Autophagy in spinal ligament fibroblasts: evidence and possible implications for ossification of the posterior longitudinal ligament

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Abstract

Background

The molecular mechanisms of ossification of the posterior longitudinal ligament (OPLL) remains to be elucidated. The aim of the present study was to investigate the autophagy of spinal ligament fibroblasts derived from patients with OPLL and to examine whether autophagy associated gene expression was correlated with the expression of osteogenic differentiation genes.

Methods

Expression of autophagy associated genes was detected in 21 samples from patients with OPLL patients and 16 non-OPLL patients. The correlation of autophagy associated gene expression and the expression of osteogenic differentiation genes was analyzed by Pearson's correlation. The expression of autophagy associated genes of fibroblasts was assessed by reverse transcription-quantitative Polymerase Chain Reaction (RT-qPCR), western blotting and immunofluorescence. The rate of autophagy was assessed by flow cytometry. After knockdown using small interfering RNA targeting Beclin1, the expression of osteogenic differentiation genes were compared in spinal ligament fibroblasts.

Results

In clinical specimens, mRNA expression levels of microtubule-associated protein 1 light chain 3 and Beclin1 were higher in the OPLL group compared with the non-OPLL group. Pearson correlation analysis demonstrated that Beclin1 expression was positively correlated with expression of osteocalcin (OCN) ($r = 0.8233$, $P < 0.001$), alkaline phosphatase, biomineralization associated (ALP) ($r = 0.7821$, $P < 0.001$) and collagen type 1 (COL I) ($r = 0.6078$, $P = 0.001$). Consistently, the upregulation of autophagy associated genes in fibroblasts from patients with OPLL were further confirmed by western blotting and immunofluorescence. The rate of autophagy was also increased in fibroblasts from patients with OPLL. Furthermore, knockdown of Beclin1 led to a decrease in the expression of OCN, ALP and COL I by 63.2% ($P < 0.01$), 52% ($P < 0.01$) and 53.2% ($P < 0.01$) in ligament fibroblasts from patients with OPLL, respectively.

Conclusions

Beclin1-mediated autophagy was involved in the osteogenic differentiation of ligament fibroblasts, and promoted the development of OPLL.

Introduction

Bone formation is regulated according to a genetically controlled time course and spatial relationships. Under pathological conditions, bones can form at extra-skeletal sites, which is known as heterotopic ossification. Ossification of the posterior longitudinal ligament (OPLL) is characterized by heterotopic ossification in the spine ligament [1, 2].

At present, several genetic and non-genetic factors are reported to be involved in the pathological progress of OPLL. At the tissue level, OPLL is characterized by an unknown mixture of pathological endochondral and intramembranous ossification [1]. At the cellular and molecular levels, aberrant levels of fibroblast growth factor-23[3], leptin [4], dickkopf-1 and sclerostin[5], as well as mechanical stress signaling have been identified as contributing factors in the development and progress of OPLL[6–8].

Autophagy, a macromolecular degradation process, plays a pivotal role in the maintenance of cell differentiation [9]. Autophagy of osteocytes was first confirmed by Zahm *et al* [10], and subsequent studies have reported that autophagic receptor and Unc-51 like autophagy activating kinase 1 (ULK1) have an important effect on the activity and differentiation of osteoblasts [11–13]. Consistently, another study demonstrated that osteoblastic differentiation was dependent on Beclin1 dependent autophagy [14]. Microtubule-associated protein 1 light chain 3 (LC3), a lipidated protein specifically associated with the membranes of the autophagosomes, was also correlated with osteoblastic differentiation [15]. Furthermore, our previous results revealed that the level of osteocyte autophagy was associated with bone loss in ovariectomized rats and aged rats [15–17]. At present, it is generally accepted that autophagy plays an essential role in the osteogenic differentiation of mesenchymal stem cells [14] and bone homeostasis [18]. However, autophagy of spinal ligament fibroblast has not yet been investigated and the underlying role of autophagy in OPLL processes remains unknown.

Based on the aforementioned data, it is speculated that autophagy is associated with the differentiation of osteoblasts and bone formation. We hypothesized that ossification of ligament fibroblasts may be attributed to abnormal autophagy. To investigate this hypothesis, autophagy was assessed by reverse transcription-quantitative (RT-q)PCR, transmission electron microscopy, western blotting and immunofluorescence in ligament clinical specimens and ligament fibroblasts. Subsequently, Pearson's correlation was used to compare the expression of osteogenic-specific genes with autophagy. In addition, small interfering (si)RNA was used to knockdown the expression of Beclin1 in spinal ligament fibroblasts, following which the expression levels of osteocalcin (OCN), alkaline phosphatase, biomineralization associated (ALP) and collagen type 1 (COL I) were compared in OPLL ligament fibroblasts and non-OPLL ligament fibroblasts.

Materials And Methods

Characterization of patients. The present study was approved by The Fifth Affiliated Hospital, Southern Medical University, Shanghai Tenth People's Hospital, Tongji University and Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (approval no. XHEC-D-2015-112). Spinal ligament tissues were obtained during surgery using intraoperative aseptic techniques. Patients who underwent

anterior cervical decompression surgery were enrolled in this study. A total of 37 posterior longitudinal ligament specimens were obtained from The Fifth Affiliated Hospital, Southern Medical University and Shanghai Tenth People's Hospital, Tongji University. The OPLL group included patients with a radiographic diagnosis of OPLL involving the cervical spine, as well as symptoms, such as neck pain and numbness in the extremities. By contrast, the non-OPLL group included patients with cervical spinal fractures, who had undergone anterior decompression surgery. Non-OPLL patients did not have OPLL, cervical spondylosis or stenosis. The inclusion and exclusion criteria for patients referenced previous method[19, 20]. Demographics of these patients are provided in Table I. All experiments were performed in The Fifth Affiliated Hospital, Southern Medical University and Shanghai Tenth People's Hospital, Tongji University. All patients provided written informed consent between January 2015 and June 2017, and the research was approved by The Fifth Affiliated Hospital, Southern Medical University Ethics Committee.

Reagents and antibodies. Monodansylcadaverine (MDC) was purchased from Sigma-Aldrich; Merck KGaA. Polyclonal antibodies against Beclin1, and LC3 were obtained from Cell Signaling Technologies, Inc. β-actin, vimentin and keratin antibodies were obtained from Abcam.

Measurement of cell viability. Cell viability was examined using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium (Beyotime Institute of Biotechnology), according to the manufacturer's protocols. Briefly, the ligament fibroblasts were plated in 96-well culture plates and cultured in osteogenic differentiating medium for 1, 2, 3, 4, 5, 6, 7 or 8 days. Cell viability assays were performed and the absorbance of optical densities were measured at each time point and detected by a microplate spectrophotometer at 450 nm.

Specimen processing and cell isolation. During the anterior cervical decompression surgery, 37 posterior longitudinal ligament specimens were obtained. To avoid contamination with osteoblasts or osteocytes, the ligaments were extracted carefully, cut up into 1 mm^2 pieces and washed with PBS several times. Subsequently, the specimens were divided into two parts. One was stored in liquid nitrogen for RT-qPCR analysis. The remaining were washed with normal saline, plated in 35-mm culture dishes, and maintained in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum (Gibco, USA). All assays were carried out on fifth passage cell cultures.

RT-qPCR. Total RNA was obtained from the posterior longitudinal ligament specimens or the ligament fibroblasts, according to our previous study design. Expression of Beclin1, LC3, ULK1, COL I, OCN and ALP were examined by RT-qPCR, according to our previous method [16]. For PCR amplification, specific oligonucleotide primers of rat sequences were designed on the basis of sequences in GenBank (Table II).

Western blotting. The protein expression of Beclin1 and LC3-II/I was detected by western blotting, according to our previous paper [15]. Briefly, total protein was extracted using a western blot kit (Beyotime Institute of Biotechnology). Each sample (50 μg/well) was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel for Beclin1 protein and 12% sodium dodecyl sulfate-polyacrylamide gel for LC3 protein. Next, separated proteins were transferred to polyvinylidene difluoride membranes, followed by

blocking with 5% non-fat milk for 1 h. Next, the polyvinylidene difluoride membranes were incubated with anti-Beclin1(Cat.#3495; CST, USA) (dilution 1:500) or anti-LC3 antibodies (Cat. #83506; CST, USA) (dilution 1:200) and detected using the ECL detection kit (Beyotime Institute of Biotechnology). The blots were quantified by densitometry using Image Lab version 2.1 software (Bio-Rad Laboratories, Inc.).

Knockdown of Beclin1 in ligament fibroblasts. siRNAs specifically targeting Beclin1 were constructed and designed by superbiotek (Shanghai, China). To target Beclin1, the following sequences were used: Forward, 5'- GAGCGAUGGUAGUUCUGGAGG-3' and reverse, 3'-UCCAGAACUACCAUCGCUCUG-5'. A missense siRNA vector (lack of complementary sequences) served as a non-silencing control (siControl). All transfections were carried out using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Levels of autophagy and apoptosis quantified by flow cytometry. The rates of apoptosis were analyzed by flow cytometry in the present study, according to our previous method [16]. The rates of autophagy were analyzed by flow cytometry according to the method of Bursch et al [21] and Shen et al [22]. Briefly, the cells were incubated with 0.05 mM MDC (Cat. #30432; Sigma-Aldrich, USA) in PBS at 37 °C for 10 minutes and then the intracellular MDC was measured by flow cytometry within 30 minutes. The autophagy incidence was determined as the percent of MDC positive cells automatically analyzed using FlowJo software (Tree Star, San Carlos, CA) and the unstained cells were used as control.

Immunofluorescence for vimentin and keratin. The ligament fibroblasts were seeded on 6-well plates with sterile glass cover slip, fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 for 20 min. Subsequently, the ligament fibroblasts were incubated with primary anti-vimentin (Cat.#5741; CST, USA) (dilution 1:200) or anti-keratin antibodies (Cat.#13063; CST, USA) (dilution 1:200) for 2 h. This was followed by incubation with an Alexa Fluor® 488-conjugated secondary antibody (Cat. #4408; CST, USA) (dilution 1:200) for 1 h, and images were taken with a fluorescence microscope (Olympus Corporation).

Immunofluorescence for LC3 and Beclin1 protein. The ligament fibroblasts were seeded on 6-well plates with sterile glass cover slips and transfection of LC3 protein fused with green fluorescent protein plasmid (GFP-LC3) was carried out using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), as previously described [23]. Subsequently, cells were incubated for 72 h and then fixed with 4% paraformaldehyde for 15 min, followed by incubation with anti-Beclin1(Cat.#3495; CST, USA) (dilution 1:200) in 5% bovine serum albumin (Cat. # ST023; Beyotime, China) overnight at 4˚C. Subsequently, the ligament fibroblasts were incubated with Alexa Fluor® 594-conjugated secondary antibody (Cat.#8889; CST, USA) (1:100) and then observed by fluorescence microscopy (Olympus Corporation).

Statistical analysis. All data were from at least three independent experiments and presented as the mean ± SD. Differences in mRNA expression among the OPLL and non-OPLL groups were analyzed using a t-test, with SPSS 13 (SPSS, Inc.). Multiple comparisons of data among the groups were determined by the one-way ANOVA followed by Dunnett's test. Pearson correlation coefficients were used to analyze the

correlation between parameters of autophagy level and osteogenic makers. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

High expression of Beclin1 and LC3 mRNA in OPLL tissues. In this part of the study, expression levels of different autophagy markers in the spinal ligament tissues from OPLL and non-OPLL patients were investigated. The present results reported that the expression of Beclin1 was significantly higher in the OPLL group compared with the non-OPLL group (Fig. 1A). Similarly, compared with the non-OPLL group, there was also increased expression of LC3 observed in the OPLL group (Fig. 1B). However, the expression level of ULK1 in the OPLL tissue was similar to that of the mean level of non-OPLL tissues (Fig. 1C).

Correlation between rate of autophagy and osteogenic differentiation markers. Next, correlation was analyzed between the expression of autophagy-related genes (LC3 and Beclin1 mRNA) and osteogenic differentiation-related genes. Pearson correlation analysis demonstrated that the level of Beclin1 mRNA expression was positively correlated with the expression of ALP ($r = 0.7821$, $P < 0.001$) (Fig. 1D), COL I ($r = 0.6078$, $P = 0.001$) (Fig. 1E) and OCN ($r = 0.8233$, $P < 0.001$) (Fig. 1F). However, the expression of LC3 mRNA was not correlated with ALP ($r = 0.1189$, $P = 0.6076$) (Fig. 1G), COL I ($r=-0.07361$, $P = 0.7512$) (Fig. 1H) and OCN ($r = 0.11$, $P = 0.635$) (Fig. 1I).

Identification of ligament fibroblasts and growth characterization. Next, ligament fibroblasts were isolated from tissue for further study (from OPLL and non-OPLL patients). After a 1-week tissue fragment culture, slender cells could be observed surrounding the isolated tissue fragments. They were multi-angled in shape and also had relatively long cell antennas. As shown in Fig. 2A, cells derived from the cervical posterior longitudinal ligaments of both OPLL and non-OPLL patients showed similar fibroblast-like morphological characters in size, nucleus and shape (Fig. 2A). To further identify ligament fibroblasts, immunofluorescence staining was performed to evaluate the expression of vimentin and keratin. As expected, vimentin was generally expressed in the cytoplasm of the cells derived from both OPLL and non-OPLL ligament fibroblasts, and keratin was not detected in these ligament fibroblasts (Fig. 2B), which indicated that cells derived from patients with OPLL and non-OPLL patients showed similar fibroblast-like morphological characters in size, nucleus and shape (Fig. 2C).

Different expression of autophagy-specific gene makers in cells from OPLL and non-OPLL patients. In order to further confirm the results observed at the tissue level, flow cytometry assay, RT-qPCR, and western blotting and were performed in ligament fibroblast cells. As shown in Fig. 3A, the flow cytometry assay indicated that the rates of autophagy in OPLL-derived ligament fibroblasts was 1.6-fold of those in the non-OPLL-deprived ligament fibroblasts. Consistent with the aforementioned results, RT-qPCR showed that the mRNA expression of LC3 and Beclin1 increased in ligament fibroblasts from patients with OPLL compared with fibroblasts from non-OPLL patients (Fig. 3A). Next, Beclin1 and LC3 protein expression was detected by western blotting, the expression levels of LC3 and Beclin1 proteins were significantly

higher in ligament fibroblasts from patients with OPLL compared with the control (Fig. 3B). Consistently, fluorescence microscopy showed that there was increased Beclin1 and LC3 protein dots in the cytoplasm of ligament fibroblasts from patients with OPLL compared with the group of ligament fibroblasts from non-OPLL patients (Fig. 4). Together, these data indicated that autophagy was upregulated in ligament fibroblasts from patients with OPLL.

Role of Beclin1 in osteogenic differentiation of ligament fibroblasts. The aforementioned data indicated that Beclin1 was closely correlated with osteogenic differentiation markers and the expression of Beclin1 was significantly upregulated in the spinal ligament fibroblasts derived from patients with OPLL. Therefore, a specific gene interference technique was used, targeting Beclin1, to suppress the expression of Beclin1 in ligament fibroblasts. After 72 h of transfection in the ligament fibroblasts from patients with OPLL, it was found that Beclin1 expression decreased significantly by 61% ($P < 0.0001$) in the transfected cells, as measured by RT-qPCR (Fig. 5A). However, in the ligament fibroblasts from non-OPLL patients, there was no significant difference in Beclin1 expression among groups ($p = 0.173$) (Fig. 5A). The low efficiency in the knockdown of Beclin1 in the non-OPLL group could be because the basal autophagy level was low in the ligament fibroblasts from non-OPLL patients.

Subsequently, to clarify whether there are different roles of Beclin1 in osteogenic differentiation, mRNA expression of OCN, COL I and ALP was detected and then compared between the OPLL group and the non-OPLL group. Before transfection, the expressions levels of OCN (Fig. 5B), COL I (Fig. 5C) and ALP (Fig. 5D) were 2.71 ($P < 0.001$), 3.31 ($P < 0.001$) and 2.71 ($P < 0.001$) fold of those in the non-OPLL cells, respectively. However, after transfection for 72 h, the expression of OCN, ALP and COL I was reduced by 63.2% ($P < 0.01$), 52% ($P < 0.01$) and 53.2% ($P < 0.01$) in ligament fibroblasts from patients with OPLL compared with 3.1% ($P > 0.01$), 6.8% ($P > 0.01$) and 10.7% ($P > 0.01$) in the non-OPLL patients, respectively (Fig. 5B&C&D).

Discussion

OPLL, which is characterized by ectopic bone formation in the spinal ligament, is a multi-factorial disease involving genetic, physical and neurological disorders [1, 19]. The pathogenesis of OPLL is not completely understood. Current research suggests that OPLL is possibly associated with abnormal expression of connexin43 [20], PERK [19] and TGF-β [24]. However, these results were not consistent and further research is needed to elucidate the major genes that cause the susceptibility to OPLL.

In the present study, expression of ULK1, Beclin1 and LC3 were measured, which showed that Beclin1 and LC3 expression was significantly increased in OPLL tissues compared with non-OPLL tissues. Next, the association between the expression of Beclin1, LC3 and the osteogenic differentiation makers in 21 patients with OPLL were analyzed. As a result, it was found that Beclin1 mRNA was positively correlated with osteogenic differentiation makers. However, LC3 expression was not significantly correlated with osteogenic differentiation makers. There are two possible explanations for this First, LC3 protein comprises a soluble LC3I protein and LC3II protein, and LC3 mRNA level is not a very reliable

measurement for changes in autophagy. Secondly, the small number of ligament tissue samples included in the present study may limit the interpretation of these results. Therefore, large-sample studies should be conducted to confirm the role of LC3 in OPLL in the future.

Next, ligament fibroblasts were isolated from tissues for further study (from OPLL and non-OPLL patients). Consistently, there was a higher level of Beclin1 and LC3 mRNA expression in the posterior longitudinal ligament fibroblasts derived from patients with OPLL compared with those derived from non-OPLL patients. Analysis of cell growth and morphology demonstrated that no difference was observed in cells from OPLL and non-OPLL patients *in vitro*. Although, it was not consistent with a previous study that reported that cells from patients with OPLL were larger in size and nucleus [19]. The main reason for this could be that cells were derived from different populations. Previous data suggests that autophagy is essential for osteogenic differentiation [18]. To confirm the results at the tissue level, the mRNA and protein expression of autophagy related genes were further analyzed in ligament fibroblasts derived from OPLL and non-OPLL patients. Consistently, the present results indicated that the level of autophagy in ligament fibroblasts from patients with OPLL were higher compared with those derived from non-OPLL patients. These data suggested that autophagy was increased in ligament fibroblasts, in particular in ligament fibroblasts from patients with OPLL.

Fibroblast cells can differentiate into osteoblasts or osteocytes [25]. Therefore, the expression levels of three marker genes related to osteogenic differentiation, OCN, ALP and COL I, were assessed. It was found that the mRNA expression of osteogenic differentiation markers OCN, COL I and ALP were significantly upregulated in the OPLL group [26] compared with the control. Furthermore, analysis of gene expression demonstrated a higher mRNA and protein expression of Beclin1 and LC3II in the fibroblasts from patients with OPLL. Notably, Beclin1 expression was correlated with the expression of osteogenic differentiation markers OCN, ALP and COL I in patients with OPLL. Consistently, a recent study reported that Beclin1 plays an important role in the differentiation of osteoclasts and chondrocytes [9]. To confirm the role of Beclin1 in fibroblasts, siRNA targeting Beclin1 was designed and used to downregulate the expression of Beclin1. As expected, a significant decrease in the expression of Beclin1 was observed in the fibroblasts from patients with OPLL, while a slight reduction of Beclin1 expression, which was not significantly different, was found in the non-OPLL group. These results is consistent with previous researches that the effect of siRNA is dependent on its relative expression level[19, 20]. Similarly, in ligament fibroblasts derived from patients with OPLL, the expression levels of OCN, ALP and COL I were significantly suppressed by knocking down Beclin1 compared with the control. However, no significant difference was observed in fibroblasts from non-OPLL patients. The present results suggested that autophagy was involved in the process of osteogenic differentiation in OPLL. The possible underlying mechanism could be that autophagy can hydrolyze organophosphates and offer phosphonic acid for the deposition of hydroxyapatite crystals on collagen fibers and subsequently lead to osteogenesis development [27].

Although our research is the first to provide data demonstrating that autophagy plays a role in the development of OPLL. However, OPLL is a multifactorial disease in which complex genetic and

environmental factors interact. Obviously, the cell biological data in vitro provides very limited evidence. Furthermore, control group was taken from the patients having cervical spinal fracture. However, some of them may develop OPLL thereafter due to aging, diabetes mellitus, obesity and susceptibility genetic loci. Thus, further research using strict inclusion and exclusion criteria is indispensable to confirm the findings of this study.

To conclude, Beclin1-mediated autophagy was involved in the osteogenic differentiation of ligament fibroblasts, and promoted the development of OPLL.

Conclusions

Our findings suggested that autophagy is upregulated in spinal ligament fibroblasts derived from OPLL patients and Beclin1-mediated autophagy was involved in the osteogenic differentiation of ligament fibroblasts, and promoted the development of OPLL.

Abbreviations

OPLL: ossification of the posterior longitudinal ligament; RT-qPCR: reverse transcription-quantitative Polymerase Chain Reaction; OCN: osteocalcin; ALP: alkaline phosphatase; COL I: collagen type 1; ULK1: Unc-51 like autophagy activating kinase 1; LC3: Microtubule-associated protein 1 light chain 3; siRNA: small interfering RNA; MDC: Monodansylcadaverine;

Declarations

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None.

Informed consent

Not applicable.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Yuehua Yang and Zunwen Lin designed research; Yuehua Yang and Jiangwei Cheng analyzed data; Yuehua Yang, Sheng Ding, Wei-wei Mao, and Sheng Shi performed research; Yuehua Yang wrote the paper; Biru Liang and Zunwen Lin developed software necessary to perform and record experiments.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Care and Use Committee of Southern Medical University and were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Yan L, Gao R, Liu Y, He B, Lv S, Hao D. The Pathogenesis of Ossification of the Posterior Longitudinal Ligament. Aging Dis. 2017;8:570–82.
2. Nam DC, Lee HJ, Lee CJ, Hwang SC. Molecular Pathophysiology of Ossification of the Posterior Longitudinal Ligament (OPLL). Biomol Ther (Seoul). 2019;27:342–8.
3. Kawaguchi Y, Kitajima I, Nakano M, Yasuda T, Seki S, Suzuki K, Yahara Y, Makino H, Ujihara Y, Ueno T, Kimura T. Increase of the Serum FGF-23 in Ossification of the Posterior Longitudinal Ligament. Global Spine J. 2019;9:492–8.
4. Feng B, Cao S, Zhai J, Ren Y, Hu J, Tian Y, Weng X. Roles and mechanisms of leptin in osteogenic stimulation in cervical ossification of the posterior longitudinal ligament. J Orthop Surg Res. 2018;13:165.

5. Kashii M, Matuso Y, Sugiura T, Fujimori T, Nagamoto Y, Makino T, Kaito T, Ebina K, Iwasaki M, Yoshikawa H. Circulating sclerostin and dickkopf-1 levels in ossification of the posterior longitudinal ligament of the spine. J Bone Miner Metab. 2016;34:315–24.
6. Shi L, Miao J, Chen D, Shi J, Chen Y. Endoplasmic reticulum stress regulates mechanical stress-induced ossification of posterior longitudinal ligament. Eur Spine J. 2019;28:2249–56.
7. Chen S, Zhu H, Wang G, Xie Z, Wang J, Chen J. Combined use of leptin and mechanical stress has osteogenic effects on ossification of the posterior longitudinal ligament. Eur Spine J. 2018;27:1757–66.
8. Chen D, Chen Y, Li T, Shi L, Pan M. Role of Cx43-Mediated NFsmall ka, CyrillicB Signaling Pathway in Ossification of Posterior Longitudinal Ligament: An In Vivo and In Vitro Study. Spine (Phila Pa 1976). 2017;42:E1334-E41.
9. Arai A, Kim S, Goldshteyn V, Kim T, Park NH, Wang CY, Kim RH. Beclin1 Modulates Bone Homeostasis by Regulating Osteoclast and Chondrocyte Differentiation. J Bone Miner Res. 2019;34:1753–66.
10. Zahm AM, Bohensky J, Adams CS, Shapiro IM, Srinivas V. Bone cell autophagy is regulated by environmental factors. Cells Tissues Organs. 2011;194:274–8.
11. Kirkin V, Lamark T, Sou YS, Bjorkoy G, Nunn JL, Bruun JA, Shvets E, McEwan DG, Clausen TH, Wild P, Bilusic I, Theurillat JP, Overvatn A, Ishii T, Elazar Z, Komatsu M, Dikic I, Johansen T. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol Cell. 2009;33:505–16.
12. Whitehouse CA, Waters S, Marchbank K, Horner A, McGowan NW, Jovanovic JV, Xavier GM, Kashima TG, Cobourne MT, Richards GO, Sharpe PT, Skerry TM, Grigoriadis AE, Solomon E. Neighbor of Brca1 gene (Nbr1) functions as a negative regulator of postnatal osteoblastic bone formation and p38 MAPK activity. Proc Natl Acad Sci U S A. 2010;107:12913–8.
13. Cai ZY, Yang B, Shi YX, Zhang WL, Liu F, Zhao W, Yang MW. High glucose downregulates the effects of autophagy on osteoclastogenesis via the AMPK/mTOR/ULK1 pathway. Biochem Biophys Res Commun. 2018;503:428–35.
14. Vidoni C, Ferraresi A, Secomandi E, Vallino L, Gardin C, Zavan B, Mortellaro C, Isidoro C. Autophagy drives osteogenic differentiation of human gingival mesenchymal stem cells. Cell Commun Signal. 2019;17:98.
15. Yang Y, Zheng X, Li B, Jiang S, Jiang L. Increased activity of osteocyte autophagy in ovariectomized rats and its correlation with oxidative stress status and bone loss. Biochem Biophys Res Commun. 2014;451:86–92.
16. Yang YH, Chen K, Li B, Chen JW, Zheng XF, Wang YR, Jiang SD, Jiang LS. Estradiol inhibits osteoblast apoptosis via promotion of autophagy through the ER-ERK-mTOR pathway. Apoptosis. 2013;18:1363–75.
17. Chen K, Yang YH, Jiang SD, Jiang LS. Decreased activity of osteocyte autophagy with aging may contribute to the bone loss in senile population. Histochem Cell Biol. 2014;142:285–95.
18. Jaber FA, Khan NM, Ansari MY, Al-Adlaan AA, Hussein NJ, Safadi FF. Autophagy plays an essential role in bone homeostasis. J Cell Physiol. 2019;234:12105–15.

19. Chen Y, Wang X, Yang H, Miao J, Liu X, Chen D. Upregulated expression of PERK in spinal ligament fibroblasts from the patients with ossification of the posterior longitudinal ligament. Eur Spine J. 2014;23:447–54.
20. Yang HS, Lu XH, Chen DY, Yuan W, Yang LL, He HL, Chen Y. Upregulated expression of connexin43 in spinal ligament fibroblasts derived from patients presenting ossification of the posterior longitudinal ligament. Spine (Phila Pa 1976). 2011;36:2267–74.
21. Bursch W, Hochegger K, Torok L, Marian B, Ellinger A, Hermann RS. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. J Cell Sci. 2000;113(Pt 7):1189–98.
22. Shen C, Yan J, Jiang LS, Dai LY. Autophagy in rat annulus fibrosus cells: evidence and possible implications. Arthritis Res Ther. 2011;13:R132.
23. Dash R, Azab B, Quinn BA, Shen X, Wang XY, Das SK, Rahmani M, Wei J, Hedvat M, Dent P, Dmitriev IP, Curiel DT, Grant S, Wu B, Stebbins JL, Pellecchia M, Reed JC, Sarkar D, Fisher PB. Apogossypol derivative BI-97C1 (Sabutoclax) targeting Mcl-1 sensitizes prostate cancer cells to mda-7/IL-24-mediated toxicity. Proc Natl Acad Sci U S A. 2011;108:8785–90.
24. Chen G, Zhang W, Li YP, Ren JG, Xu N, Liu H, Wang FQ, Sun ZJ, Jia J, Zhao YF. Hypoxia-induced autophagy in endothelial cells: a double-edged sword in the progression of infantile haemangioma? Cardiovasc Res. 2013;98:437–48.
25. Tanno M, Furukawa KI, Ueyama K, Harata S, Motomura S. Uniaxial cyclic stretch induces osteogenic differentiation and synthesis of bone morphogenetic proteins of spinal ligament cells derived from patients with ossification of the posterior longitudinal ligaments. Bone. 2003;33:475–84.
26. Wang Y, Wang J, Zheng J, Yu M, Cai L, Zhang S, Li J. Ectopic osteogenesis by type I collagen loaded with a novel synthesized PTH-related peptide-1 in vivo. J Biomed Mater Res A 2019.
27. Haberzettl P, Hill BG. Oxidized lipids activate autophagy in a JNK-dependent manner by stimulating the endoplasmic reticulum stress response. Redox Biol. 2013;1:56–64.

Tables

Table.1 Tissue samples used in our study and the clinicopathologic characteristics of patients. Patient demographic characteristic are summarized in Table.1

OPLL			Non-OPLL		
Code	Segment/type	sex/age	Code	Cervical fracture Segment	sex/age
1	C3/4/local	M/55	1	C2/3	M/36
2	C4/5/local	F/57	2	C5/6	M/29
3	C4/5/6/ continuous	M/45	3	C5/6	F/63
4	C5/6/ local	F/41	4	C6/7	M/60
5	C4/5/6 continuous	F/48	5	C4/5	F/36
6	C5/6/ local	F/56	6	C3/4	M/33
7	C5/6/local	M/43	7	C5/6	F/40
8	C5/6/local	M/56	8	C5/6	M/51
9	C4/5/6/ mixed	F/41	9	C5/6/7	M/39
10	C4/5/6/local	F/53	10	C6/7	F/51
11	C2/3/local	M/47	11	C7/T1	F/60
12	C3/4/local	M/50	12	C5/6/7	F/46
13	C5/6/local	F/46	13	C4/5	M/37
14	C5/6/local	F/43	14	C4/5/6	M/50
15	C5/6/7/ continuous	M/44	15	C4/5	F/26
16	C4/5/6/7/ segmental	F/40	16	C5/6	M/17
17	C5/6/local	F/48			
18	C5/6/local	M/50			
19	C4/5/local	M/51			
20	C4/5/6/7/continuous	F/42			
21	C3/4/5/ mixed	M/59			

Table 2 Primer sequences of Beclin1 LC3, ULK1, OCN,ALP, COL I and β-actin.

Index	Primer sequences
Beclin1	
Forward	5′- CAGGAACTCACAGCTCCATT-3′
Reverse	5′- CATCAGATGCCTCCCCAATC-3′
LC3	
Forward	5′-AGCCACCTGCCACTCCTGAC-3′
Reverse	5′-ACCTTCCCTGCTGCCCTCAC-3′
ULK1	
Forward	5′- GAGTCGGAGTCGGAGTCGGATC -3′
Reverse	5′- CGAACTTGCCCACGGTCTCTG -3′
OCN	
Forward	5′-AGGGCAGCGAGGTAGTGA-3′
Reverse	5′-CCTGAAAGCCGATGTGGT-3′
ALP	
Forward	5′- GTGGACTATGCTCACAACAA -3′
Reverse	5′-GGAGAAATACGTTCGCTAGA-3′
COL I	
Forward	5′-CGAAGACATCCCACCAATC-3′
Reverse	5′-ATCACGTCATCGCACAACA-3′
β-actin	
Forward	5′- CTCCATCCTGGCCTCGCTGT -3′
Reverse	5′- GCTGTCACCTTCACCGTTCC -3′

Figures

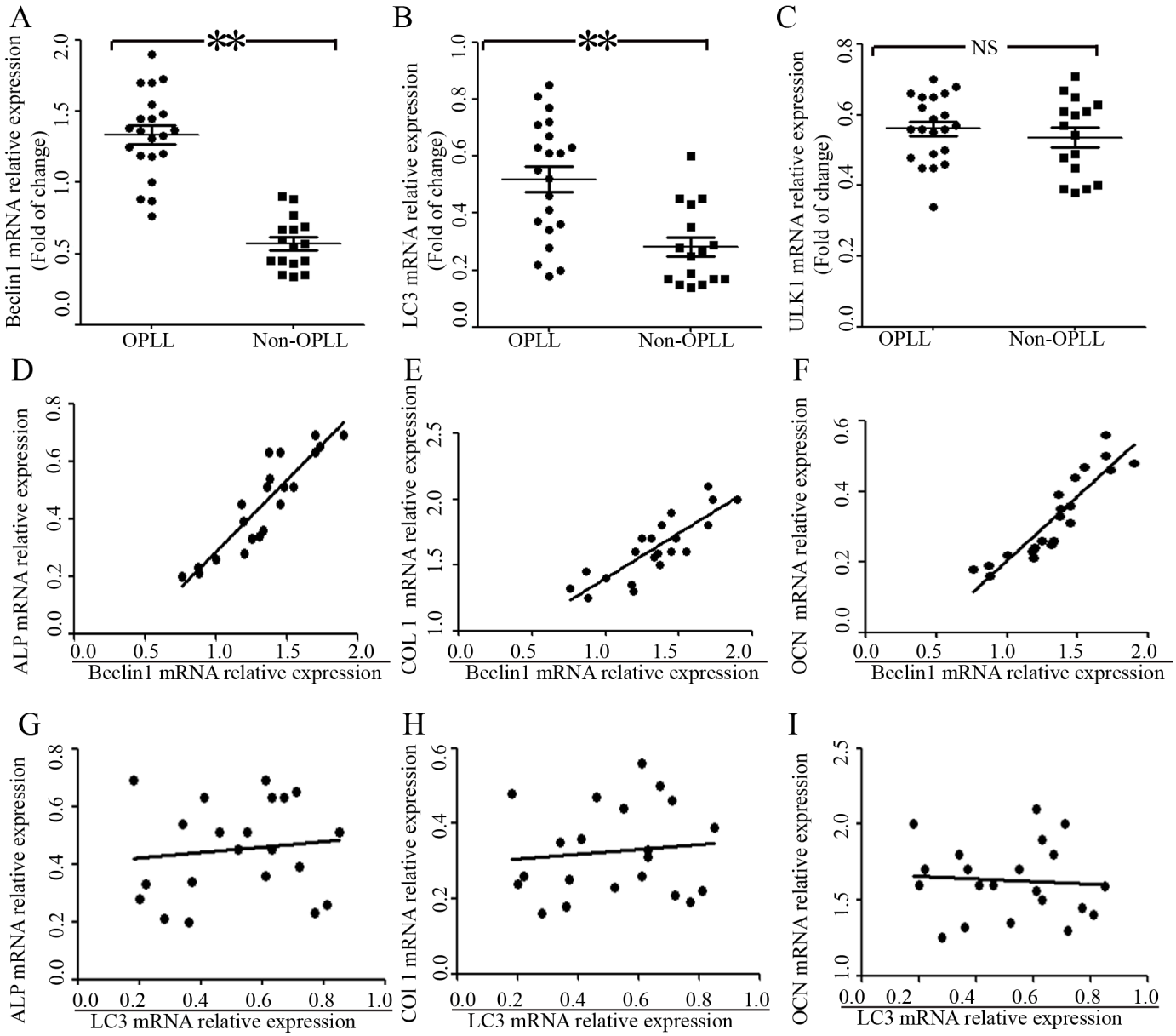

Figure 1

Expression of Beclin1 and LC3 mRNA in OPLL tissues and correlation between autophagy level and osteogenic differentiation markers. Reverse transcription-quantitative PCR assay to measure the mRNA expression of (A) Beclin1, (B) LC3 and (C) ULK1 in spinal ligament tissues from patients with OPLL and non-OPLL patients. The relative gene expression was normalized against β-actin. *P<0.05, **P<0.01. Pearson's correlation was used to analyze the correlation between Beclin1 level and osteogenic biomarkers (D) ALP, (E) COL 1 and (F) OCN. Correlation between LC3 level and (G) ALP, (H) COL 1 and (I) OCN. LC3, microtubule-associated protein 1 light chain 3; OPLL, ossification of the posterior longitudinal ligament; ULK1, Unc-51 like autophagy activating kinase 1; ALP, alkaline phosphatase, biomineralization associated; COL 1, collagen type 1; OCN, osteocalcin.

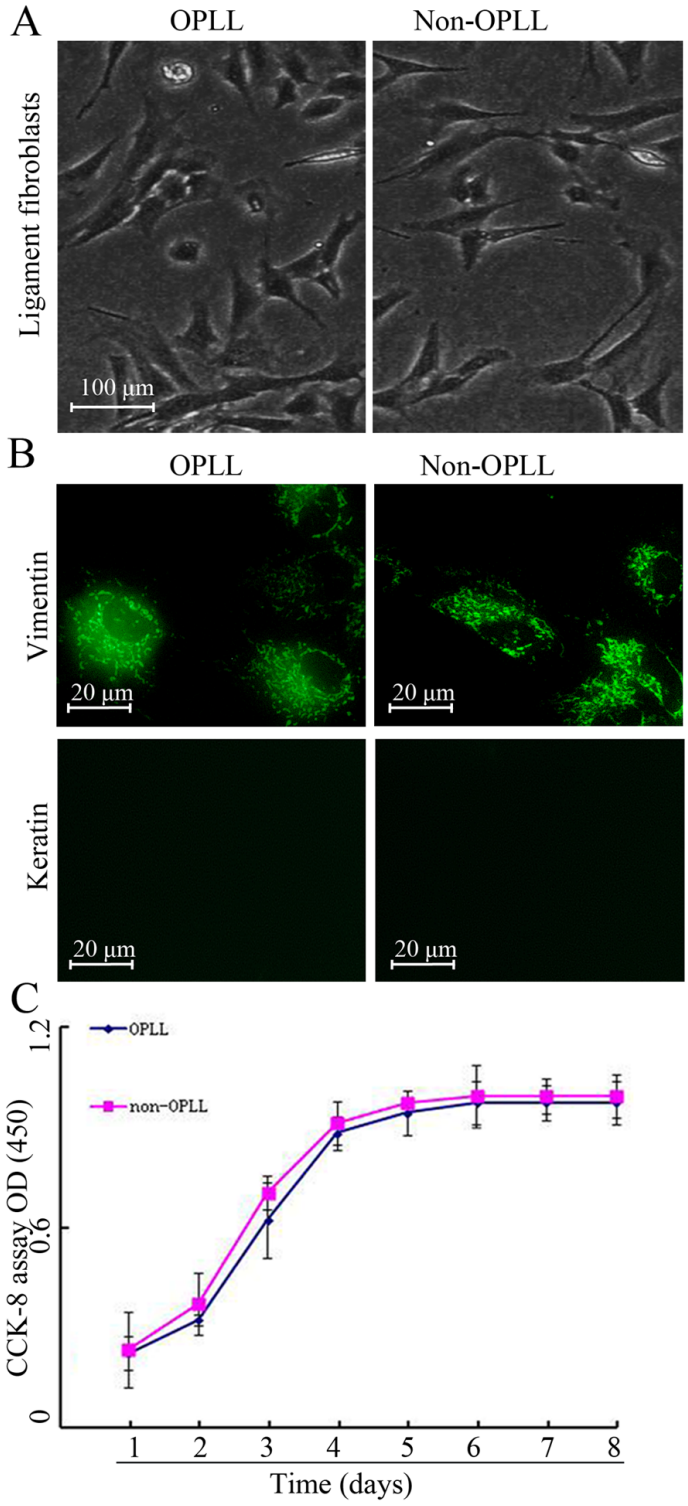

Figure 2

Identification of ligament fibroblasts and growth characterization. (A) Optical microscopy analysis of ligament fibroblasts. The cells were multi-angled in shape and also had relatively long cell antennas. (B) Representative fluorescent images showing immunofluorescence assay for vimentin and keratin expression. Scale bar, 20 μm. (C) CCK-8 assay measuring cell viability, cells were cultured in DMEM for 1,

2, 3, 4, 5, 6, 7 or 8 days. *P<0.05 OPLL vs. non-OPLL. OPLL, ossification of the posterior longitudinal ligament.

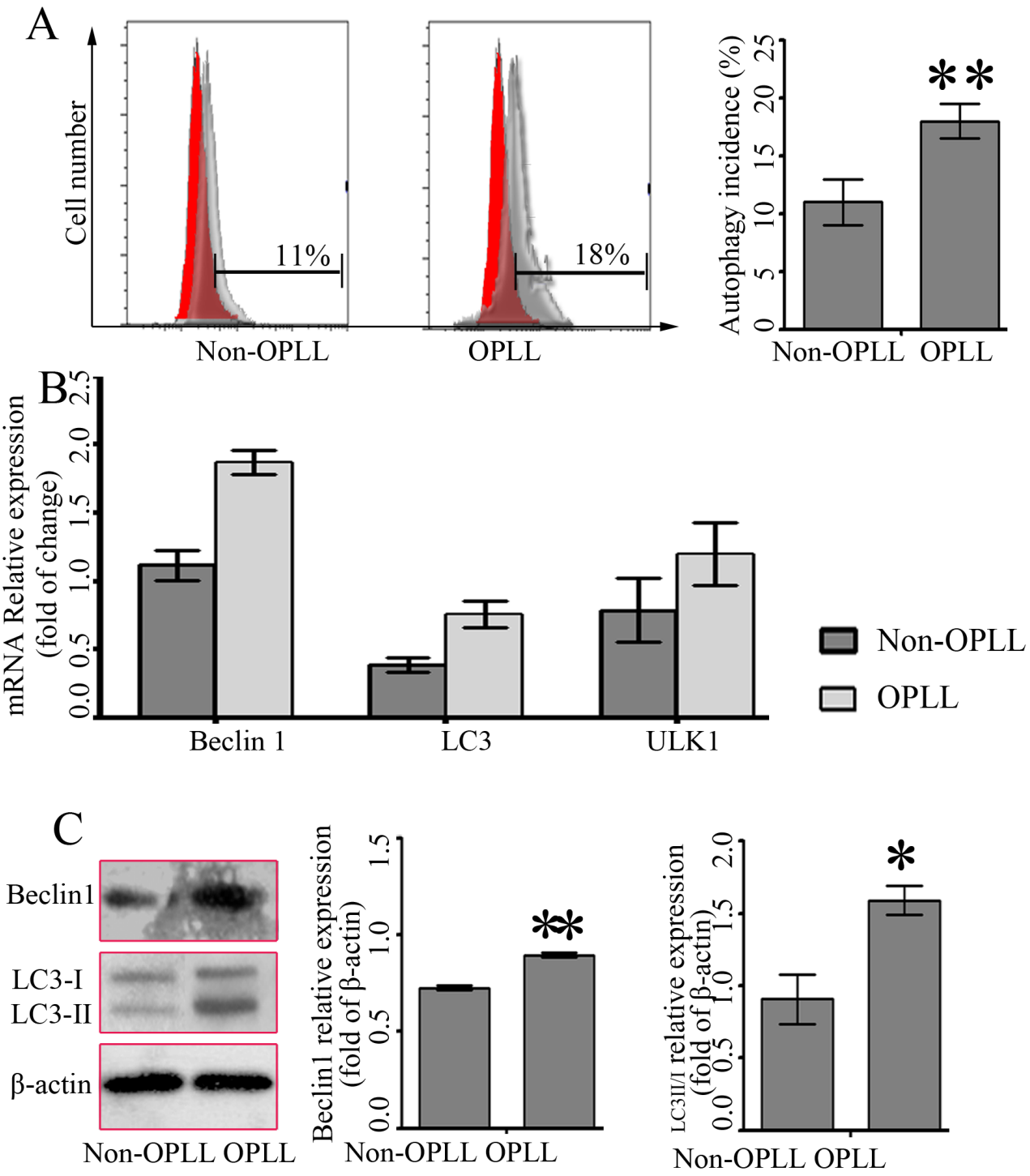

Figure 3

(A) Flow cytometry was used to measure autophagy, which was the percentage of staining density of monodansylcadaverine. (B) RT-qPCR assay to measure the mRNA expression of Beclin1 and LC3 in ligament fibroblasts from patients with OPLL and non-OPLL patients. (C) Western blotting to measure

protein expression of LC3 and Beclin1 in ligament fibroblasts from patients with OPLL and non-OPLL patients. Data is presented as the mean ± SD of at least three independent experiments. *P<0.05, **P<0.01 OPLL vs. non-OPLL. OPLL, ossification of the posterior longitudinal ligament; RT-qPCR, reverse transcription-quantitative PCR; LC3, microtubule-associated protein 1 light chain 3.

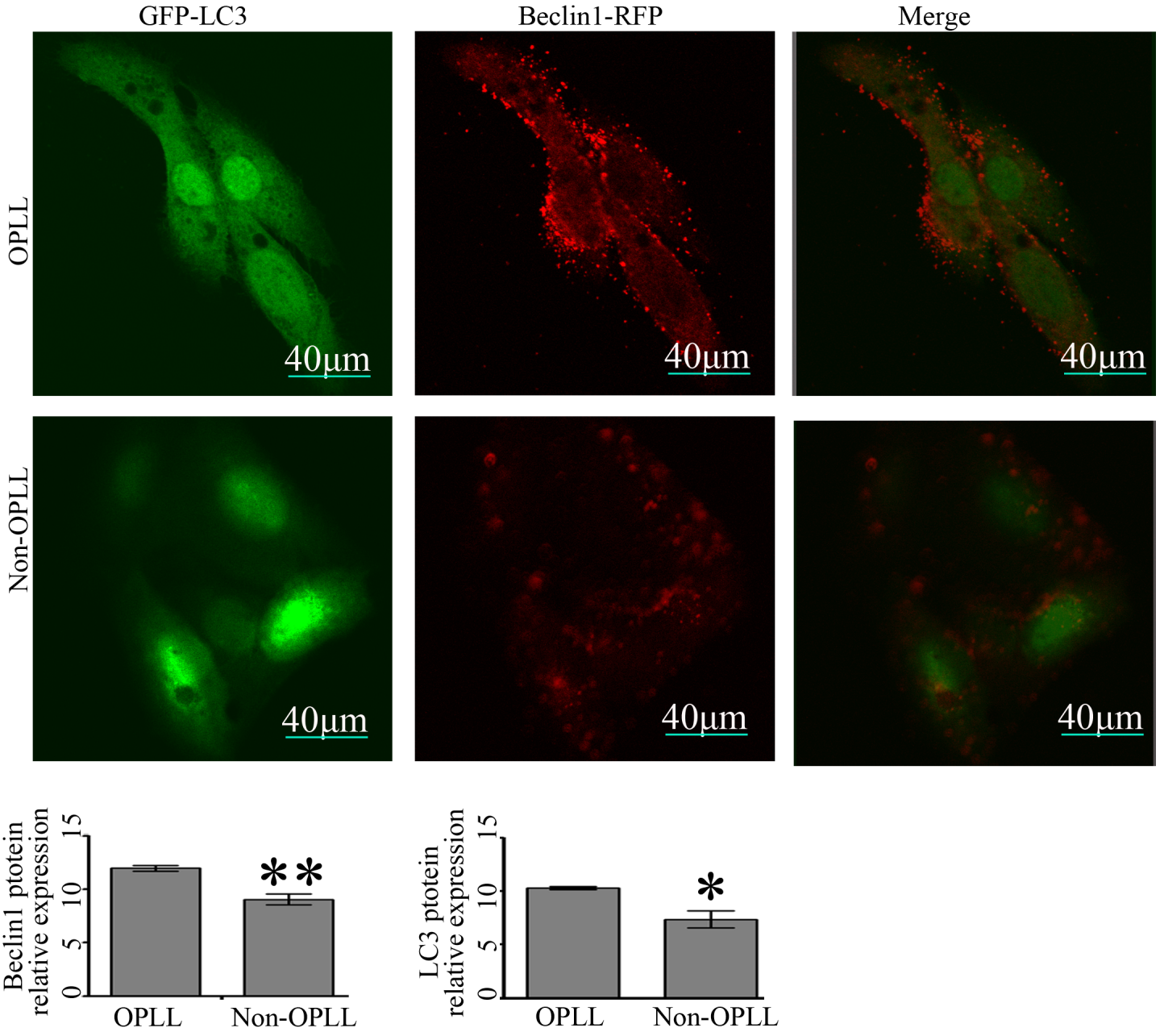

Figure 4

Representative fluorescence images showing immunofluorescence assay for LC3 protein (green), Beclin1 (red), and merged images under the two different conditions. Scale bar, 40 μm. There was an increase in the accumulation of Beclin1 protein dots and LC3-labeled vacuoles in the cytoplasm of ligament fibroblasts from patients with OPLL. Data is presented as the mean ± SD of at least three independent experiments. *P<0.05, **P<0.01 OPLL vs. non-OPLL. OPLL, ossification of the posterior longitudinal

ligament; RT-qPCR, reverse transcription-quantitative PCR; LC3, microtubule-associated protein 1 light chain 3.

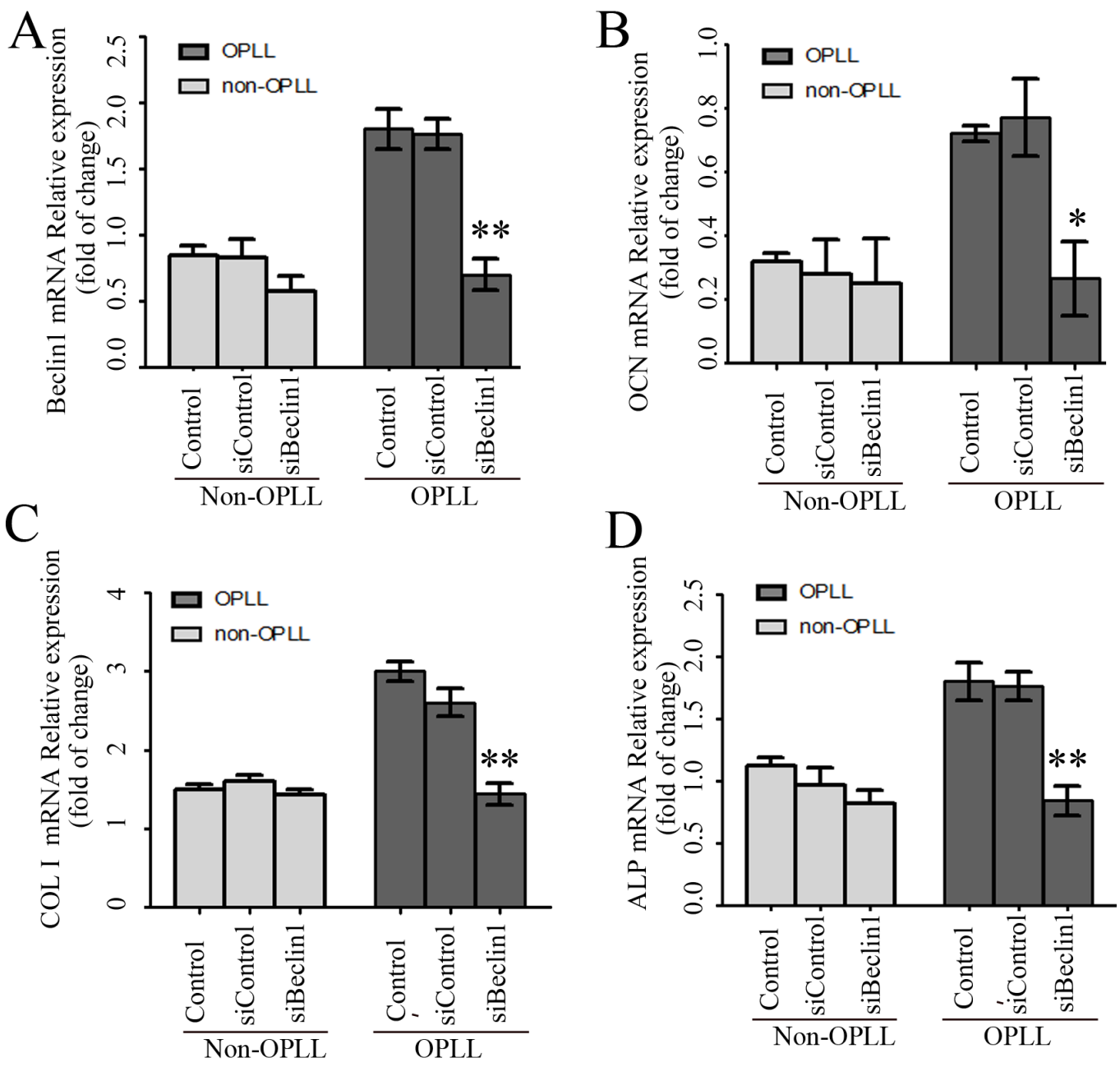

Figure 5

Role of Beclin1 in osteogenic differentiation of ligament fibroblasts. RT-qPCR assay to measure the mRNA expression of (A) Beclin1, (B) OCN, (C) COL I and (D) ALP in OPLL and non-OPLL cells transfected with siBeclin1 or siControl for 72 h. In OPLL cells, after knockdown by siBeclin1, the expression of Beclin1, OCN, COL I and ALP genes were reduced by 63.2% ($P<0.01$), 52% ($P<0.01$) and 53.2% ($P<0.01$), respectively. However, no significant difference was observed between the siBeclin1, siControl and control groups in non-OPLL cells. OPLL, ossification of the posterior longitudinal ligament; RT-qPCR, reverse

transcription-quantitative PCR; ALP, alkaline phosphatase, biomineralization associated; COL 1, collagen type 1; OCN, osteocalcin; si, small interfering.