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Full Length Article

Long non-coding RNA MALAT1 functions as miR-1 sponge to regulate Connexin 43-mediated ossification of the posterior longitudinal ligament

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ABSTRACT

Ossification of the posterior longitudinal ligament (OPLL) is the major cause for several deteriorate bone and joint diseases. Its development is a highly organized dynamic process as modulated by various physiological and pathophysiological factors. Both long non-coding RNAs (lncRNAs) and small non-coding RNAs (miRNAs) have been postulated to involve into almost all the biological conditions. Here, we applied high through-put transcriptome screening to unveil lncRNAs highly regulated under OPLL condition. siRNA assay in combination with western blot and quantitative PCR deciphered the lncRNA and miRNA functions in OPLL and their underlying mechanism. Here we identified an lncRNA, named Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) engaged into the development of OPLL by indirectly targeting Connexin 43 (Cx43) gene. As previously reported, Cx43 is one of the main proteins contributing to OPLL partially through enhancing inflammatory signaling. On top of that, we provided another regulatory layer that MALAT1 served as the upstream effector governing the transcription of Cx43 gene. Perturbation of MALAT1 significantly inhibited Cx43 expression, inflammation, and osteogenesis. Mechanistically, *in silico* analysis and experimental validation both confirmed that microRNA-1 (miR-1) was the mediator connecting MALAT1-Cx43 axis: overexpression of miR-1 diminished Cx43 expression and OPLL process; meanwhile, MALAT1 acted as miR-1 sponge to inhibit its suppressive transcription effect on downstream ossification related genes. Knock-down of MALAT1 released sequestered miR-1, which repressed Cx43 expression and associated OPLL. Likewise, induced OPLL caused by overexpression of MALAT1 can be ameliorated by enhanced miR-1 function, knock-down of Cx43 or inhibition of inflammation. More importantly, further validation using patient ligament samples from non-OPLL and OPLL individuals identified MALAT1-miR-1-Cx43 regulatory axis. Collectively, we found a novel mechanism through lncRNA-miRNA interaction that provides more insights into understanding the development of OPLL.

1. Introduction

Ossification of the posterior longitudinal ligament (OPLL) features the pathological development progress in various bone or joint deterioration diseases. It is the condition that posterior longitudinal ligament structure becomes less flexible due to ectopic bone formation in the cervical spinal ligament [1–3]. So far, surgery plus anti-inflammatory and pain-control medication are the most effective treatments, although the clinical outcomes are usually unsatisfactory due to the recurrence or worsen of the symptoms, which manifest our lack of deep knowledge about the pathological process during the development of OPLL and less understanding of the etiology leading to this disease.

Studies from other groups and our previous results mapped several novels, potential mechanisms underlying this disease using both *in vivo* tissues and *in vitro* primary cultured fibroblasts from non-OPLL and OPLL patients [4–8]. Various molecular signaling pathways were brought into our attention, *i.e.* oxidative stress [8], inflammation [8,9], mitogen-activated protein kinases (MAPK) pathways [7]. Pro-inflammatory cytokines (*i.e.* Tumor necrosis factor (TNF)α and Interlukin-6 (IL-6) [8]) and key transcription factors [10] are tightly involved during the development of OPLL, *i.e.* nuclear factor (NF)-kB is one of the major nuclear factors facilitating the osteogenesis by enhancing inflammation response [8]. During our previous studies, we found that a gap-junction protein, Connexin 43 (Cx43) was crucial mediating the ossification of ligament fibroblasts [5–8]. Its transcription and translation were up-regulated by OPLL risk factors, such as

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Table 1
MALAT1 siRNA target position and sequence.

MALAT1 (NR_002819.4)	siRNA target position and sequence
Site 2594	5′-GCATTGGACTTTGAGTTAA-3′
Malat1-1-F	CCGGGCATTGGACTTTGAGTTAACTCGAGTTAACTCAAAGTCCAATGCTTTTT
Malat1-1-R	AATTAAAAAGCATTGGACTTTGAGTTAACTCGAGTTAACTCAAAGTCCAATGC
Site 4210	5′-CCAGAGAACTTAAAGTCTT-3′
Malat1-2-F	CCGGCCAGAGAACTTAAAGTCTTCTCGAGAAGACTTTAAGTTCTCTGGTTTTT
Malat1-2-R	AATTAAAAACCAGAGAACTTAAAGTCTTCTCGAGAAGACTTTAAGTTCTCTGG
Site 4971	5′-GCTCCTTGGTGAATTGATA-3′
Malat1-3-F	CCGGGCTCCTTGGTGAATTGATACTCGAGTATCAATTCACCAAGGAGCTTTTT
Malat1-3-R	AATTAAAAAGCTCCTTGGTGAATTGATACTCGAGTATCAATTCACCAAGGAGC

Table 2
Cx43 siRNA target position and sequence.

Cx43 NM_000165.4	siRNA target position and sequence
Site 548	5′-CCTGGCTCATGTGTTCTAT-3′
Cx43-1-F	CCGGCCTGGCTCATGTGTTCTATCTCGAGATAGAACACATGAGCCAGGTTTTT
Cx43-1-R	AATTAAAAACCTGGCTCATGTGTTCTATCTCGAGATAGAACACATGAGCCAGG
Site 976	5′-GCGTTAAGGATCGGGTTAA-3′
Cx43-2-F	CCGGGCGTTAAGGATCGGGTTAACTCGAGTTAACCCGATCCTTAACGCTTTTT
Cx43-2-R	AATTAAAAAGCGTTAAGGATCGGGTTAACTCGAGTTAACCCGATCCTTAACGC
Site 1166	5′-CCGCAATTACAACAAGCAA-3′
Cx43-3-F	CCGGCCGCAATTACAACAAGCAACTCGAGTTGCTTGTTGTAATTGCGGTTTTT
Cx43-3-R	AATTAAAAACCGCAATTACAACAAGCAACTCGAGTTGCTTGTTGTAATTGCGG

Table 3
has-miR-1-3p inhibitor and mimics sequence.

Name	Sequence
Has-miR-1-3p inhibitor	5′-AUACAUACUUCUUUACAUUCCA-3′
Has-miR-1-3p mimics	5′-UGGAAUGUAAAGAAGUAUGUAU-3′
	5′-AUACAUACUUCUUUACAUUCUU-3′
Inhibitor NC	5′-CAGUACUUUUGUGUAGUACAA-3′
Mimics NC	5′-UUGUACUACACAAAAGUACUG-3′

Table 5
qPCR primers.

Name	Sequence
Cx43-F	5′ TATTTCAATGGCTGCTCCTC 3′
Cx43-R	5′ ATGGCTAGTGGCTGTAATTC 3′
COL1A1-F	5′ GGCCAAGACGAAGACATCCC 3′
COL1A1-R	5′ GTTGTCGCAGACGCAGATCC 3′
RUNX2-F	5′ ATAGCGTGGTTCTCTTTG 3′
RUNX2-R	5′ TAATGGTGTGGACTTCTC 3′
OPG-F	5′ TTTACGCCTAACTGGCTTAGTG 3′
OPG-R	5′ AGCTGGAAAGTCTGTTCTTGTG 3′
OCN-F	5′ GCAGCGAGGTAGTGAAGAGAC 3′
OCN-R	5′ GAAAGAAGGGTGCCTGGAGAG 3′
ALP-F	5′ AAGGAGGAAGCCTGGGAAG 3′
ALP-R	5′ TCAGTGGTGGAGCCAAGTC 3′
NF-kB p65-F	5′ GAATGGCTCGTCTGTAGTG 3′
NF-kB p65-R	5′ TGGTATCTGTGCTCCTCTC 3′
MALAT1-F	5′ TGAAGAGGCAATGTCCATC 3′
MALAT1-R	5′ ATACCTGTCTGAGGCAAAC 3′
GAPDH-F	5′ CACCCACTCCTCCACCTTTG 3′
GAPDH-R	5′ CCACCACCCTGTTGCTGTAG 3′

mechanical stress [5]. Mechanistically, Cx43 activation was not only tightly associated with enhanced oxidative stress, inflammatory signaling, and consequent osteogenesis, it also directly coordinated the forementioned pathways. Despite the large amount of research focusing on this pathological condition, deeper and updated work regardingly is still urgently needed, especially in Cx43-assosiated OPLL incidence.

Non-coding RNAs includes, but not limited to long non-coding RNAs (lncRNAs) and microRNAs (miRNAs or miR), depending on the transcription length [11]. miRNAs are non-transcriptional RNA transcript with ~22 nucleotides. They bind to their target through seed-sequence complementary principle to inhibit mRNA transcription [12]. lncRNAs, on the other hand, are non-coding RNAs with a length of > 200 nucleotides. Based on their subcellular locations, they can function as genetic/epigenetic modifiers, transcription factor decoy, miRNA sponges *etc.* [11]. Both miRNA and lncRNA participate almost all the biological conditions, including osteogenesis [12–14]. However, there is very limited information about their roles in ossification of posterior longitudinal ligament tissues. Several studies have indicated the post-transcriptional regulation of Cx43 expression through miRNAs in

Table 4
miRNA qPCR primers.

Name	Sequence
hsa-miR-613 sense[a]	5′-ACACTCCAGCTGGG AGGAATGTTCCTTC-3′
hsa-miR-613 antisense[b]	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGCAAA-3′
hsa-miR-206 sense[a]	5′-ACACTCCAGCTGGG TGGAATGTAAGGAAGT-3′
hsa-miR-206 antisense[b]	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCACACC-3′
hsa-miR-1 sense[a]	5′-ACACTCCAGCTGGG TGGAATGTAAAGAAGT-3′
hsa-miR-1 antisense[b]	5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTACATA-3′
hsa-U6 F	5′-CTCGCTTCGGCAGCACA-3′
hsa-U6 R	5′-AACGCTTCACGAATTTGCGT-3′

[a] Together with TRP primer for quantification.
[b] For reverse-transcription reaction.

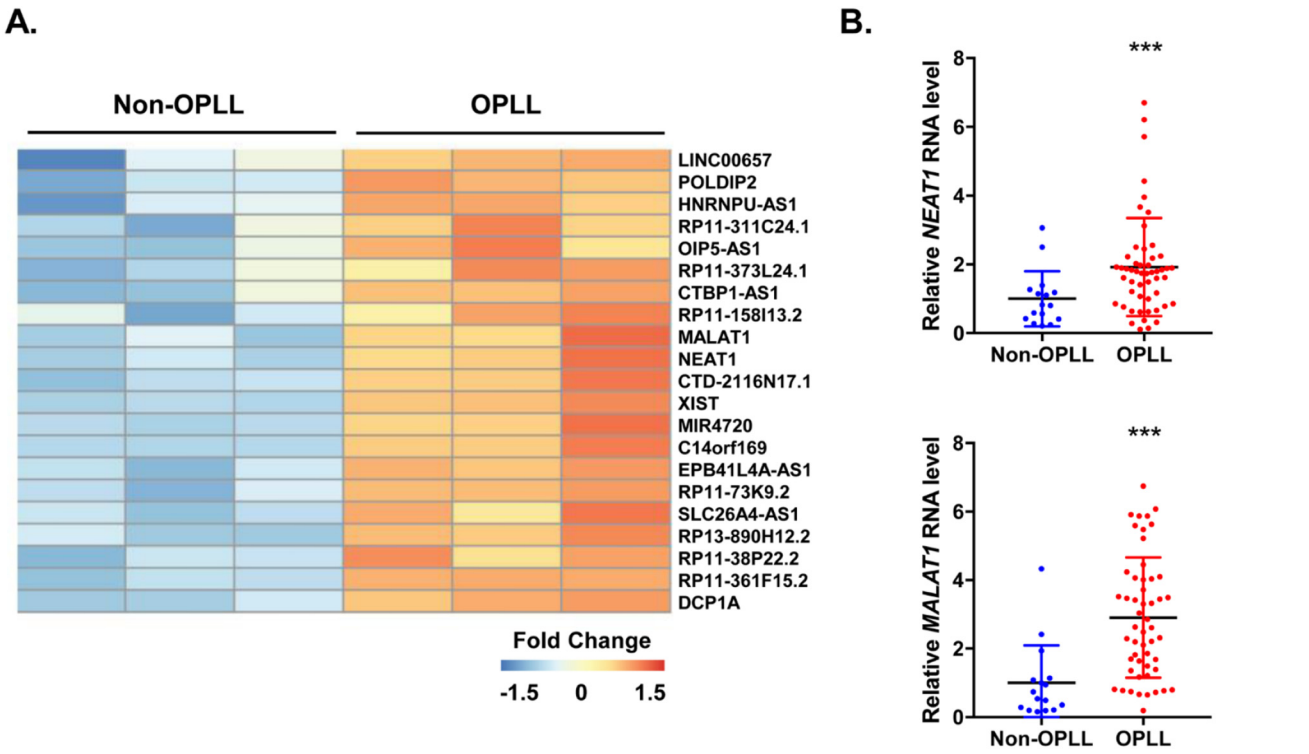

Fig. 1. Identification of highly-upregulated lncRNAs in OPLL ligament samples. A) LncRNA microarray showed the top 21 up-regulated lncRNA in OPLL samples. N = 3 in each group. (B) qPCR quantification of MALAT1 and NEAT1 from non-OPLL (n = 16) and OPLL patients (n = 52) samples. p*** < 0.001 compared to non-OPLL.

various different systems [15–19]. miR-1 exacerbates arrhythmogenesis through targeting Cx43 in heart and therefore presents as a strong anti-arrhythmia target [20]. miR-130a shows the similar cardiovascular function inducing arrhythmia through Cx43 [16]. During the differentiation of mammary cancer stem-like cells, miR-206 represses Cx43 to facilitated prosurvival signaling [21]. Nevertheless, none of them have specifically addressed miRNA regulatory machinery on Cx43 in OPLL. So far, there is no study reporting the regulation of Cx43 by lncRNAs.

Thus, we applied high through-put lncRNA microarray to screen non-OPLL and OPLL patient samples identifying differentially expressed lncRNA profiles, in together with *in silico* analysis and biological validation to reveal potential lncRNA-miRNA-Cx43 regulatory connections.

2. Material and method

2.1. Patient information

In total 16 control individuals (non-OPLL) and 52 OPLL patients undergoing anterior cervical decompression surgery were enrolled into this study. The OPLL was diagnosed by a series of clinical examinations including X-ray photographs, computed tomography, and magnetic resonance imaging of the cervical spine. The detailed information was listed in Supplemental Table 1. Informed consent was obtained from each patient.

2.2. Primary ligament fibroblasts cell culture

Posterior longitudinal ligament specimens were harvested during the anterior cervical decompression surgery [5]. The ligament tissues were carefully dissected from a nonossified 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. Afterwards, 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. Before the analysis of osteogenic differentiation, the OPLL cells were stimulated with osteogenic induction medium as DMEM culture mediate supplemented with 10% FBS, 25 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 10 nM dexamethasone (Gibico). Cells were maintained for 1–2 weeks, with media replaced every 3 days.

2.3. lncRNA microarray

Total RNA was isolated from tissue samples from three subjects of control and three of OPLL subjects using RNA isolation kit and library purification kit (QIAGEN). The total RNA were then applied to library preparation procedure and Agilent Human lncRNA/mRNA (4 × 180 K) microarray system to profile differentially expression of lncRNA/mRNAs. The expression data of all six samples were then normalized using quantile algorithm. Top ranked differentially expressed lncRNAs were filtered through p value (cutoff value 0.05) and fold change (cut off 2 and -2) through the Agilent GeneSpring Software (Agilent). The lncRNAs with low or invariable expression level between two groups were filtered out. All human lncRNAs were annotated by public lncRNA database ENSEML (https://useast.ensembl.org/). All the data was log 2-scale transformed. While the entire differentially expressed lncRNA need to be further studied and may be summarized elsewhere, around 20 top-ranked lncRNA were selected in this study for validation using qRT-PCR method.

2.4. Construction of gene silencing/overexpression systems

Standard procedures for plasmid construction, lentivirus production and concentration determination were performed following previous protocol [22]. Briefly, siRNAs targeting either Cx43 or MALAT1 were designed, synthesized (see Tables 1 and 2 for sequence), and further

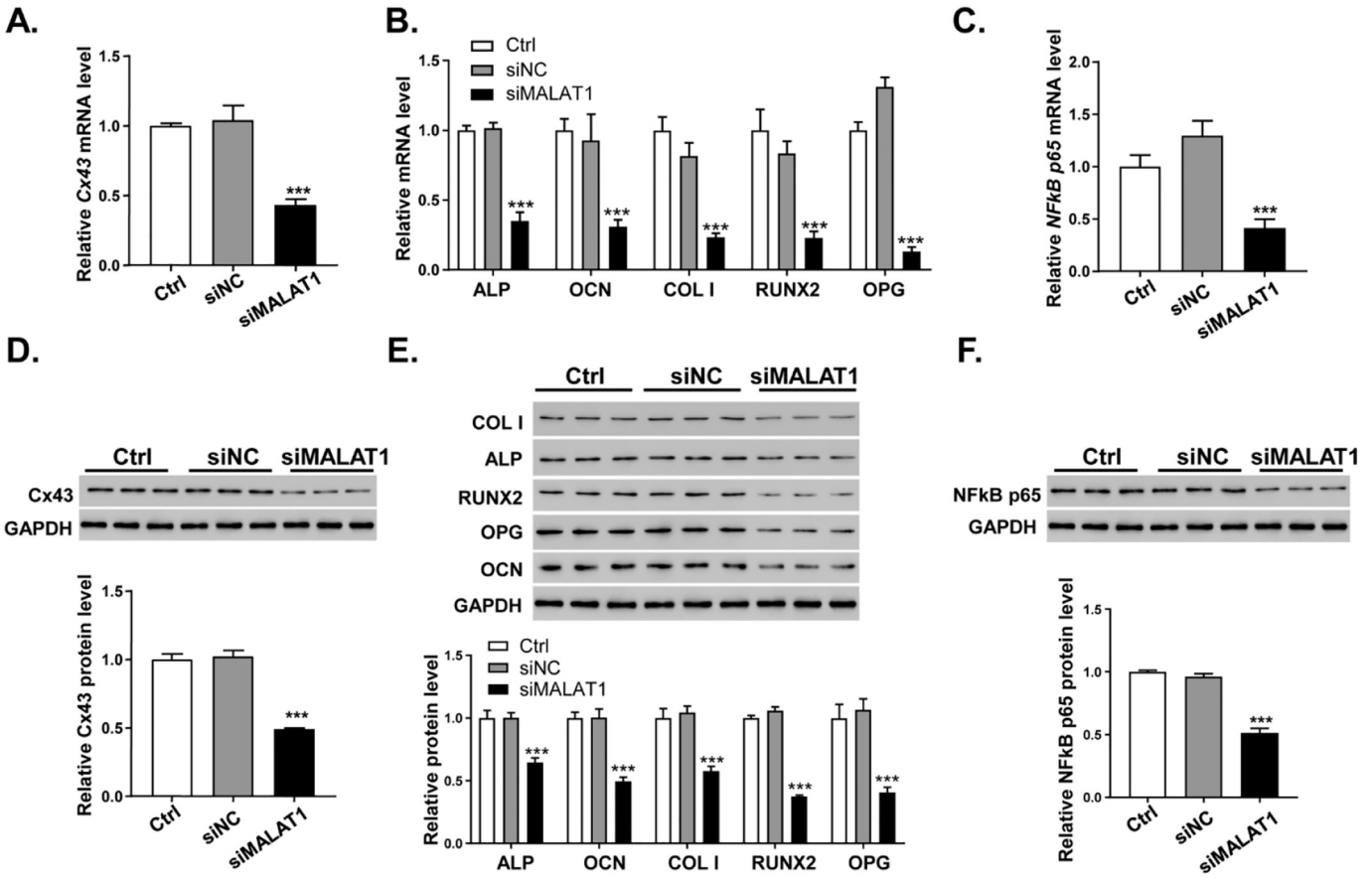

Fig. 2. Knock-down of lncRNA MALAT1 ameliorated inflammation signaling and Cx43-mediated ossification. (A&D) Infection of lentivirus with siRNA targeting MALAT1 decreased mRNA and protein levels of Cx43 in OPLL cells, as compared with scramble (siNC). There is no difference between control (Ctrl) and scramble (siNC). (B&E) Knock-down of MALAT1 inhibited osteogenesis pathway molecules expression in OPLL cells, from both mRNA and protein levels. (C&F) Knock-down of MALAT1 suppressed NFkB subunit p65 expression. N = 3 from each group, p*** < 0.001 compared to siNC.

cloned into pLKO.1 puro plasmid (Addgene). HEK293T cells (293T cells) were cultured until 90% confluence and transfected with modified pLKO.1, psPAX2, and pMD2G using Lipofectamine 2000 (Invitrogen). 72 h after the transfection, cells were harvested for virus collection.

Full-length MALAT1 (NR_002819.4) was amplified and cloned into pcDNA 3.1(+) expression plasmid. The potential mutation was verified by Sanger sequencing. A certain amount of MALAT1-expression plasmid was transfected into primary fibroblasts using Lipofectamine 2000 for 48–72 h.

2.5. miRNA inhibitors and mimics

Both miR-1 inhibitor and mimics were designed and synthesized (JRDUN Biotechnology, Shanghai, China) please see Table 3 for detailed sequence. 100 pmol of inhibitor/mimics was added into the culture medium for 24 h.

2.6. Western blot analysis

Third passage of OPLL and non-OPLL 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 X TBST buffer. The blots were then incubated with 1st antibody overnight at 4 °C (Cx43, Abcam, Ab11370, 1:6000; collagen I, Abcam, Ab34710, 1:1000; ALP, Abcam, Ab67228, 1:500; OCN, Abcam, Ab76690, 1:2000; OPG, Abcam, Ab73400, 1:1000; RUNX2, Abcam, Ab23981, 1:1000; NFkB p65, Cell Signaling Tech, #8242, 1:1000; phosphorylated NF-kB p65 (S536) antibody, Abcam Ab86299. 1:1000; GAPDH, Cell Signaling Tech, #5174, 1:2000). Finally, the membranes were washed with 1 X TBST for three times and incubated with ECL (Millipore) for image scanning. The density of each protein band was analyzed by ImageJ.

2.7. RNA isolation and quantitative PCR

RNA from OPLL and non-OPLL cells was extracted using TRIzol (Invitrogen) according to manufacturer's protocol. 1 μg of isolated RNA was applied for first-strand cDNA transcript (Invitrogen) and further used in quantitative PCR. A 25 μl SYBR 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 were listed in Table 5.

For detection of miRNA, miRNA-specific reverse-transcript primer was used to synthesize cDNA complementary to miRNA. Afterwards, a specific forward primer together with a universal terminal reverse primer were added into SYBR green mix to perform quantitative PCR. The primer sequences were listed in Table 4.

2.8. CCK8 cell proliferation assay

Cell proliferation assay was performed accordingly to manufacture's instruction (CP002, SABioscience). Briefly, 3×10^3 OPLL cells were seeded into each well in 96 well plates. On the next day, cells were treated as indicated in different experiments and mixed with Cell Counting Kit-8 (CCK-8) in the ratio of 1:10 using non-FBS culture medium at 0 h, 24 h, 48 h, and 72 h after treatment. Then these cells

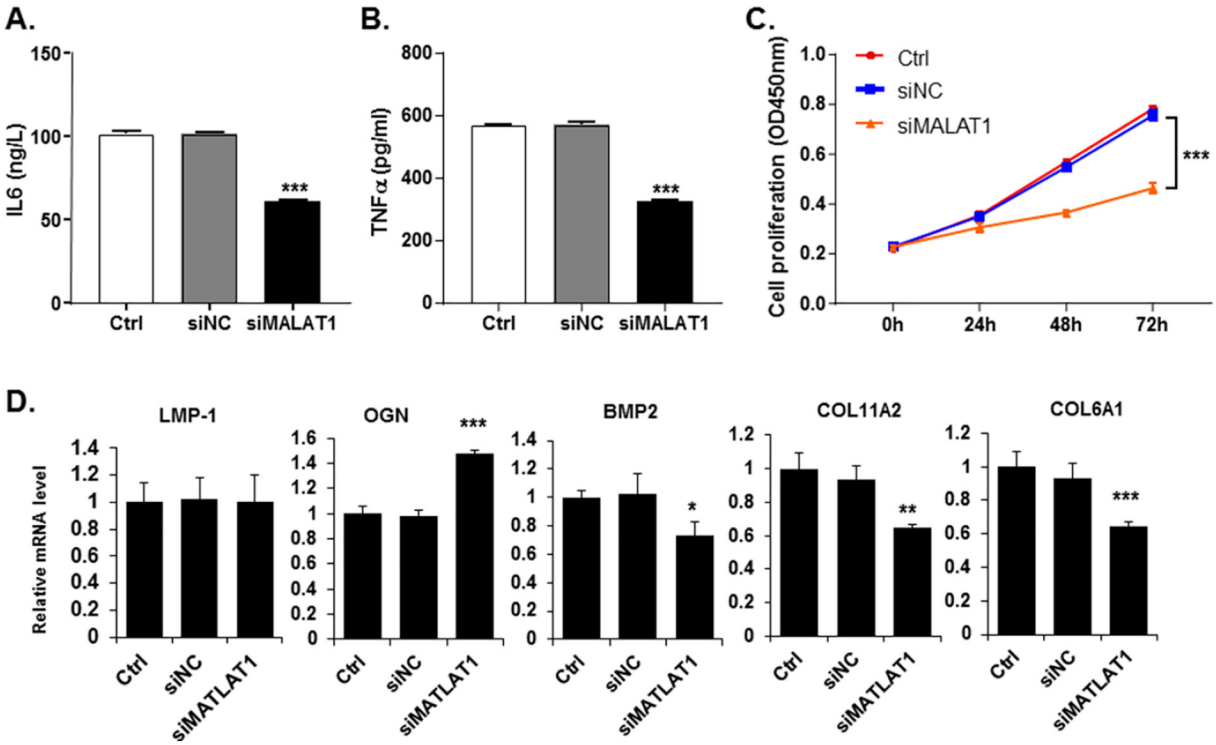

Fig. 3. Inhibition of MALAT1 expression suppressed secretion of inflammatory cytokines and cell proliferation. Detection of IL6 (A) and TNFα (B) production from OPLL cells with or without siRNA infection. (C) Cell proliferation rate of OPLL cells was assessed from time 0 h, 24 h, 48 h, and 72 h after siRNA infection. (D) mRNA levels of differentiation marker genes in si MALAT1 OPLL cells, as compared with scramble (siNC). N = 3 in each group, p*** < 0.001 compared to siNC. In C, the comparison was between siNC and siMALAT1 at 72 h.

were further incubated for 1 h at 37 °C and the absorbance was recorded at 450 nm. Triplet for each sample was performed.

2.9. The enzyme-linked immunosorbent assay for detection of IL1 and TNFα

Assays were performed following manufacture's instruction (R&D). Briefly, cells were collected with PBS with repeated freeze-thaw to release the intracellular components, centrifuged, and supernatant was collected for assay. Dilute and add samples and standard to 96 well with appropriate negative and positive control. Incubate for 30 min at 37 °C, wash five times with wash buffer then incubate with enzyme for another 30 min at 37 °C. Afterwards, wash again for five times and stop the development after 15 min incubation. The absorbance was read under 450 nm wavelength and sample concentration was calculated according to standard curve.

2.10. Dual luciferase reporter assay

Human primary ligament fibroblasts were transfected with either empty vector or pGL3 plasmid (Promega) containing 3′ UTR of Cx43. Internal control vector pRL-CMV (Promega) was co-transfected with miR-1 inhibitor (100 pmol) or mimics (100 pmol) using lipofectamine 2000 at the same time. The relative luciferase activity was normalized to Renilla luciferase activity 48 h after the transfection. Each test was repeated triplicate.

2.11. Computational prediction of miRNA-lncRNA and miRNA-mRNA interaction

miRNA-lncRNA interactions was predicted based on StarBase v2.0 (http://starbase.sysu.edu.cn/) with high-stringency criterion [23,24]. Two potential binding sites between miR-1 and MALAT1 were indicated with high argonaute 2-CLIP-seq read number (631.007 and 1466 respectively). Similarly, two binding sites for each miR-206 and miR-613 were predicted interaction with MALAT1 using the same criterion. Analysis from microRNA.org (http://34.236.212.39/microrna/home.do) showed that Cx43 was one of the potential targets of miR-1, preserving two binding sites with relative high negative mirSVR score.

2.12. Statistical analysis

Data were presented as mean ± SD. Statistical analysis was conducted using unpaired Student's *t*-test between two groups. For comparison of more than two groups, one-way ANOVA followed by post-test was performed. $p < 0.05$ was considered statistical significant.

3. Results

3.1. MALAT1 was highly upregulated in ligament tissues from OPLL patients

We screened the lncRNA expression patterns in three OPLL patients' ligament tissues by using lncRNA microarray. Fig. 1A listed the top 21 upregulated lncRNAs in OPLL patients. Of note, LncRNA Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1), Nuclear Enriched Abundant Transcript 1 (NEAT1), and X-inactive specific transcript (XIST) were top-ranked lncRNAs as differentially up-regulated. Further, we chose MALAT1 and NEAT1 as the representative to verify the microarray data quality from a larger population of 52 OPLL patient and 16 healthy control (Fig. 1B). Both of them were significantly induced as shown in both microarray and by qPCR. MALAT1 has been comprehensively studied in many biological processes and disease conditions. It exists in both cytoplasm and nuclear compartments and based on its cellular localization, MALAT1 can act as miRNA sponge (cytoplasm) or chromatin modulator (nuclear). So far, only two

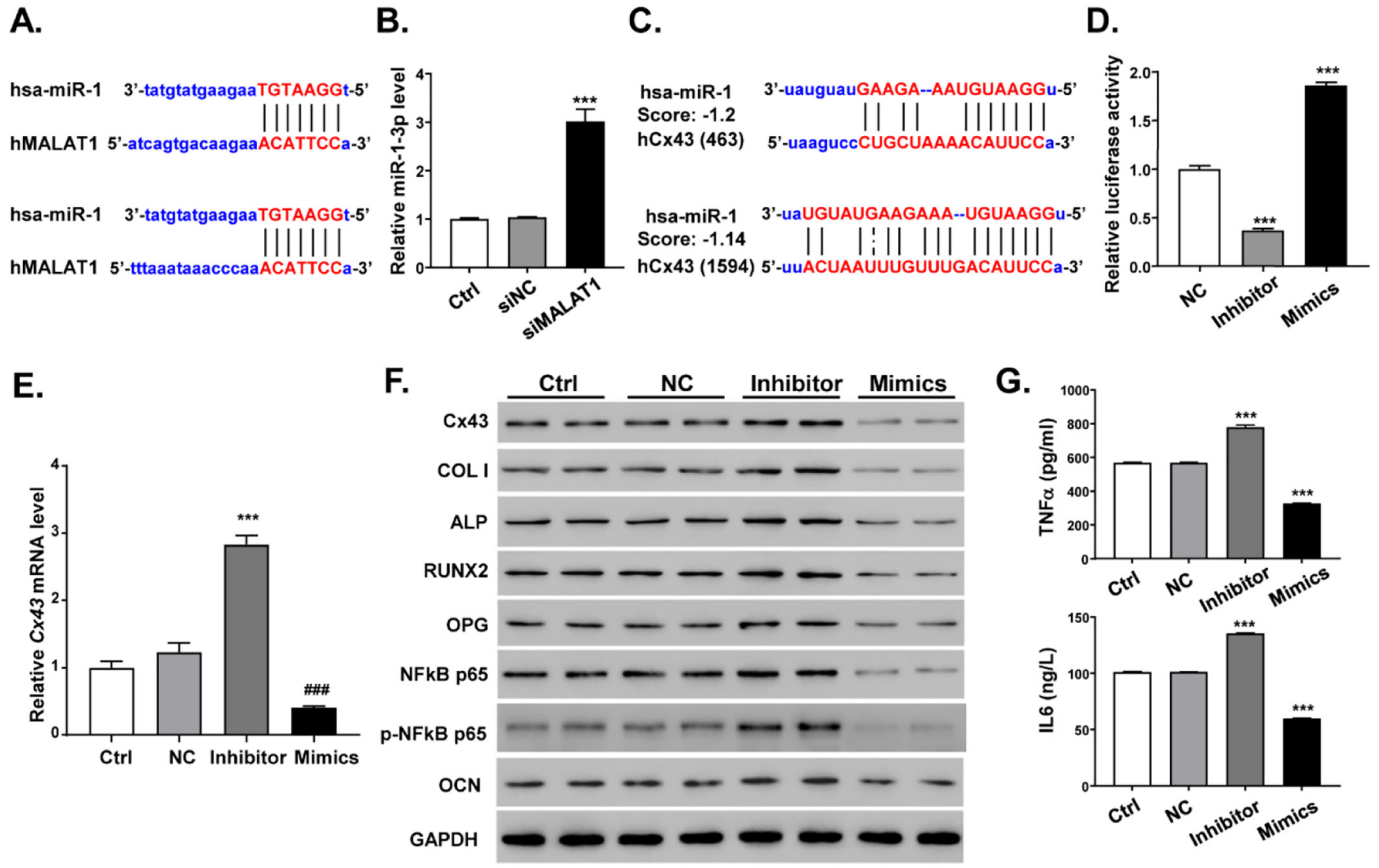

Fig. 4. miR-1 was under the regulation of MALAT1 and mediated ossification of OPLL cells. (A) Interaction prediction between miR-1 and MALAT1 based on StarBase v2.0. Two complementary sites were predicted between human miR-1 (hsa-miR-1) and human MALAT1 (hMALAT1). (B) Increased level of miR-1-3p in OPLL cells after siRNA knock-down of MALAT1. (C) Binding prediction between miR-1 and Cx43 from microRNA.org. Two sites were indicated with relatively high negative score. (D) Cx43 3′-UTR luciferase activity under the treatment with miR-1 inhibitor or mimics in OPLL cells. (E) The mRNA level change of Cx43 after miR-1 inhibitor or mimics treatment in OPLL cells. (F) Protein expressions involved in osteogenic and inflammatory pathways under miR-1 inhibitor or mimics treatment in OPLL cells. (G) Production of TNFα and IL6 from OPLL cells treated with miR-1 inhibitor or mimics. N = 3 from each group, ***$p < 0.001$ compared to siNC or NC. ### indicated the comparison between NC and Mimics.

different groups reported the osteogenic function of MALAT1 in osteoblastic cells and aortic valve interstitial cells. Given its highly dynamic regulation in OPLL tissue, cellular compartment, literature knowledge, and as the top regulated lncRNA from our microarray dataset, we chose MALAT1 as the candidate lncRNA to decipher its role in OPLL.

3.2. Deletion of MALAT1 inhibited expression of Cx43, osteogenesis, inflammation, and cell proliferation in OPLL fibroblasts

In order to study the function of MALAT1 in OPLL, we isolated primary fibroblasts from ligament tissue of non-OPLL and OPLL individuals serving as the *in vitro* test system. As shown in Supplemental Fig. 1 bright field pictures, fibroblasts from OPLL patients exerted different morphology with larger cell size and nucleus as compared to non-OPLL individuals. Our previous study has proved a negative association between Vimentin expression and the ossification of non-OPLL cells under mechanical stress (MS) [25]. Likewise, immunocytochemistry staining of the primary culture cells confirmed the same decrease of Vimentin expression in OPLL cells (Supplemental Fig. 1).

To further explore the function of MALAT1, we constructed a lentivirus system to delivery siRNAs targeting MALAT1 into OPLL fibroblasts. All of the three MALAT1 siRNAs showed significant suppressive efficacy on endogenous MALAT1 transcript (Supplemental Fig. 2A). We chose MALAT1-3 in the rest of the study since it yielded the most drastic reduction. With the suppression of MALAT1, both mRNA and protein levels of Cx43 were decreased (Fig. 2A&D) compared to scramble group. Meanwhile, the proteins and transcription factor related to osteogenesis, *i.e.* alkaline phosphatase (ALP), osteocalcin (OCN), collagen I (COL I), osteoprotegerin (OPG), and runt-related transcription factor 2 (RUNX2) were all reduced after knock-down of MALAT1 (Fig. 2B&E), indicating a inhibition of osteogenesis pertinent to lncRNA MALAT1. During the development of OPLL, it is proved that inflammation contributes to this process through either maintaining or initiation machinery [8]. Likewise, inhibition of MALAT1 reduced inflammatory signaling as represented by decreased NF-κB p65 subunit and phosphorylation (Fig. 2C&F, Supplemental Fig. 2B). As a consequence, both inflammatory cytokines IL6 and TNFα production within OPLL fibroblasts were decreased (Fig. 3A&B). CCK8 cell proliferation assay indicated that knock-down of MALAT1 also inhibited cell proliferation (Fig. 3C), which is the symbol of suppressed trans-differentiation. We have performed measurement of cell proliferation in both OPLL cells and non-OPLL cells (Supplemental Fig. 3C) but the effect of MATLAT1 was only observed in OPLL cells. This data suggested the inflammation regulated by MATLAT1 takes place specifically in OPLL cells.

Therefore, inhibition of MALAT1 would be beneficial to ameliorate the progress of osteogenesis in OPLL fibroblasts.

3.3. miR-1 was down-regulated by MALAT1 and modulated Cx43-associated OPLL

Depending on the subcellular location, lncRNAs existing within

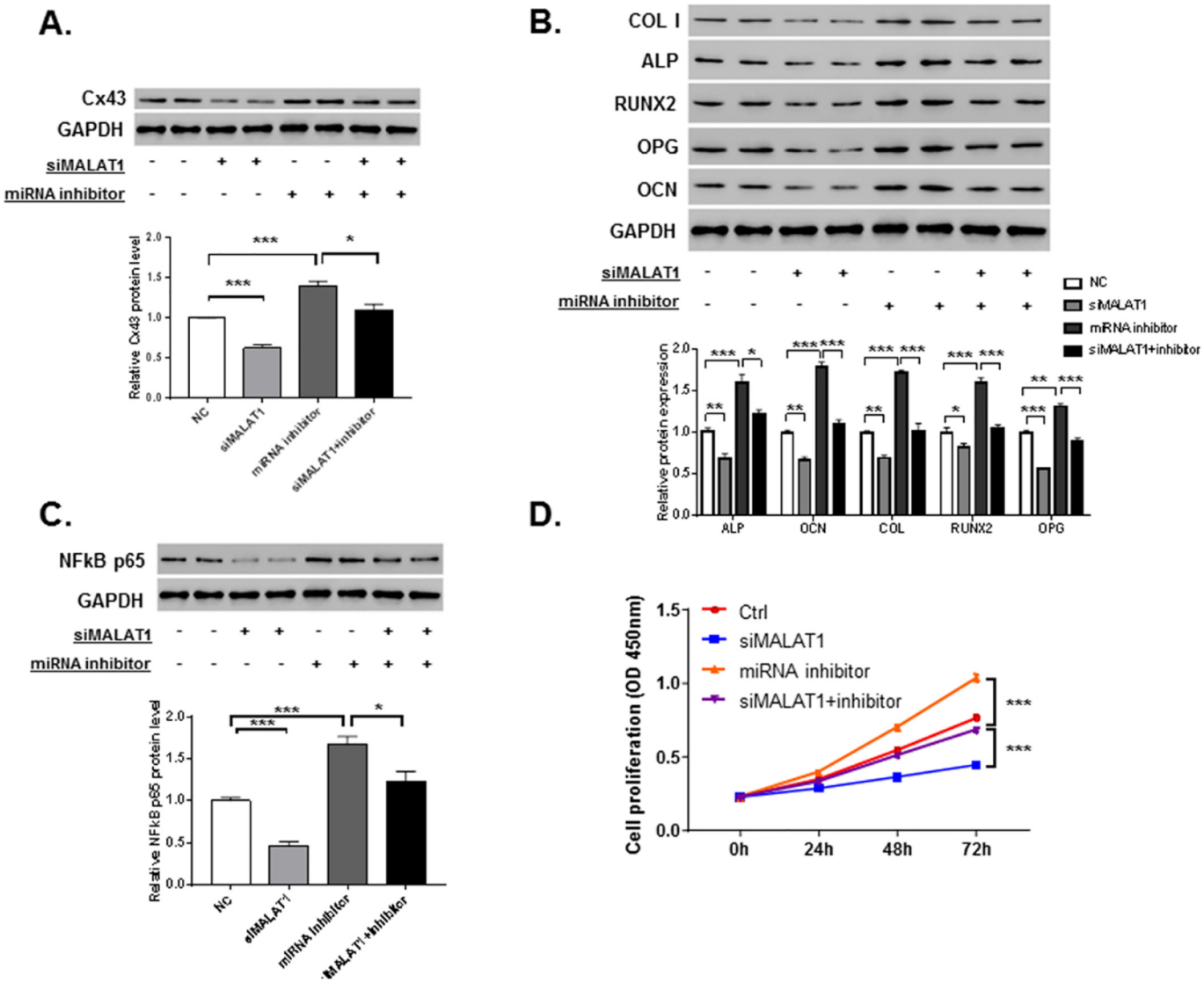

Fig. 5. Suppression of miR-1 activity reversed the reduction of osteogenesis caused by knock-down of MALAT1. (A) Cx43 protein expression with incubation of siMALAT1 and/or miR-1 inhibitor. (B) Osteogenic gene expressions under the treatment with siMALAT1 and/or miR-1 inhibitor. (C) Expression of NF-κB p65 subunit with siMALAT1 and/or miR-1 inhibitor. (D) Cell proliferation from time 0 h to 72 h after the incubation with siMALAT1 and/or miR-1 inhibitor. N = 3 from each group, *p < 0.05, **p < 0.01, ***p < 0.001 as indicated.

cytoplasmic compartment can act as miRNA sponge to fine tune the miRNA-mRNA regulatory axis. MALAT1 has been extensively reported binding to a variety of miRNAs [26]. Based on the StarBase v2.0 analysis, we found miR-1 (Fig. 4A), miR-206, and miR-613 (Supplemental Fig. 3D) can potential serve as seeds for MALAT1 sponge. Further validated by experiments, only miR-1 was up-regulated after MALAT1 knock-down (Fig. 4B and Supplemental Fig. 3E). Thus, we focused on delineating the function of miR-1 in the following studies. As calculated by microRNA.org, hsa-miR-1 had great potential to bind to the 3′ UTR of Cx43 gene, the core molecule linking MALAT1 and OPLL process. Two targeting sites were indicated with a relative high negative mirSVR score (Fig. 4C). Further validated by dual-luciferase-reporter assay, miR-1 inhibitor reduced, while its mimics enhanced Cx43 3′-UTR luciferase activity (Fig. 4D, Supplemental Fig. 4), indicating a direct targeting of miR-1 to Cx43 gene. In line with these findings, the Cx43 mRNA levels were accordingly altered under the treatment with miR-1 inhibitor or mimics (Fig. 4E). Consequentially, protein levels of osteogenic and inflammatory makers including NF-κB p65 phosphorylation were changed (Fig. 4F and Supplemental Fig. 5) attendant with inflammatory cytokines shifts (Fig. 4G).

3.4. Anti-osteogenic function of miR-1 was sequestered by MALAT1 sponge

As predicated before (Fig. 4A), there was great potential interaction between MALAT1 and miR-1 based on their sequence complement, free energy *etc.* Knock-down of MALAT1 not only released trapped miR-1 (Fig. 4B), but also enhanced miR-1 downstream targeting, *i.e.* inhibition of Cx43 (Fig. 5A, compared between “miRNA inhibitor” and “siMALAT1 + miRNA inhibitor”). Functionally, the reversed expression of Cx43 in this case further augmented osteogenesis (Fig. 5B), inflammation such as NF-κB p65 and its phosphorylation (Fig. 5C and Supplemental Fig. 6), and attendant cell proliferation (Fig. 5D).

Reciprocally, overexpression of MALAT1 (Supplemental Fig. 6) accelerated osteogenesis (Fig. 6C&D) and related inflammatory signaling (Fig. 6E&F). Based on the proposed functional mechanisms, this augmentation can be inhibited by: 1) activation of miR-1 with its mimics to counteract the sponge effect from MALAT1 (Fig. 6C&D&E&F); 2) knock-down of downstream effector Cx43 (Fig. 6 and Supplemental Fig. 7); 3) chemically inhibition of NF-kB signaling with Ammonium pyrrolidinedithiocarbamate (PDTC) (Fig. 6C&D&E&F).

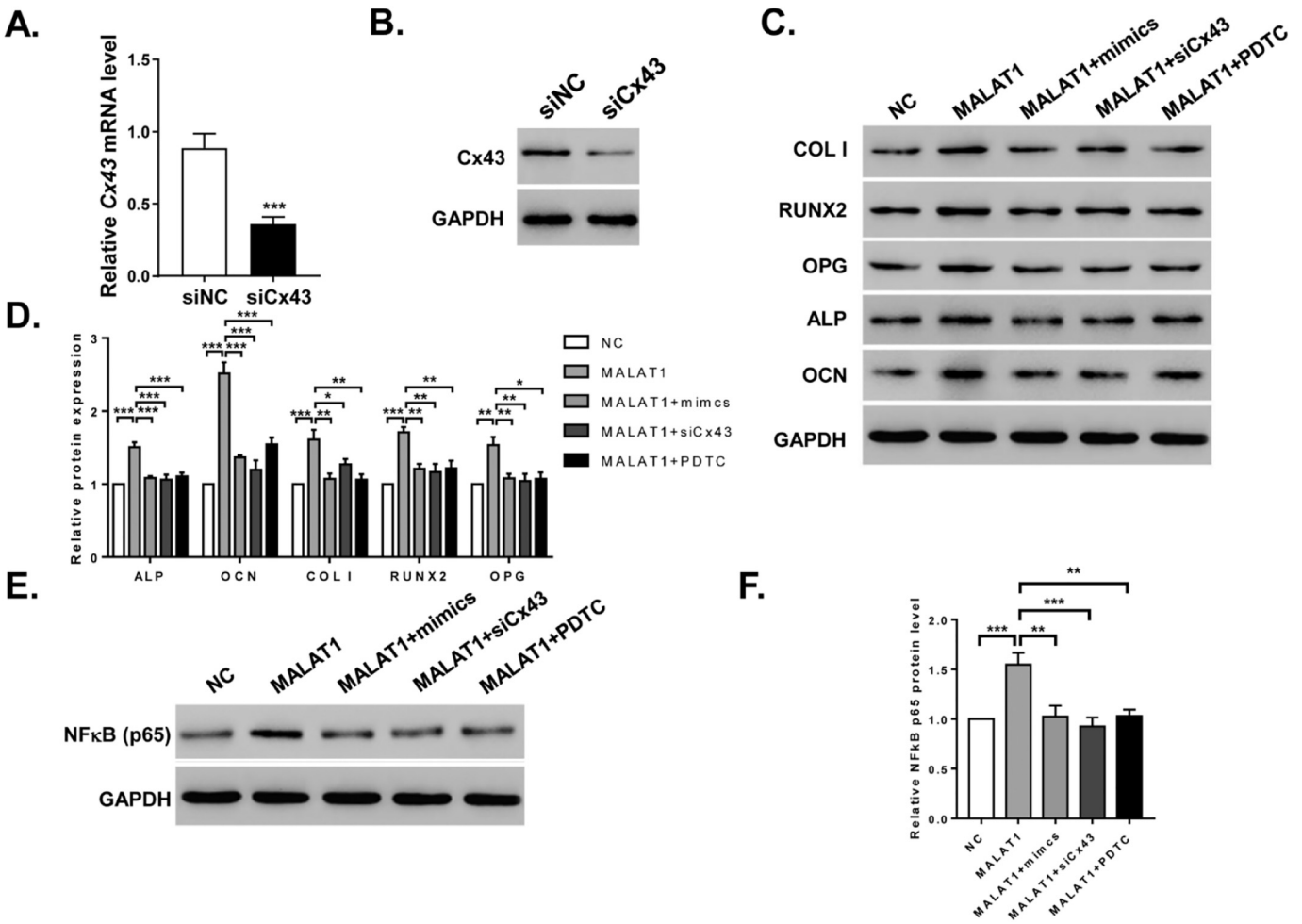

Fig. 6. Overexpression of MALAT1 augmented ossification process, which was suppressed by miR-1 mimics, knock-down of Cx43, or NF-κB selective inhibitor. (A&B) Expression of Cx43 mRNA and protein levels after infection with Cx43 siRNA. (C&D) Expression of osteogenesis related proteins under concurrently overexpression with MALAT1 and miR-1 mimics, siCx43, or 10 μmol/L PDTC (NF-κB inhibitor). (E&F) Expression of p65 protein under the same conditions as C&D. N = 3 from each group, *p < 0.05, **p < 0.01, ***p < 0.001 compared to groups were indicated.

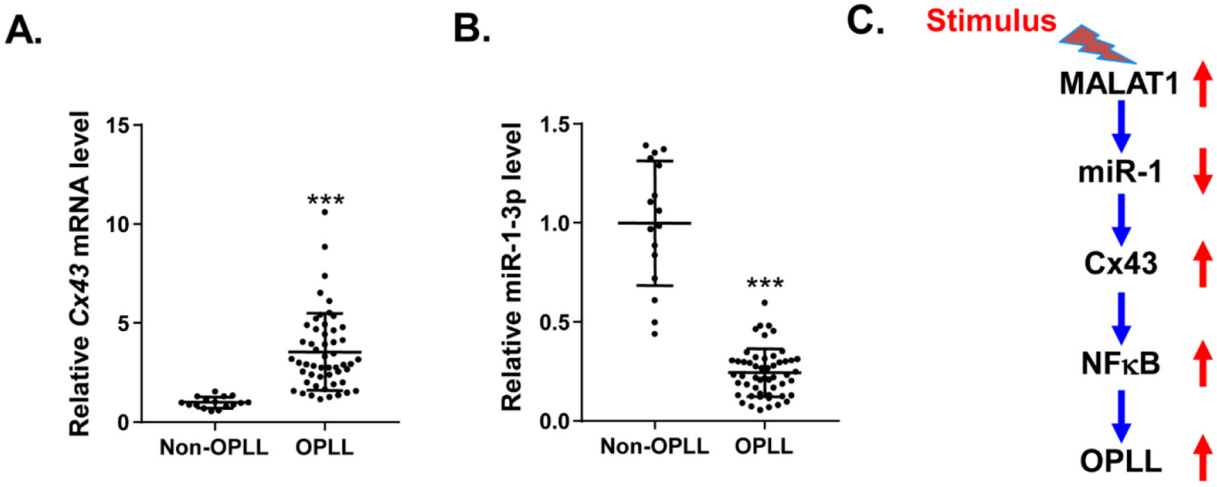

Fig. 7. Expression of Cx43, MALAT1, and miR-1 from OPLL patient tissues. Cx43 mRNA (A), and miR-1-3p expression (B) between non-OPLL and OPLL patient ligament tissues. N = 16 in non-OPLL; N = 52 in OPLL group; ***p < 0.001 compared to non-OPLL group. (C) Schematic illustration of MALAT1-induced ossification of posterior longitudinal ligament fibroblasts. During the development of OPLL, miR-1-targeting Cx43 is suppressed leading to the induction of Cx43 and associated inflammation and osteogenesis. This is partially due to the increase of lncRNA MALAT1 under OPLL stimuli, which acts as miR-1 sponge to block its inhibitory effect.

3.5. MALAT1-miR-1-Cx43 regulatory axis in OPLL patient tissues

To confirm our hypothesis, we have examined forementioned molecules in ligament tissues from a larger population of non-OPLL (16) and OPLL (52) patients. In line with the *in vitro* results, both MALAT1 and Cx43 mRNA were significantly alleviated in OPLL patients (Figs. 7A, 1B), with suppression of miR-1 level as compared to non-OPLL individuals (Fig. 7C). Therefore, we proposed that lncRNA MALAT1 regulated Cx43 associated ossification of the posterior longitudinal ligament fibroblasts through acting as miR-1 sponge. As expected, miR-1 directly targeted Cx43 to modulate this disease process (Fig. 7D).

4. Discussion

lncRNAs preserve a broad range of functions during various developmental and pathological courses [11]. As a comprehensively studied lncRNA, MALAT1 has been identified crucial in several vital conditions [26]. For the first time, we reported the osteogenic effect of MALAT1 during the development of ossification of the posterior longitudinal ligament tissues. In OPLL patient tissues, MALAT1 level was significantly elevated as compared to normal individuals. *In vitro* overexpression of MALAT1 recapitulated ossification development found *in vivo*. On the contrary, perturbation of MALAT1 transcript in primary fibroblast from OPLL patients ameliorated Cx43 expression and its associated ossification. MALAT1 is largely, but not exclusively expressed within nuclear. Multiple previous works mentioned that MALAT1 is also existed in the cytoplasmic compartment [26] and based on its subcellular localization, it is believed that this part of MALAT1 functions as miRNAs sponge to sequester miRNA's transcriptional suppression function, those including but not limited to miR-30a [27], miR-101 [28], miR-204 [29], miR-206 [30], miR-133 [31], and miR-200c [32]. So far, only one study indicated that MALAT1 can affect the osteolysis in ultra-high molecular weight polyethylene-treated hFOB 1.19 cells through interaction with anti-osteolysis miR-22 targeting pro-osteolysis indicators, receptor activator of nuclear factor-kB ligand (RANKL) [33]. Although inconsistent with our observation, we believe that MALAT1 exerts diverse functions in different system, such as ligament fibroblast in our case. Moreover, *in silico* analysis indicated the existence of multiply counterpart miRNAs for MALAT1, which might potentially decipher opposite effect of MALAT1 through binding to different miRNAs. In this scenario, miR-1, other than miR-206 or miR-613 (Supplemental Fig. 3) was the mediator in-between MALAT1 and Cx43 and eventually led to suppression of osteogenesis.

The relationship between miR-1 and Cx43 has been extensively discussed in multiply systems. miR-1 has been implicated in the determination of myogenesis [34,35]. It was shown by Anderson et al. for the first time that miR-1 and miR-206 inhibited the expression of Cx43 during myoblast differentiation [35]. Further, Yang et al. applied *in vivo* arrhythmic rodent model to prove the antiarrhythmic function of miR-1 [20]. Following their studies, several groups showed the similar cardiovascular function of miR-1 in the models of viral myocarditis [19] and ventricular septal defects [36]. Apart from being the downstream effector of MALAT1, miR-1 itself was sufficient to modulate Cx43 expression and associated osteogenesis in ligament fibroblasts. In line with the change of MALAT1 in human samples, miR-1 was significantly inhibited in OPLL patient samples. Similarly, *in vitro* inhibition of miR-1 by its specific inhibitor augmented Cx43 expression, mimicking the development of OPLL *in vivo*. Meanwhile, blocking of MALAT1 using siRNA can potentially release the sequestered miRNAs, including miR-1 and rescue the OPLL process. Likewise, enhancing miR-1 function or knock-down of Cx43 both ameliorated MALAT1-induced OPLL development.

Besides, it is evidential that miRNAs can in return modulate its lncRNA sponge expression through the same sequence complementary. For example, miR-1 suppressed breast cancer development by down-regulating MALAT1 and K-ras [37]. Thus, it is possible that under the current situation, activation of miR-1 not only directly influences Cx43 target transcription, but also affects upstream MALAT1 in a feedback manner and indirectly alters Cx43-associated osteogenesis.

As a hallmark feature, inflammation is accompanied during the progress of OPLL, both *in vivo* and *in vitro* [8]. Similarly, knock-down of MALAT1 or activation of miR-1 also rescued the pro-inflammatory signaling and the production of inflammatory cytokines during ossification, indicating the broad targets of MALAT1-miR-1 regulatory machinery.

Except for MALAT1, we have identified a list of lncRNAs highly up-regulated in OPLL patients. Although only MALAT1 has limited reports on its role in osteogenesis, it is worthwhile investigating the other candidates' functions based on their known knowledge in other systems.

Collectively, we found the first lncRNA being involved into the process of OPLL development. Acting as a miRNA sponge, MALAT1 indirectly targeted Cx43 gene through sequestering miR-1-Cx43 inhibitory interaction and eventually led the incidence of ossification of ligament fibroblasts.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.06.019.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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