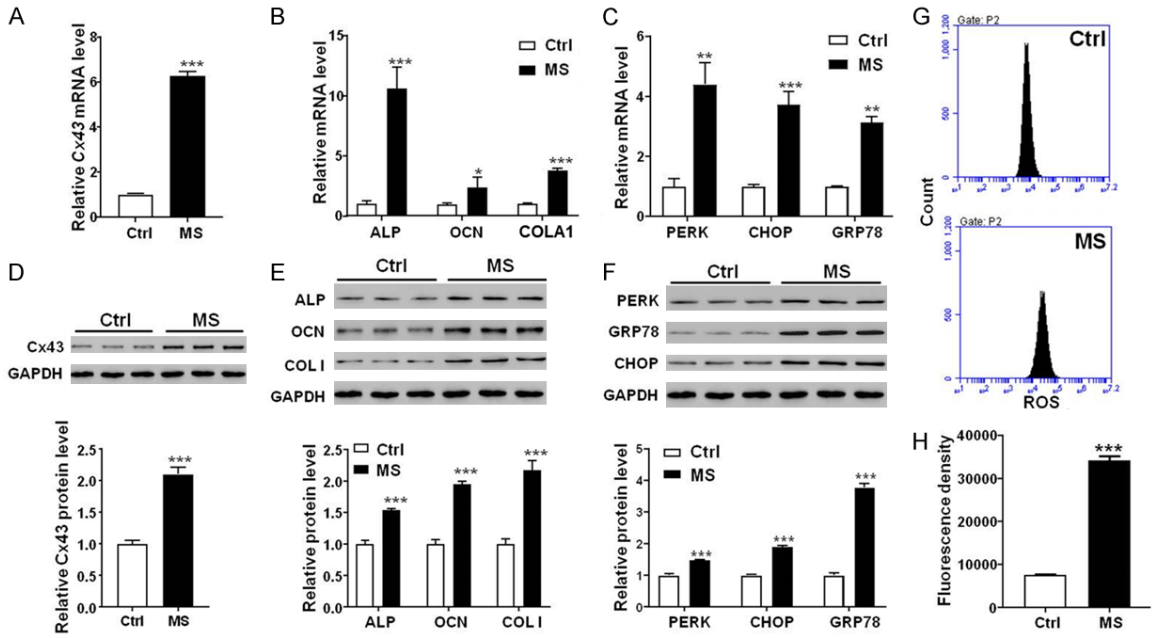

Figure 2. Mechanical stress triggers ossification, ER stress, ROS production, and Cx43 expression. (A-C) mRNA expression levels of CX43 (A), ossification markers (B) and ER stress markers (C) in mechanical stress (MS) treated OPLL cells as compared with non-treatment control (Ctrl). (D-F) Protein expression levels of Cx43 (D), ossification markers (E) and ER stress markers (F) in MS treated cells. (G, H) Representative flow cytometry images (G) and quantification (H) of reactive oxygen species (ROS) levels in MS treated cells. Differences between two samples were conducted using an unpaired Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001; Ctrl group vs. MS group.

(**Figure 1E**) levels. To assess the status of ER stress in OPLL cells, we compared some hallmark marker genes, such as PERK, GRP78, CHOP, X-box binding protein 1 (XBT-1), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). As illustrated in **Figure 1C**, **1F-H**, all of these ER stress markers examined were significantly induced at the mRNA and protein levels.

MS increases ER stress and oxidative stress during ossification

MS is one of the major risk factors leading to OPLL, partially through the up-regulation of Cx43 expression. In accordance with our previous findings [7], MS induced the expression of Cx43 (**Figure 2A** and **2D**) and ossification markers (**Figure 2B** and **2E**) in OPLL cells. Similar to OPLL, ER stress was induced by MS treatment, as indicated by increases in the levels of ER stress markers (**Figure 2C** and **2F**). Accumulating evidence suggests that ER stress and ROS generation are closely linked events [22], and as expected, we observed the accumulation of ROS in OPLL cells following MS (**Figure 2G** and **2H**).

Ameliorated ER stress coincides with Cx43 perturbation

An siRNA targeting Cx43 (siCx43) was transfected into OPLL cells predisposed to ER stress by MS. Compared with the scramble group (siNC), siCx43 inhibited the expression of endogenous Cx43 (**Figure 3A** and **3C**), while there was no difference in its levels between the non-transfection and siNC groups. Inhibition of Cx43 suppressed the expression of ER stress markers (**Figure 3B** and **3D**). Accordingly, blockage of Cx43 expression ameliorated MS-induced ROS generation (**Figure 3E** and **3F**).

Cx43 over-expression induces ER stress and oxidative stress

We performed a gain-of-function assay by over-expressing Cx43 in OPLL cells. At the mRNA and protein levels, the over-expression vector significantly induced Cx43 expression (**Figure 4A** and **4C**) compared with empty vector (Vector group). Meanwhile, we found Cx43 over-expression drastically increased ER stress (**Figure 4B** and **4D**) and oxidative stress (**Figure 4E** and **4F**).

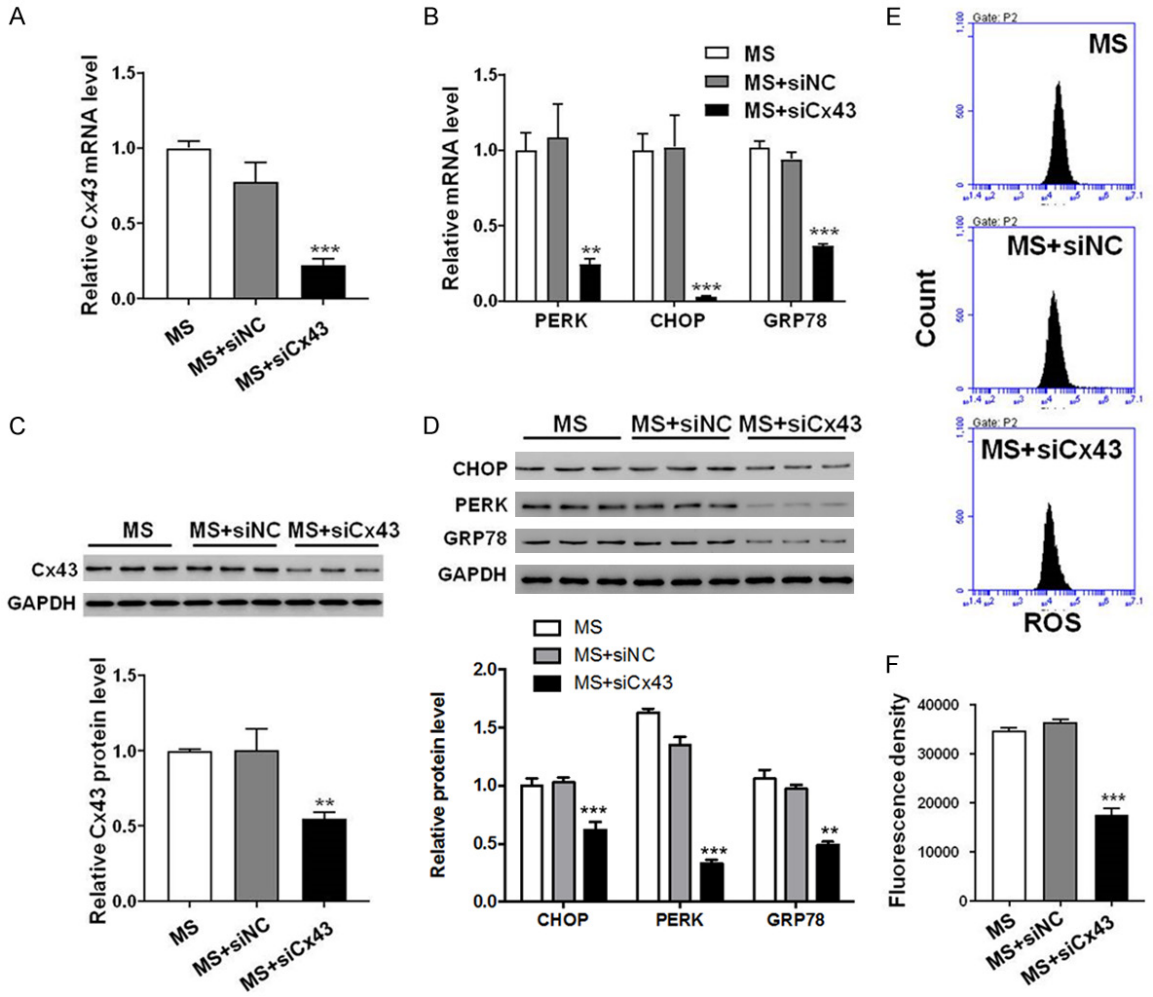

Figure 3. Cx43 silencing dramatically attenuates ER stress and ROS generation. (A, B) mRNA expression levels of CX43 (A) and ER stress markers (B) in MS-treated OPLL cells with Cx43 silenced and as compared to non-transfected control and non-specific siRNA control (NC). (C, D) Protein expression levels of Cx43 (C), and ER stress markers (D) in Cx43 silenced cells. (E, F) Levels of ROS as assessed by flow cytometry (E) followed with quantification (F) in MS-treated cells that are transfected with siRNAs. n=3 in each group. Differences were conducted using ANOVA followed by a post hoc test. **P*<0.05, ***P*<0.01, ****P*<0.001; MS+siNC (scramble) group vs. MS+siCx43 group.

Inhibition of ER stress blocks ossification and the related MAPK signaling pathway

Given the significant augmentation of ER stress during the development of OPLL, we blocked this excessive response using chemical treatment with 4-phenylbutyrate (4-PBA; Sigma-Aldrich, St. Louis, MO; P21005), an ER stress inhibitor and observed that it not only dramatically ameliorated MS-induced ER stress and ossification in OPLL cells (**Figure 5A** and **5B**) but also inhibited the MS-induced MAPK signaling pathway (**Figure 5C**). In parallel, 4-PBA also improved the ROS generation induced by MS (**Figure 5D** and **5E**). Similarly, siRNA induced silencing of the major ER stress transducer PERK (**Figure 6A** and **6B**) significantly attenuated MS-induced Cx43 expression (**Figure 6C**) and the expression of ossification markers (**Figure 6D**).

Activation of ER stress mobilizes the MAPK pathway and ossification of OPLL cells

In order to confirm the role Cx43 plays in ER stress stimulation, ER stress activation was induced by a 12/15-Lipoxygenase inhibitor in OPLL cells (**Figure 7A**). As expected, promoted ER stress could enhance ossification (**Figure 7B**), the MAPK signaling pathway (**Figure 7C**),

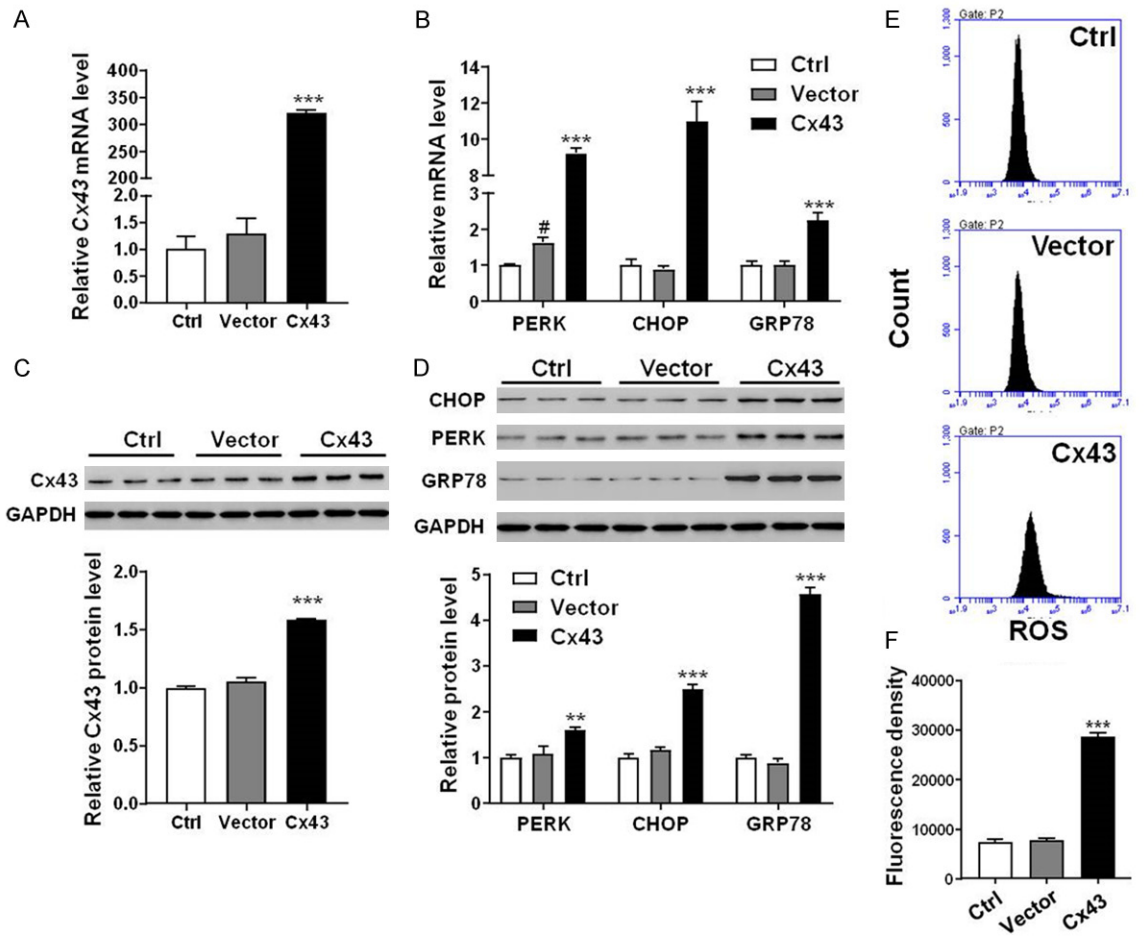

Figure 4. Cx43 over-expression augments ER stress and oxidative stress. (A, B) mRNA expression levels of CX43 (A) and ER stress markers (B) in MS-treated OPLL cells with Cx43 over-expressed as compared to non-transfected control and empty vector control (Vector). (C, D) Protein expression levels of Cx43 (C), and ER stress markers (D) in Cx43 over-expressed cells. (E, F) Levels of ROS as assessed by flow cytometry (E) followed with quantification (F) in MS-treated cells that are transfected with over-expressing vectors. n=3 in each group. Differences were conducted using ANOVA followed by a post hoc test. **P*<0.05, ***P*<0.01, ****P*<0.001; Vector (empty vector) group vs.Cx43 over-expressed (Cx43) group.

as well as the ROS production (**Figure 7D** and **7E**) in OPLL cells under MS. As illustrated above, MS- and OPLL-related ossification partially rely on crosstalk between Cx43 and the ER stress response. Upon treatment with the ER stress activator, we observed that Cx43 knockdown, although not entirely, attenuated the augmentation of ER stress (**Figure 7A**), the associated ossification (**Figure 7B**), and ROS generation (**Figure 7D** and **7E**).

Assessment of Cx43 and PERK expression in human OPLL samples

We analyzed the expression of Cx43 and PERK in non-OPLL and OPLL human samples. Echoing our *in vitro* study results, Cx43 (**Figure 8A**) and PERK (**Figure 8B**) mRNA levels and protein levels (**Figure 8C**) were significantly induced in human ligament tissues from OPLL patients as measured from a relatively large population pool.

Discussion

OPLL is a common disease within East Asian populations where the morbidity of subjects over 65 years of age is 20-34% [23]. Due to a lack of understanding of its underlying mechanisms, so far, the only effective treatment is surgery, but patients undergoing surgery may still experience later deterioration because of

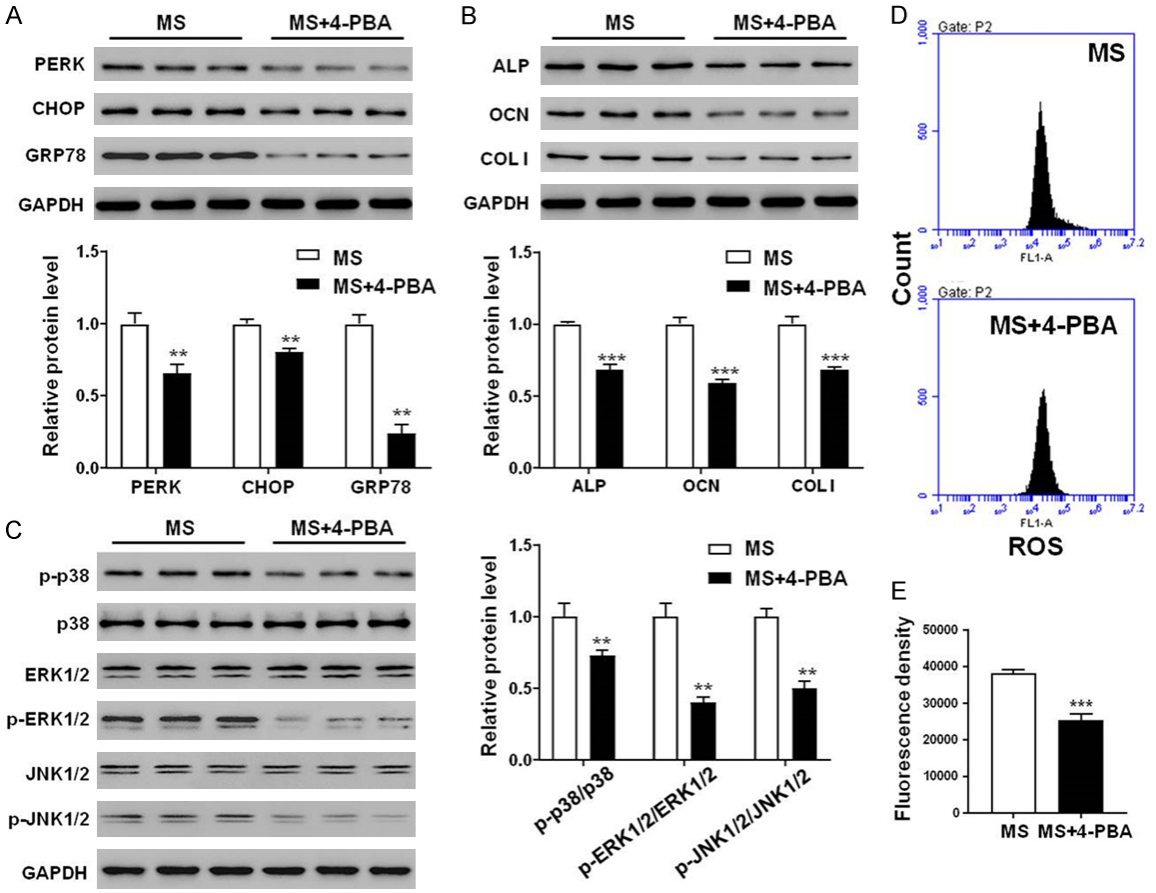

Figure 5. Inhibition of ER stress ameliorates MS-induced ossification. (A) Protein expression levels of ER stress markers in OPLL cells treated with the ER stress inhibitor 4-PBA. (B) Protein levels of ossification markers in OPLL cells treated with 4-PBA. (C) Protein levels of MAPK signaling pathway components in OPLL cells. (D, E) ROS generation levels in 4-PBA treated OPLL cells as assessed by flow cytometry (D) followed with quantification (E). n=3 in each group. Differences between two samples were conducted using an unpaired Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001; MS group vs. MS+4-PBA group.

disease progression [24]. In this study, we uncovered a previously undefined contributor, namely, the ER stress response, to the development of OPLL, and more importantly, identified a strong link between ER stress and Cx43-mediated OPLL development.

On the basis of our previous studies and literature, the expression of OCN demonstrates osteogenesis, ALP provides phosphonic acid for the deposition of hydroxyapatite crystals, and COL I is essential for bone formation and bone strength. These three markers may represent different stages of bone formation [2, 25]. In line with our previous findings, OPLL cells and non-OPLL cells exposed to MS undergo a bone formation-like ossification process, as indicated by the increased expression of ALP, OCN, and COLA1 genes and the coded proteins [7, 8, 13]. The induction of osteogenesis is a complex process involving the interaction of new protein synthesis, the actions of various stress response mechanisms, and related signaling pathways. Under pathological conditions, detrimental factors disturb this homeostasis and result in imbalanced and excessive stress responses. Several studies have demonstrated that the ER stress response is closely related to osteogenesis in osteoblasts, periodontal ligament cells, and other cell types [26-28]. For the first time, our study comprehensively reports the robust activation of the ER stress response under pathological osteogenic development in posterior longitudinal ligament fibroblasts, which are believed to be vital players in the incidence of OPLL.

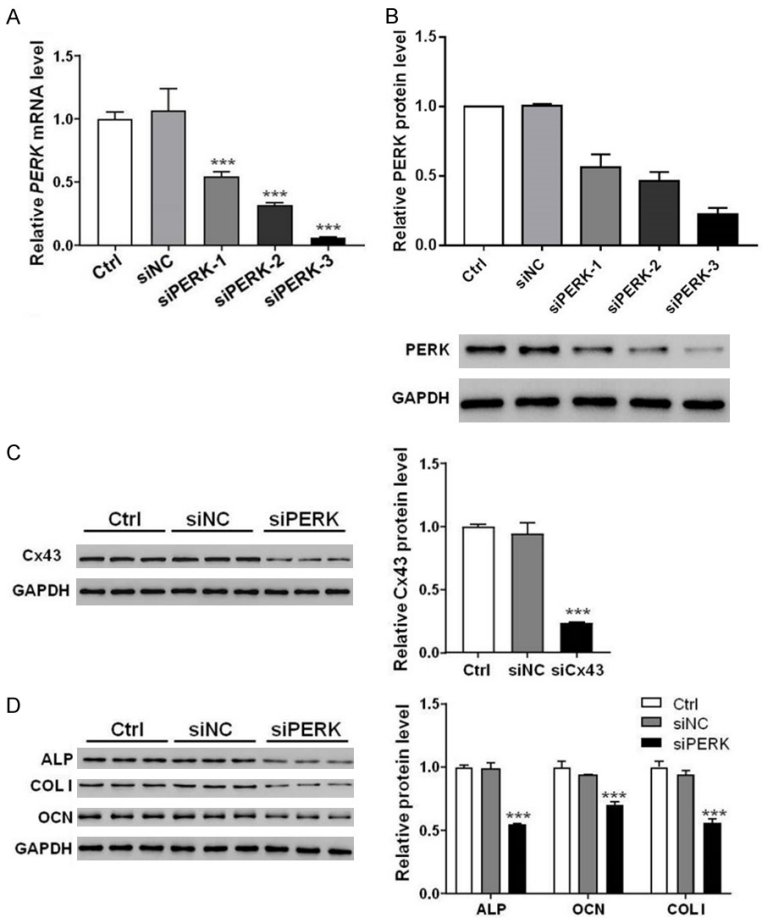

Figure 6. PERK silencing suppresses ossification and Cx43 expression. (A, B) mRNA (A) and protein (B) expression levels of PERK in OPLL cells after transfected with PERK siRNAs. (C, D) Protein levels of Cx43 (C) and ossification markers (D) in PERK silenced OPLL cells. n=3 in each group. Differences were conducted using ANOVA followed by a post hoc test. **P*<0.05, ***P*<0.01, ****P*<0.001; siNC (scramble) group vs. siPERK group.

PERK is a major transducer of the ER stress response. It contains a luminal regulatory domain and a cytoplasmic eIF2a kinase domain, which is conserved among the four-member family of kinases that regulate translation initiation in mammals [29, 30]. A high concentration of calcium within the ER lumen inhibits the dimerization and activation of PERK by promoting the binding of PERK with ER chaperones [31, 32]. CHOP is a multifunctional transcription factor in the ER stress response that is necessary for bone formation [33] and mediates ATF4-induced cell apoptosis [34]. Inhibition of the ER stress response, either using a chemical compound or siRNA targeting PERK, significantly repressed the induction of oxidative stress and consequent ossification of ligament fibroblasts, which is in accordance with our previous findings [19]. On the contrary, chemical activation of ER stress significantly augmented the ossification of ligament cells. Together, these observations emphasize the positive association of the ER stress response with osteogenesis.

Cx43 protein plays a major role in the development of OPLL, as confirmed by our previous studies [7, 13-15]. We have shown that Cx43 promotes OPLL partially through the MAPK pathway and the NF-κB inflammation signaling pathway. Given the excessive expression of Cx43 protein during osteogenesis identified previously, we speculated that it might also trigger the ER stress response, which is specifically responsible for the removal of incorrectly folded proteins. As expected, the manipulation of Cx43 expression altered the ER stress response accordingly in both gain- and loss-of-function tests. That being said, the ER stress response acts as a surveillance mechanism to control Cx43-mediated osteogenesis during the development of OPLL. However, whether or not the ER stress response specifically controls Cx43-mediated signaling remains poorly understood. On the basis of our preliminary data (**Figure 6C**), the amelioration of an excessive ER stress response controlled Cx43 expression and downstream signaling. Evidentially, as we proposed previously, the p38 MAPK pathway is involved in Cx43-mediated OPLL [14]. The ER stress response also alters MAPK signaling together with modification of Cx43 expression. This indicates that ER stress might potentially modulate Cx43-related pathways in OPLL in a more specific way. However, this regulatory mechanism requires further investigation.

To further confirm our observations from the *in vitro* study, we examined Cx43 and PERK

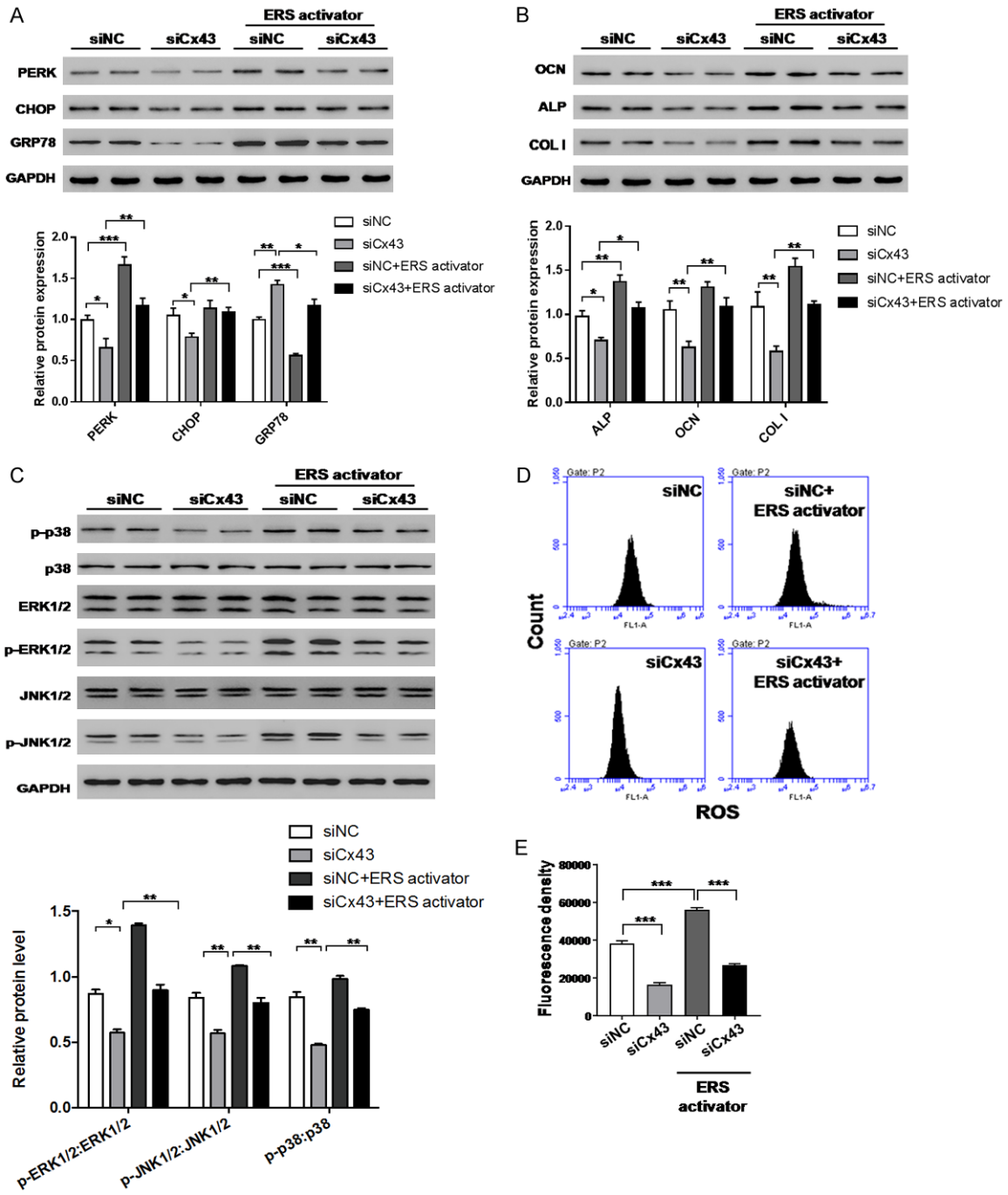

Figure 7. Cx43 knockdown inhibits MAPK pathway activation, ER stress-induced ossification, and oxidative stress. (A-C) Protein expression levels of ER stress markers (A), ossification markers (B) and a few key components of the MAPK signaling pathway (C) in Cx43 silenced OPLL cells with or without ER stress activation. (D, E) ROS generation levels in Cx43 silenced OPLL cells with or without ER stress activation as assessed by flow cytometry (D) followed with quantification (E). n=3 in each group. Differences were conducted using ANOVA followed by a post hoc test. **P*<0.05, ***P*<0.01, ****P*<0.001; comparisons within groups are indicated.

expression in 52 OPLL patients. Compared with non-OPLL individuals, there was a significant up-regulation of Cx43 and PERK mRNA levels. This is the first screening of genes in a large population of patients with OPLL, the findings of which were in line with our previous observations of Cx43 induction in a smaller group of patients [14]. The increase of PERK mRNA

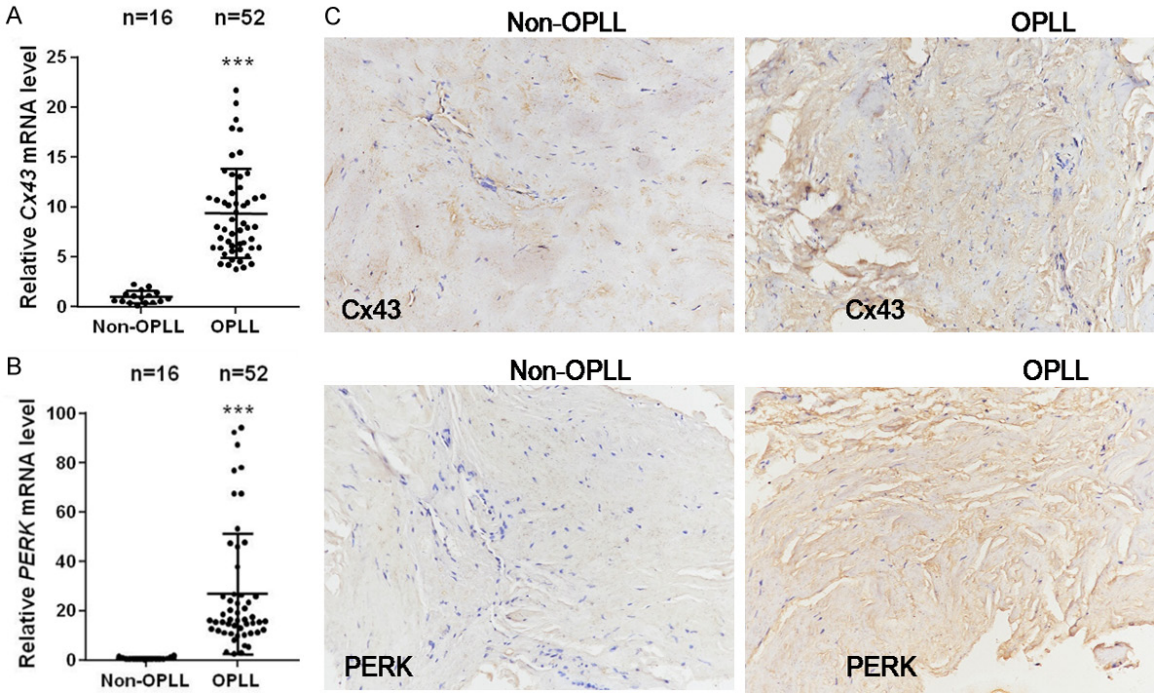

Figure 8. Expression of Cx43 and PERK in non-OPLL and OPLL tissues. A, B: mRNA expression levels of CX43 and PERK as compared in ligament tissue from non-OPLL and OPLL patients. Sample numbers are indicated at the top of each group. C: Representative images of immunohistochemical (IHC) stains of Cx43 and PERK in ligament tissue as indicated. Differences between two samples were conducted using an unpaired Student's *t*-test. ***P<0.001; OPLL group vs. non-OPLL group.

expression further confirms our data from an *in vitro* cell culture system and provides an insight into the role of ER stress as an underlying mechanism of OPLL and as a potential therapeutic target.

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Disclosure of conflict of interest

None.

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