Inflammation, Vol. 42, No. 2, April 2019 (© 2018)
DOI: 10.1007/s10753-018-0920-6

ORIGINAL ARTICLE

CPEB1 Expression Correlates with Severity of Posttraumatic Ankle Osteoarthritis and Aggravates Catabolic Effect of IL-1β on Chondrocytes

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Abstract— Most cases of posttraumatic ankle osteoarthritis (PTAOA) represent a sequela of ankle fractures. The cytoplasmic polyadenylation element-binding protein 1 (CPEB1) is an RNA binding protein that controls protein expression. Here, we report the previously unappreciated association of CPEB1 with PTAOA. We found that CPEB1 was upregulated in articular cartilage from patients with PTAOA. Additionally, its expression level positively correlated with disease severity. In human primary chondrocytes cultured *in vitro*, CPEB1 was upregulated when treated with pro-inflammatory cytokines, *i.e.*, IL-1β and TNF-α, suggesting that the observed CPEB1 upregulation in articular cartilage of PTAOA patients may be attributed to local inflammatory milieu. Functionally, CPEB1 overexpression aggravated the catabolic effect of IL-1β on chondrocytes *in vitro*, and *vice versa*, its knockdown reduced this effect, together implying a detrimental role of CPEB1 involved in OA progression. In sum, our study identifies CPEB1 as a potential regulator of disease progression of PTAOA.

KEY WORDS: CPEB1; correlation; posttraumatic ankle osteoarthritis; chondrocyte; IL-1β; catabolism.

INTRODUCTION

Osteoarthritis (OA) is the most common arthritis and a leading cause of disability worldwide [15]. OA is featured by progressive cartilage degeneration and also involves other pathological changes, including the disorder of matrix catabolism [28]. Although compared with hip and knee OA, ankle OA is much less common, and its prevalence and incidence continue to increase [27, 31]. Ankle OA is mainly derived from posttraumatic origin, and 65%–80% of cases are posttraumatic ankle osteoarthritis (PTAOA), representing a sequela of trauma [11, 33]. The ankle trauma that are likely to cause OA include fracture of the tibia, fibula, and talus as well as ankle ligament lesions [33]. Several risk factors for the development of PTAOA have been documented, such as severity and type of facture, the degree of cartilage injury, extent of fracture reduction, increasing age, obesity, *etc.*, with the magnitude of the initial trauma to the articular surface being the most important predisposition [5, 12]. Regardless of the aforementioned etiology, however, the molecular marker and mechanisms that underlie the pathogenesis of PTAOA are poorly understood.

Lei Li and Jiaping Lan are the co-first authors contributed to this study.

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Abbreviations: PTAOA, Posttraumatic ankle osteoarthritis; CPEB1, Cytoplasmic polyadenylation element-binding protein 1; UTR, Untranslated region; MMPs, Matrix metalloproteinases; siRNA, Small interfering RNA; MEFs, Mouse embryo fibroblasts

The cytoplasmic polyadenylation element-binding protein 1 (CPEB1) is an RNA-binding protein that binds the 3′ untranslated region (UTR) cytoplasmic polyadenylation element of its target mRNAs and regulates their translation through cytoplasmic changes in poly(A) tail length [32]. CPEB1 can either mediate the extension or removal of poly(A) tail, and by so doing, it consequently promotes or represses translation, respectively [14]. Due to its wide range of target mRNAs, to date, CPEB1 has been implicated in numerous biological activities and diseases, such as cell cycle progression [8, 25], cellular differentiation and senescence [7, 30], energy metabolism and insulin resistance [1, 4], inflammatory immune response [13], neurological abnormalities [2, 32], and cancer metastasis [24]. In the present study, we explored whether CPEP1 is associated with PTAOA, and we further examined its role in chondrocyte catabolism induced by IL-1β. Our findings indicate that CPEB1 expression is upregulated in articular cartilage of PTAOA patients and its expression level positively correlates with disease severity, and that CPEB1 aggravates IL-1β-induced catabolism of chondrocytes *in vitro*, together implying a potential role of CPEB1 in participating in disease pathogenesis.

MATERIALS AND METHODS

Ethics Committee Approval

The study design and sampling procedure were approved by the Medical Ethical Committee of Suining Central Hospital. The informed consents were obtained from all patients who consulted our institution for ankle OA evaluation and underwent amputation.

Antibodies and Reagents

The antibodies and reagents were purchased from the following sources: anti-CPEB1 (Invitrogen, PA1-1100), anti-β-Actin (Cell Signaling, 4967), anti-MMP-3 (Novus, NBP1-82431), anti-MMP-13 (Novus, NBP1-45723), anti-ADAMTS-5 (Abcam, ab41037), goat anti-rabbit IgG-HRP (Invitrogen, 65-6120), goat anti-mouse IgG-HRP (Invitrogen, 65-6520), and goat anti-rabbit IgG-peroxidase (Sigma-Aldrich, A0545). Recombinant human IL-1β (201-LB) and recombinant human TNF-α (210-TA) were purchased from R&D Systems.

Patients and Sampling

Exclusion criteria includes rheumatoid ankle OA, ligamentous posttraumatic ankle, and other inflammatory secondary ankle OA. The enrolled patients met the inclusion criteria of symptomatic posttraumatic ankle OA and were diagnosed with primary to end-stage ankle OA (37 cases; 20 female, 17 male; average age 58.4 years). The ankle articular cartilage tissues were obtained from the destructive area of tibia plateau when these patients underwent total ankle arthroplasty (OA group). On the other hand, normal articular cartilage of ankle joints (normal group) was obtained from patients who underwent amputation due to accidents and had no history of OA in ankle or other sites (21 cases; 12 female, 9 male; average age 56.5 years).

Improved Mankin Grading of Ankle OA

The histological sections were prepared as previously reported [26]. Briefly, the ankle articular cartilage samples were fixed in 4% paraformaldehyde, decalcified in 0.2 M EDTA (pH 8.0), and followed by being dehydrated in mixed solution of ethanol and xylene and embedded in paraffin. Serial sections with 5-μm thickness were subjected to H&E and Safranin-O/Fast Green staining. Samples were then divided into four groups (normal, mild, moderate, and severe) based on osteoarthritic changes assessed by the improved Mankin grading [34]. The grading criteria is described in detail as follows: score 0, cartilage with smooth surface and regular chondrocyte distribution; scores 1–4, cartilage with fibrillation surface, proteoglycan loss, and intact zonal structure; scores 5–8, cartilage with clefts protruding to the middle zone and clusters of proliferating chondrocytes; and score ≥ 9, cartilage with clefts protruding to the deep zone and clusters of proliferating chondrocytes but lose of the tangential zone.

Immunohistochemistry

Immunohistochemistry was performed as adapted to previous study [3]. Briefly, paraffin-embedded cartilage sections with 5-μm thickness were deparaffinized, and then, antigen retrieval was conducted in 10-mM citrate buffer (pH 6.0) for 15 min. Sections were blocked in 3% H_20_2 for 10 min and further blocked with 5% goat serum for 30 min at room temperature. After blocking, sections were incubated with isotype IgG or primary anti-CPEB1 (1:200 dilution) overnight at 4 °C in a humidifying box. After mild washing, sections were further incubated with secondary antibodies conjugated with peroxidase (1:2000 dilution) for 1 h at room temperature. The immunoreactivity of

cartilage sections was assessed by staining with diaminobenzidine (DAB) for 2 min, followed by counterstained with hematoxylin for 1 min. Sections were eventually visualized under a light microscope. As previously described [6, 19], the IHC score of the expression of CPEB1 was semiquantitatively evaluated based on the intensity and proposition of chondrocytes displaying nuclear-cytoplasmic staining. The score system is described as follows: 1, weak; 2, moderate; and 3, strong intensity staining in more than 10% of cells. Total 12 stochastic regions of each group were analyzed, and the mean IHC score was calculated based on the following equation: [(value of weak-intensity pixels) (value of intermediate-intensity pixels) (value of strong-intensity pixels)]/total value of pixels.

Isolation and Culture of Chondrocytes

Human primary articular chondrocytes were isolated from normal articular cartilage of ankle joints of patients, who underwent amputation due to accidents as described above. The isolation was performed as previously described [23]. Briefly, articular cartilage samples were dissected from ankle joint surfaces and rinsed in sterile PBS. Next, cartilage samples were cut into small pieces with a sterile surgical blade in a biosafety cabinet and then incubated in 1.5 mg/ml pronase solution (Roche) at 37 °C for 2 h and fully digested overnight at 37 °C in 1.5 mg/ml collagenase II solution (Sigma-Aldrich) with a condition of continuous agitation. The final product was filtered *via* a 70-μm size cell strainer (BD Biosciences) to obtain single-cell suspension. Cells were seeded in culture dish with a density of 20,000 cells/cm^2 in a humidified incubator at 37 °C with 5% CO_2. DMEM/F12 (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 1% L-glutamine (Invitrogen) was used as medium for culturing chondrocytes. Chondrocytes were maintained in a monolayer status throughout.

Treatment and Transfection of Chondrocytes

Chondrocytes were seeded with nearly 30% confluent 24 h before treatment. Chondrocytes were treated with titrated concentrations of human recombinant IL-1β or TNF-α for another 24 h. For CPEB1 overexpression, the coding sequence of human CPEB1 was cloned into pCAG vector and was transfected into chondrocytes with Cytofect™ Cell Line Transfection Kit (Cell Applications, TF104K). pCAG vector transfection was used as expression control. For CPEB1 depletion, siRNA specifically targeting scrambled sequence or CPEB1 was transfected into chondrocytes with Lipofectamine RNAiMAX (ThermoFisher Scientific) at a final concentration of 20 nM. siRNAs were purchased from Sigma-Aldrich. Forty-eight or 72 h after transfection, chondrocytes were harvested and the transfection efficacy was measured by Western blot or qRT-PCR analysis.

Western Blot Analysis

Samples of cartilage tissues were ultrasonicated prior to protein extraction. The ultrasonicated products or cultured chondrocytes were completely homogenized in RIPA lysis and extraction buffer (ThermoFisher Scientific, 89,900) added with protease inhibitor Cocktails (Sigma, S8830) on ice for 20 min. The whole lysates were centrifuged at 12,000×*g* for 10 min at 4 °C. The bottom pellets were discarded, and the supernatants containing proteins were collected. The protein concentration was determined by BCA assay according to the manufacturer's instructions (Pierce, 23225). After quantification, proteins were denatured in SDS loading sample buffer boiled for 10 min at 100 °C. Equal amount of total proteins were loaded and analyzed by 10% or 12% SDS-PAGE. Proteins were then transferred onto Immobilon-NC membranes (Millipore, HATF00010). Membranes were blocked for 1 h at room temperature with 5% bovine serum albumin soluted in TBST containing Tween 20. Membranes were then incubated sequentially with primary antibodies overnight at 4 °C and secondary antibodies for 1 h at room temperature. After washing with TBST for 30 min, protein bands were visualized by using ECL reagent kit (ThermoFisher Scientific, 32106) and automatic chemiluminescent analysis instrument (Tanon, 4600). The band intensity was quantified using ImageJ software.

RT-qPCR Analysis

The total mRNA was isolated with RNA simple total RNA kit (TIANGEN, DP419) and then immediately reverted to cDNA product using RevertAid First-strand cDNA synthesis kit (Thermo Fisher Scientific, K1621) according to the manufacturer's instructions. RT-qPCR analysis was conducted using SYBR Green PCR Master Mix (Thermo Fisher Scientific, 4367659) and CFX96 detection system (Bio-Rad) to quantify mRNA level. The mRNA level of *ACTB* gene was used as an endogenous control. Data were analyzed using the comparative Ct method and expressed as mean ± SD. Primer pairs used for amplifying targets are listed as follows: human CPEB1 forward 5′-TGTGTGCCAAAGAACCAGTG-3′, reverse 5′-ACACCACAGCTTACTCCACA-3′; human ADAMTS5 forward 5′-TCAAGAGGCTGGAGAATGGG-3′, reverse 5′-AAGTTCCTGCCGCAAGTTTT-3′; human MMP3 forward 5′-TCAAGAGGCTGGAG

AATGGG-3′, reverse 5′-AAGTTCCTGCCGCAAGTTTT-3′; human MMP13 forward 5′-AACGCCAGACAAATGTGACC-3′, reverse 5′-AGGTCATGAGAAGGGTGCTC-3′; and human β-actin forward 5′-AGGCTGTGCTATCCCTGTAC-3′, reverse 5′-AATGTCACGCACGATTTCCC-3′.

Statistical Analysis

All data were obtained from at least three independent experiments and presented as mean ± SD. The statistical significance was calculated by unpaired two-tailed Student's *t* test, unless indicated otherwise. $P < 0.05$ or $P < 0.01$ is considered to be statistically significant.

RESULTS

CPEB1 Is Upregulated in Articular Cartilage from Patients with PTAOA

To seek the possible role of CPEB1 associated with PTAOA, we first measured its mRNA level in articular cartilage from healthy individuals (normal) and patients with PTAOA. qRT-PCR analysis showed that compared with normal samples, the mRNA level of CPEB1 was significantly increased in PTAOA group (PTAOA *vs.* normal, 1.83-fold, $p = 0.0053$) (Fig. 1A). Consistent with this result, the protein level of CPEB1 was also upregulated in articular cartilage of PTAOA group, as determined by Western blot and band intensity analyses (Fig. 1B). The upregulation of CPEB1 in PTAOA was further confirmed by immunohistochemical (IHC) staining on sections of articular cartilage, in which most of it showed nuclear-cytoplasmic localization in chondrocytes (Fig. 1C), coinciding with its feature as a nuclear-cytoplasmic shuttling protein [21]. Together, these findings obtained from clinical samples indicate that CPEB1 is upregulated in articular cartilage of PTAOA patients, and suggest that it may have some relevance to PTAOA.

CPEB1 Expression Level Correlates with Disease Severity of PTAOA

Given the increased tendency of CPEB1 expression observed in PTAOA patients, we hypothesized that the expression level of CPEB1 may indicate the disease severity of PTAOA. To test this possibility, we stratified the

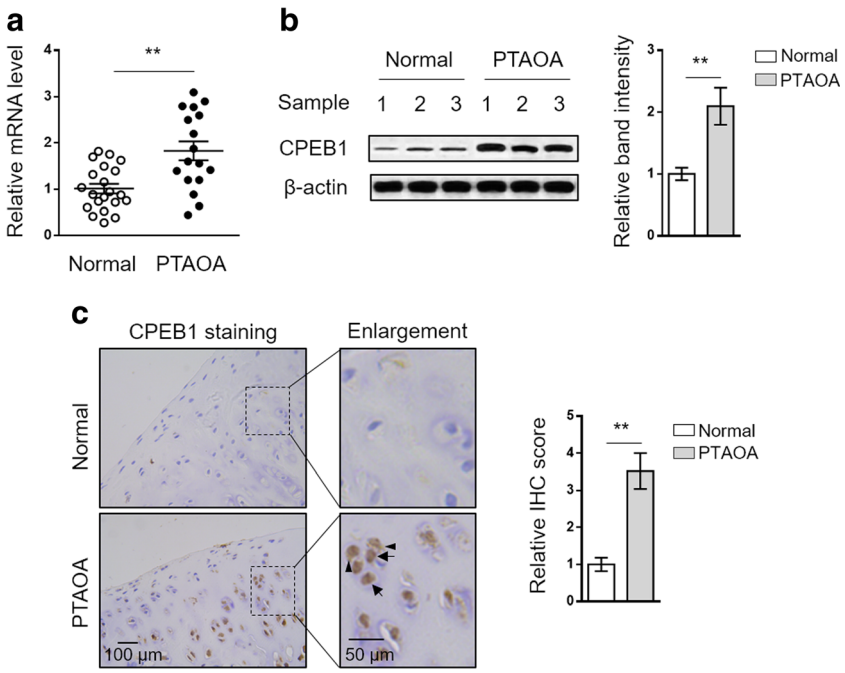

Fig. 1. CPEB1 is upregulated in articular cartilage from osteoarthritis ankle. **A** qRT-PCR analysis of CPEB1 mRNA level in ankle articular cartilage from healthy controls (normal) ($n = 21$) and posttraumatic osteoarthritis patients (PTAOA) ($n = 17$). Results relative to normal were shown. **B** Western blot analysis of CPEB1 protein level in normal and OA ankle articular cartilage. Representative images (left) and statistical analysis of band intensity were shown (right). **C** Immunohistochemical (IHC) staining of CPEB1 in normal and OA ankle articular cartilage. Representative images (left) and quantification analysis (right) were shown. Arrows indicate nuclear staining and arrowheads indicate cytoplasmic staining. β-Actin was used as a reference or loading control. Data are mean ± SD. Student's *t* test. $^{**}P < 0.01$.

recruited PTAOA patients as mild, moderate, and severe groups according to the improved Mankin grading system (Fig. 2A). The expression of CPEB1 in articular cartilage of each group was determined by IHC staining. As shown, in contrast to normal group, the staining intensity of CPEB1 (Fig. 2B) and IHC score (Fig. 2C) were gradually increased from mild to moderate to severe PTAOA. Furthermore, when pooling these data together, correlation analysis manifested that the IHC score of CPEB1 expression was positively correlated well with the Mankin grading of PTAOA ($R^2 = 0.6527$, $p < 0.01$) (Fig. 2D), thus indicating a positive correlation between CPEB1 expression level and disease severity of PTAOA.

CPEB1 Is Upregulated in Chondrocytes Treated with pro-Inflammatory Cytokines *In Vitro*

OA is now recognized as an inflammatory disease, and the secreted inflammatory molecules, such as proinflammatory cytokines (*i.e.*, IL-1β and TNF-α), are among the critical regulators of the pathophysiological processes implicated in OA development [16]. In order to establish a possible link between upregulated CPEB1 expression in articular cartilage of PTAOA and local inflammatory environment, we utilized an *in vitro* system of culturing primary human chondrocytes which were treated with IL-1β or TNF-α, and then assessed the expression change of CPEB1 at both mRNA and protein levels. As shown in Fig. 3A, B, the expression level of CPEB1 in chondrocytes was increased by IL-1β treatment in a dose-dependent manner. Moreover, similar results were obtained when chondrocytes were treated with TNF-α (Fig. 3C, D). Collectively, these data show that CPEB1 expression in chondrocytes can be upregulated when exposed to proinflammatory cytokines, at least *in vitro*, and also imply that the local inflammatory environment may contribute to the upregulation of CPEB1 observed in clinical articular cartilage of PTAOA patients.

CPEB1 Aggravates the Catabolic Effect of IL-1β on Chondrocytes *In Vitro*

Predisposing factors that potentially drive pathophysiological processes toward OA commonly activate biochemical pathways in chondrocytes, resulting in the degradation of

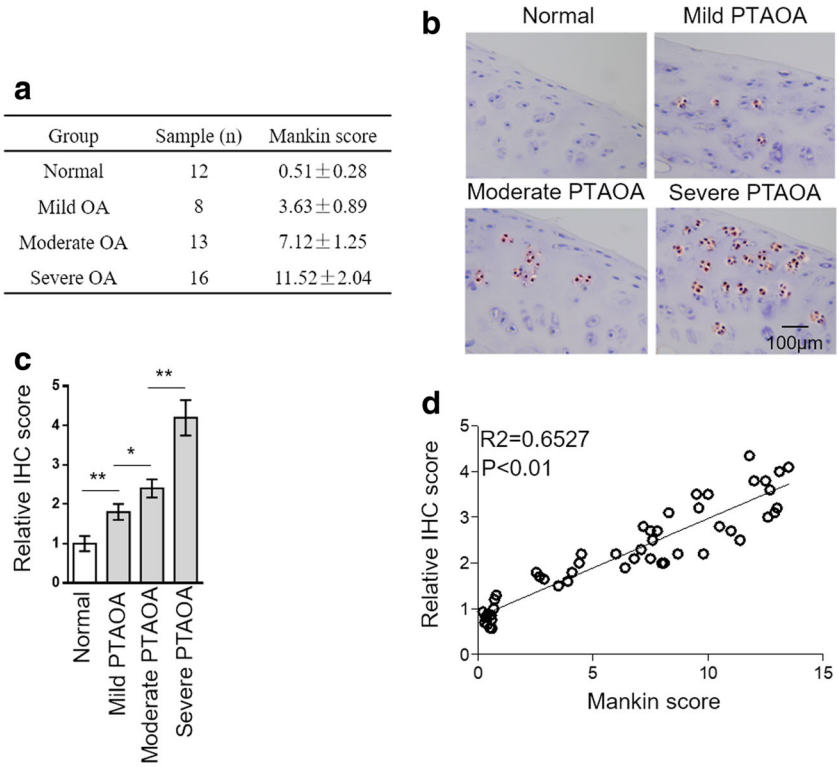

Fig. 2. CPEB1 expression level correlates disease severity. **A** Mankin score of ankle articular cartilage from normal ($n = 21$) and OA patients ($n = 17$) stratified by disease degree (mild, moderate, and severe). **B**, **C** IHC staining of CPEB1 in normal and OA ankle articular cartilage. Representative images (**B**) and quantification analysis (**C**) were shown. Scale bar 100 μm. **D** Pearson's correlation analysis between the relative value of CPEB1 staining and Mankin scoring. R^2 and P value were shown. Data are mean ± SD. Student's t test. $^{**}P < 0.01$; $^{*}P < 0.05$.

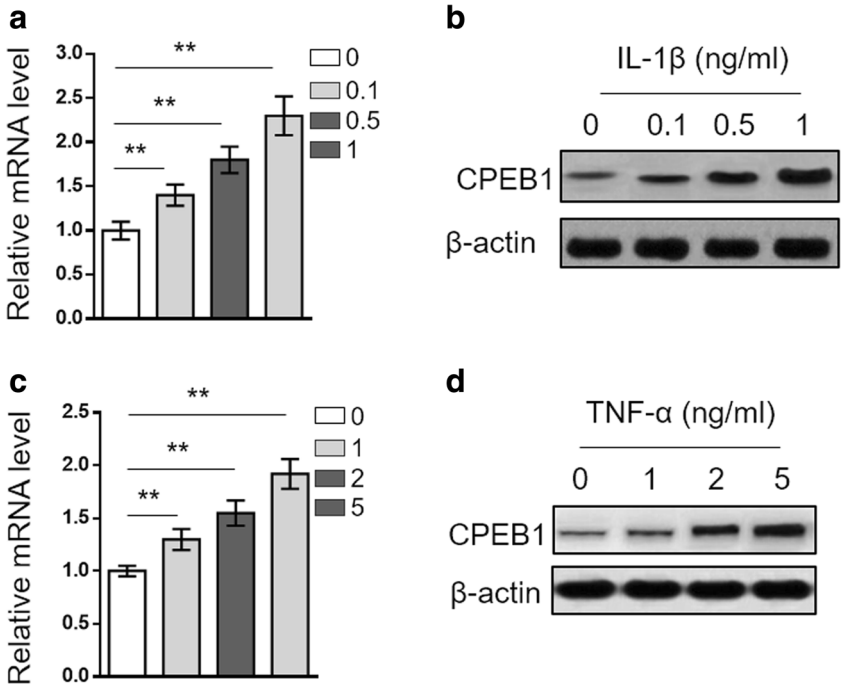

Fig. 3. CPEB1 is upregulated in chondrocytes treated with pro-inflammatory cytokines *in vitro*. **A**, **B** Relative mRNA level (**A**) and protein level (**B**) of CPEB1 in chondrocytes treated with or without increasing concentrations of IL-1β for 24 h. **C**, **D** Relative mRNA level (**C**) and protein level (**D**) of CPEB1 in chondrocytes treated with or without increasing concentrations of TNF-α for 24 h. β-Actin was used as a reference or loading control. Data are mean ± SD. Student's *t* test. $^{**}P < 0.01$.

extracellular matrix mediated by matrix metalloproteinases (MMPs) and aggrecanases (ADAMTSs) [18]. Among the members of matrix-degrading enzymes, MMP3, MMP13, and ADAMTS5 have been shown to play a crucial role in cartilage destruction in OA patients [35]. To know more about the functional role of CPEB1 in chondrocytes under pathological condition (*i.e.*, exposure to IL-1β), enforced overexpression of CPEB1 was introduced into cultured human primary chondrocytes. As shown, IL-1β treatment expectedly induced expressions of MMP3, MMP13, and ADAMTS5 in chondrocytes; although CPEB1 overexpression alone did not show obvious effect, intriguingly, it further augmented the catabolic effect of IL-1β treatment, at both mRNA and protein levels (Fig. 4A, B). These results suggest that CPEB1 is able to aggravate the catabolic effect of IL-1β on chondrocytes, at least *in vitro*. To further strengthen this notion, we depleted CPEB1 expression in chondrocytes by using small interfering RNA (siRNA) strategy. In opposite to the results of CPEB1 overexpression, CPEB1 depletion diminished the IL-1β-induced expression levels of MMP3, MMP13, and ADAMTS5, at both mRNA and protein levels (Fig. 4C, D), illustrating that the catabolic effect of IL-1β on chondrocytes is reduced when CPEB1 is absent. In conclusion, these lines of functional evidence suggest a promotive role of CPEB1 in IL-1β effect on chondrocytes and hint that this role may underlie its positive association with disease severity of PTAOA.

DISCUSSION

The development of stage-specific treatment modality for clinic PTAOA cases needs accurate evaluation of disease stage. At present, radiographic assessment using the Kellgren-Lawrence scale and histopathological classification of severity of OA cartilage lesions *via* Mankin score are frequently used [17, 34]. However, no definitive consensus about staging of PTAOA is reached [10]. Besides, the molecular marker capable of indicating the severity of PTAOA has not been reported. In the current study, we found that CPEB1 was upregulated in articular cartilage of patients with PTAOA at both mRNA and protein levels, which suggests an induction of gene expression derived from a transcriptionally regulatory mechanism. We further showed that the upregulated extent of CPEB1 expression, as detected by IHC staining on sections of articular cartilage, was positively correlated well with disease severity of PTAOA as evaluated by Mankin scoring system. These

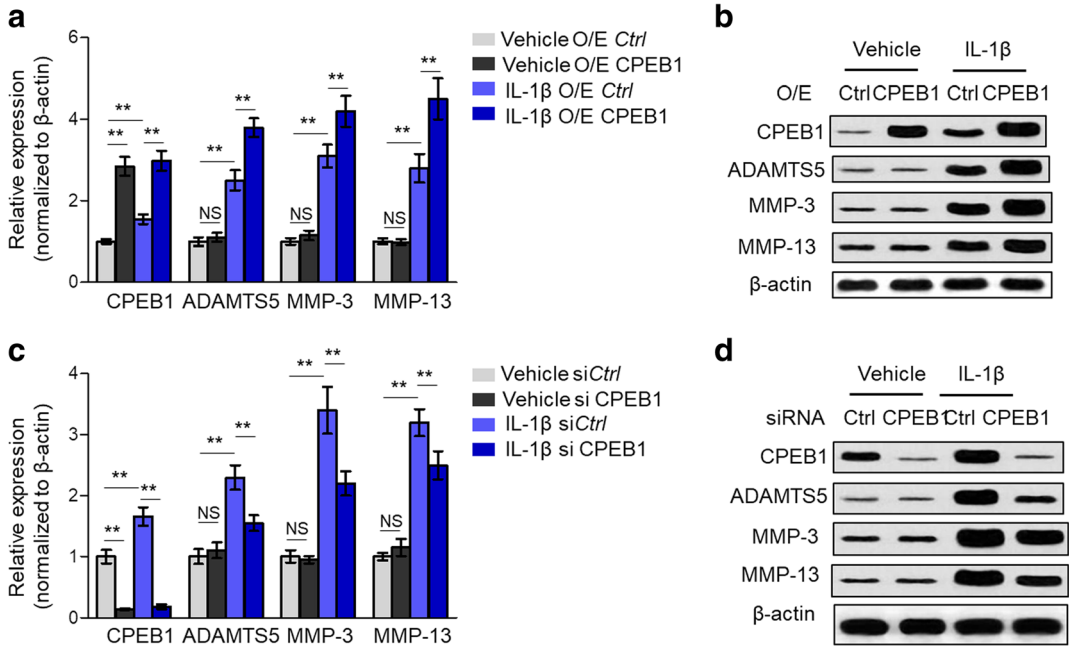

Fig. 4. CPEB1 aggravates the catabolic effect of IL-1β on chondrocytes *in vitro*. **A**, **B** mRNA levels (**A**) and protein levels (**B**) of CPEB1, ADAMTS5, MMP-3, and MMP-13 in chondrocytes stably expressing vector or CPEB1 and treated with or without 0.5 ng/ml IL-1β for 24 h. **C**, **D** mRNA levels (**C**) and protein levels (**D**) of CPEB1, ADAMTS5, MMP-3, and MMP-13 in chondrocytes transfected with siCtrl or siCPEB1 for 2 days and further treated with or without 0.5 ng/ml IL-1β for 24 h. β-Actin was used as a reference or loading control. Experiments were conducted at least three times independently. Data are mean ± SD. Student's *t* test. $^{**}P < 0.01$.

observations suggest that CPEB1 might play a role in the pathogenesis of PTAOA, and more importantly, its expression level may be used as a potential molecular indicator for evaluating the stage of PTAOA if specific criteria were made in the future and thus may hold a promise for aiding the development of precise treatment strategy for PTAOA. Nevertheless, one caution that should be observed in interpreting these observation is that due to the limited number of recruited study samples, further investigations including larger sample size are needed to consolidate the conclusion drawn from them. Additionally, whether the tendency of expression change of CPEB1 could also be observed in other types of OA merits further exploration.

In vitro cultured chondrocytes treated with inflammatory cytokines, such as IL-1β and TNF-α, exhibit pathologic changes, to some extent, reminiscent of phenotypes observed in OA chondrocytes, for instance, the highly upregulated catabolic activity [20]. Using cultured chondrocytes, we found that CPEB1 expression was induced under the treatment of inflammatory cytokines *in vitro*, which led us to suspect that the upregulation of CPEB1 observed on articular cartilage of PTAOA may be caused by local inflammatory milieu, since synovial membrane inflammation and cytokine secretion are commonly detected in OA patients [29]. However, how the treatment of inflammatory cytokines induces CPEB1 expression is currently unknown. Oppositely, two studies have shown that in mouse embryo fibroblasts (MEFs) lacking CPEB, the production of cytokines is decreased and a number of mRNAs encoding proteins involved in inflammation are dysregulated, which are related to TAK1 synthesis and NF-κB localization [9, 13], suggesting an immunoregulatory function of CPEB. Therefore, it is possible that a mutual effect and a positive feedback loop may exist between CPEB1 and inflammation mediated by production of inflammatory cytokines. Elucidating this issue may help us to understand the regulatory mechanism of CPEB1 expression.

In following functional study, we reveal the promotive role of CPEB1 in catabolic effect of IL-1β on chondrocytes *in vitro* by tactics of gain-of-function and loss-of-function. It is established that matrix-degrading enzymes mediate the degradation of extracellular matrix and promote the cartilage destruction in OA patients [22]. Based on the results of the promoted expressions of matrix-degrading enzymes by CPEB1, such as MMP3, MMP13,

and ADAMTS5 in chondrocytes, we suppose that CPEB1 may exert a detrimental role in PTAOA progression and that the upregulation of CPEB1 in articular cartilage could further aggravate this pro-arthritic effect.

In summary, this study identifies CPEB1 as a potential molecular marker associated with the severity of PTAOA and proposes that its promotive role in chondrocyte catabolism may be involved in the pathogenesis of PTAOA. Thus, manipulating the expression or activity of CPEB1 might be of benefit in interfering the pathophysiological processes of PTAOA.

COMPLIANCE WITH ETHICAL STANDARDS

The study design and sampling procedure were approved by the Medical Ethical Committee of Suining Central Hospital. The informed consents were obtained from all patients who consulted our institution for ankle OA evaluation and underwent amputation.

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