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Original Article

Regulation of cartilage damage caused by lack of Klotho with thioredoxin/peroxiredoxin (Trx/Prx) system and succedent NLRP3 activation in osteoarthritis mice

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Abstract: This present study aims to verify the underlying mechanism that anti-aging protein Klotho protects cartilages against the damage induced by oxidative stress. The Klotho expression level in the articular cartilages of mice with osteoarthritis (OA) was measured by using western blotting and quantitative real-time PCR. This work also investigated the effects of Klotho on chondrocyte functions, such as PI3K/Akt pathway, apoptosis and reactive oxygen species (ROS) production, through overexpressing Klotho in chondrocytes by transfecting with the plasmid encoding Klotho. The results showed that Klotho expression level obviously decreased in the articular cartilages of OA mice. It was also found that mechanical loading significantly reduced the expression and activity of Klotho in chondrocytes. In addition, the overexpression of Klotho suppressed chondrocyte apoptosis through thioredoxin/peroxiredoxin (Trx/Prx) family and ROS/TXNIP/NLRP3 signaling pathways. All these above findings suggest that Klotho is essential in OA progression, and may be a good target for the research and development of the drugs for OA treatment.

Keywords: Klotho, osteoarthritis, reactive oxygen species (ROS), thioredoxin/peroxiredoxin (Trx/Prx)

Introduction

Osteoarthritis (OA) featured with sub-chondral bone remodeling, synovial activation, and the progressive loss of cartilage homeostasis, is generally regarded as the primary age-relevant osteoarticular disease [1, 2]. Recently, the underlying molecular mechanism of OA was deciphered [2]. Articular chondrocyte acquisition of a $p16^{INK4A}$-dependent senescence phenotype was believed to be induced by repetitive mechanical stress and its consequent synovial inflammation [3], which is consistent with the terminal differentiation during endochondral ossification [3, 4].

Klotho, named after a Greek goddess Klotho, was identified as a mutated gene in Klotho mice in 1997, which is primarily expressed in reproductive organs, kidney and brain [5, 6]. The homozygous transgene mice have a number of phenotypes similar to premature aging syndrome, such as calcification, pulmonary emphysema, osteoporosis, short life span and skin atrophy. This present study verified the expression of Klotho in cartilages and confirmed the down-regulation of Klotho in the tissues of OA mice, as shown in a previous study [7]. In addition, the relationship between Klotho and OA was already verified in several studies [8-10]. For instance, anti-geronic secreted Klotho could not only protect against articular cartilages, but also be closely related to the suppression of OA onset; also, OA onset can be predisposed by 2 single nucleotide polymorphisms in α-Klotho, i.e. the genes that encode the anti-geronic hormone α-Klotho.

A previous study indicated that Klotho could regulate the expression and activity of forkhead-box class O (FoxO) and antioxidant system Prx/Trx through Akt signal, thereby indirectly

regulating intracellular reactive oxygen species (ROS) level [11]. Besides, when the intracellular ROS level raise, the inflammatory corpuscle, NLR Family Pyrin Domain Containing 3 (NLRP3), can be indirectly activated by activating Txnip, thus ultimately mediating the activations of caspase-1 and interleukin-11, which will induce a series of inflammatory responses [12]. More importantly, the generation of ROS was proved to play a significant role in the pathogenesis of OA because it could induce oxidative damage and the death of chondrocytes [13-17]. As a consequence, it's hypothesized that in the state of OA, the lack of Klotho can lead to the imbalance of Prx/Trx antioxidant system and eventually result in the increase of ROS level. Subsequently, with the increase of the ROS level, the inflammatory corpuscle NLRP3 will be activated, thereby mediating the downstream inflammatory responses and related apoptotic pathways.

Available literatures demonstrated that the Klotho expression level in the knee cartilages of OA mice decreased. Further studies indicated that Klotho was involved in the regulation of chondrocyte apoptosis through Trx/Prx system, ROS, apoptosis, and PI3K/Akt and NLRP3-ROS-IL-1β signaling pathways. In addition, the *in vitro* increase of Klotho could significantly alleviate the cyclic tensile strain (CTS)-induced ROS level in chondrocytes. Therefore, Klotho may be a significant factor to cause OA.

Methodology

Reagents

Monoclonal antibodies against Klotho, Prx-2, thioredoxin reductase-1 (Trxrd-1), FoxO3a, p-FoxO3a, pro-IL-1, p-FoxO, caspase-1 p20, NLRP3, and pro-caspase-1 were provided by Abcam (Cambridge, UK). Monoclonal antibodies against p-Akt (T308), p-Akt (S473), ERK1/2, p-ERK1/2 were provided by Cell Signaling Technology (Danvers, MA, USA). Fetal bovine serum (FBS) and low- and high-glucose DMEM were obtained from HyClone (Logan, UT, USA) Phosphate-buffered saline (PBS), cytoplasmic and membrane protein extraction kits, total protein extraction kit, RIPA buffer, and PMSF were obtained from Beyotime Biotechnology (Nantong, China). Apoptosis detection kit was obtained from Chemicon International, Inc. (Temecula, CA, USA). Alcian blue staining kit was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals

Twenty-four C57/6J mice weighting 19 to 21 g (3 months) were obtained from the Animal Inc. affiliated to Nanjing Medical University (Nanjing, China). These mice were randomly separated into 2 groups (control group and OA group; n=12), and then kept in 4 animal cages (6 mice per cage) in a temperature-controlled room (21-23°C), and free access to water and food. All the animal procedures were approved by the Animal Research Ethics Committee of Nanjing Medical University.

Anterior Cruciate Ligament Transaction (ACLT)

ACLT surgery was performed to induce OA in adult male C57/6J mice. Briefly, all mice were anesthetized with chloral hydrate. A medial parapatellar approach was adopted to expose the right knee joint. Then, an anterior cruciate ligament (ACL) transection was conducted with micro-scissors in the mice from the OA group, and then a positive anterior drawer sign was made to confirm the completeness of the transection. For the mice from the control group, arthrotomy was also conducted but without ACL transection. After the surgery, all mice were released from the cages for 30 min daily.

Hematoxylin and eosin (H&E) and Alcian blue stainings

At 12 week after surgery, the mice were anaesthetized, and then sacrificed by cervical dislocation. Then, the knee joint cavity was exposed by separating the patella. Next, samples were decalcified for 3 weeks by using 10% ethylenediaminetetraacetic (EDTA). After decalcifying, the samples were embedded in paraffin, were then cut into the standard 3 μm sections for H&E and Alcian blue stainings.

Immunohistochemistry

To perform the immunohistochemical analysis for Klotho, Prx-2 and Trxrd-1 expressions in articular cartilages of mice, the paraffin-embedded tissues in full-thickness were processed in this present study. A blocking serum (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) was used to incubate the

slides for 60 min. Then, the slides were incubated with the primary antibodies against Klotho, Prx-2 and Trxrd-1 for 2 h at room temperature (RT). Finally, the sections were incubated with the peroxidase-labeled secondary antibodies, followed by the streptavidin-biotin staining (DAB kit, Invitrogen, Paisley, UK).

Chondrocyte culture and tensile strain loading

For the primary culture of chondrocytes, the chondrocytes were isolated from the knee cartilages of mice. Firstly, the articular cartilage tissues were digested for 30 min using 0.25% trypsin after being cut into small pieces, followed by digesting with 0.2% Type II collagenase for 3 h. Finally, DMEM/F12 media, antibiotics and 10% FBS were used to culture the released cells.

When the confluence increased up to 80%, the cells were subjected to cyclic tensile strain for 48 h at 20% elongation with a 0.25 to 1 Hz sinusoidal curve indicated by a Flexcell1 FX-5000 Tension System following the manufacturer's instructions (Flexcell International Corporation, Burlington, NC, USA). To preserve the chondrocyte phenotype, only the cells not exceeding 2 passages were adopted.

Cell transfection

Empty vector, and lentiviral vector encoding Klotho were obtained from Genechem (Shanghai, China). Lentiviral transfection was conducted following the manufacturer's instructions. Green fluorescent protein (GFP) was used to assess the transfection efficiencies. 5 singl puromycin (Sigma-Aldrich, USA) was used to screen the stably transfected cells after 48 h.

Western blot analysis

Total protein was prepared by using RIPA buffer, and the nuclear protein was isolated by using nuclear protein extraction kit (Beyotime) strictly according to the kit instructions. The protein mixtures were separated with gel electrophoresis in sodium dodecyl sulfate-polyacrylamide (SDS-PAGE), were then transferred onto nitrocellulose membranes. Briefly, 10% SDS-PAGE was used to extract 15 μg plasma proteins from cartilage tissues and chondrocytes. Then, the proteins were electroblotted onto polyvinylidene difluoride membranes with a thickness of 0.45 mm (Millipore, Bedford, MA, USA), and were then blocked with 5% non-fat dry milk diluted in Tris-buffered saline with Tween 20 (TBST) for 1 h. Next, these membranes were incubated with Klotho, Prx-2, Trxrd-1, Akt, p-Akt (T308), p-Akt (S473), ERK1/2, p-ERK1/2, p-FoxO3a, FoxO3a, caspase-1 p20, pro-caspase-1, pro-IL-1β, IL-1β and NLRP3 antibodies at 4°C overnight. To visualize the antigen-antibody complexes, enhanced chemiluminescence assay (Thermo Scientific, Pierce, Rockford, IL, USA) was performed.

Co-immunoprecipitation

Immunoprecipitation lysis buffer (30 mM Tris-HCl, 150 mm NaCl, 1% Triton X-1002 mM EDTA, 10% glycerol, 1× protease inhibitor) was used to homogenize cartilage tissues on ice before ultrasound. After 30 minutes of incubations on ice, the lysate was separated by centrifugation, and then protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, sc-2003) were used to pre-clear the supernatant at 4°C. Then, 2 μg antibody against Txnip was used to incubate the precleared supernatant on a rocker at 4°C for 4 h. Next, after adding beads for an additional 1 h, centrifugation was performed at 1,000× g for 5 min to collect immunoprecipitates. The immunoprecipitation lysis buffer was subsequently used to wash the obtained immunoprecipitates for 3 times, followed by centrifugation. 2× sample buffer was used to re-suspend and boil the pellet to analyze the expressions of TXNIP, Trx and NLRP3 by using western blotting as above described.

Quantitative real-time PCR (QRT-PCR)

TRIzol reagent (Invitrogen) was used to extract total RNA from chondrocytes, subsequently the total RNA was reversely transcribed into cDNA by using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). SYBR Premix Ex Taq II reagents provided by TaKaRa (Dalian, China) were used to perform qRT-PCR following the manufacturer's instructions. 20 μL mixture with 2 μL cDNA was used to perform PCR. Specific primers (TaKaRa) were used to amplify Klotho cDNAs. Glyceraldehyde-3-phosphatedehydrogenase (GADPH) was used as an internal gene to normalize and calculate the gene expression by using the 2-DDCT method. The primer sequences for Klotho as fol-

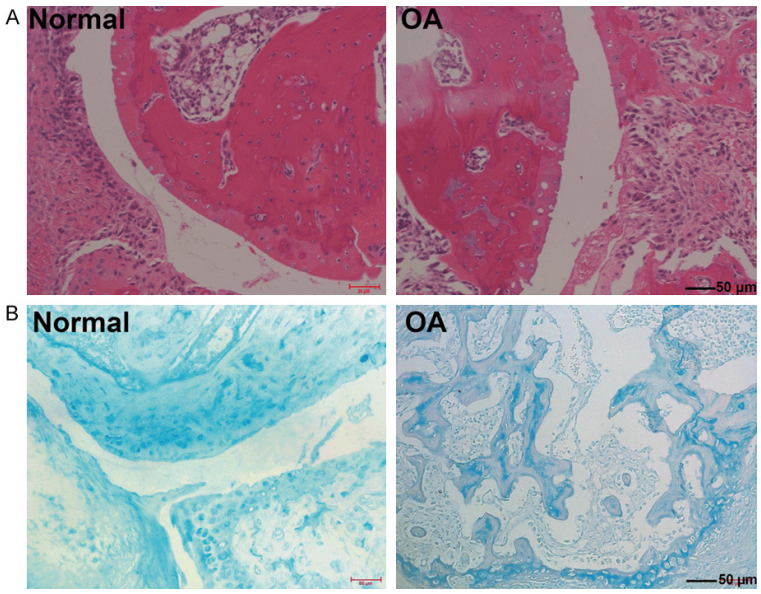

Figure 1. Hmatoxylin and eosin (H&E) and Alcian blue stainings. A. Representative photomicrographs for HE staining of pathological joint sections of normal and OA mice at 12 week after Anterior Cruciate Ligament Transaction (ACLT). B. Alcian blue staining for pathological joint sections of normal and ACLT-treated OA mice.

lows: forward, 5'-ACTACGTTCAAGTGGACACTACT-3' and reverse, 5'-GATGGCAGAGAAATCAACACAGT-3'.

Quantification of intracellular ROS level

PBS was used to wash the collected cells, and then 10 μM DCFH-DA (Molecular Probe) was used to label these cells at 37°C in the dark for 30 min using DMEM without phenol red. Then, flow cytometry (Partec, excited at 495 nm, emit at 529 nm) was used to determine the ROS levels (fluorescence intensity) in these cells after 3 times of washes with PBS. An FLx8000 fluorescent microplate reader purchased from Bio-Tek (VT, USA) was used to quantify the relative fluorescent intensities at the indicated time points. The cells were then placed in 35 mm dishes containing coverslips to visualize the intracellular ROS level by using fluorescence microscope.

In situ TUNEL staining

Apoptosis was evaluated with a TUNEL kit purchased from Roche Diagnostics Company (Mannheim, Germany). Briefly, after deparaffinization and hydration, proteinase K (20 μg/ml) was used to incubate the sections at RT for 15 min. After permeabilization, the TUNEL reaction mixture was used to incubate the slides. HRP-conjugated Fab fragments were adopted in this present study to detect the labelled DNA strand breaks.

Statistics

All data were shown as mean ± SEM of 3 independent experiments. One-way analysis of variance (ANOVA) was used to compare the difference between experimental and control groups, and $P<0.05$ was considered as significant difference.

Results

HE and Alcian blue staining results

Representative photomicrographs for HE and Alcian blue stainings of joint pathological sections of normal and OA mice were available at 12 week after ACLT. As shown in **Figure 1A** and **1B**, intact and smooth cartilages were showed on the sham-operated knee joints. Additionally, typical healthy chondrocytes were presented in the cartilages with flat cells in the superficial zone (1 or 2 layers), and columns of round cells in the middle and deep zones were observed. On the contrary, cartilage damage and disorganized chondrocytes were presented on the ACLT-operated knees of OA mice.

Expressions of Trx/Prx family members and Klotho decreased but NLRP3/caspase-1/IL-1β Axis expression increased in OA mice

Prxs widely distributed in mammalian tissues are defined as 6 small non-seleno peroxidases from a family. With the help of thioredoxin as an electron donor, Prxs can detoxify peroxides, while the thioredoxin can be subsequently reduced with Trxrd. A previous study provided the sufficient evidence that Akt and ERK played a crucial role in up-regulation of Prx-2 induced by Klotho [11]. In OA mice, anti-oxidative factors, Trxrd-1 and Prx-2 in the Trx/Prx redox control system significantly decreased (**Figure 2A**). Meanwhile, the immunostaining intensities of

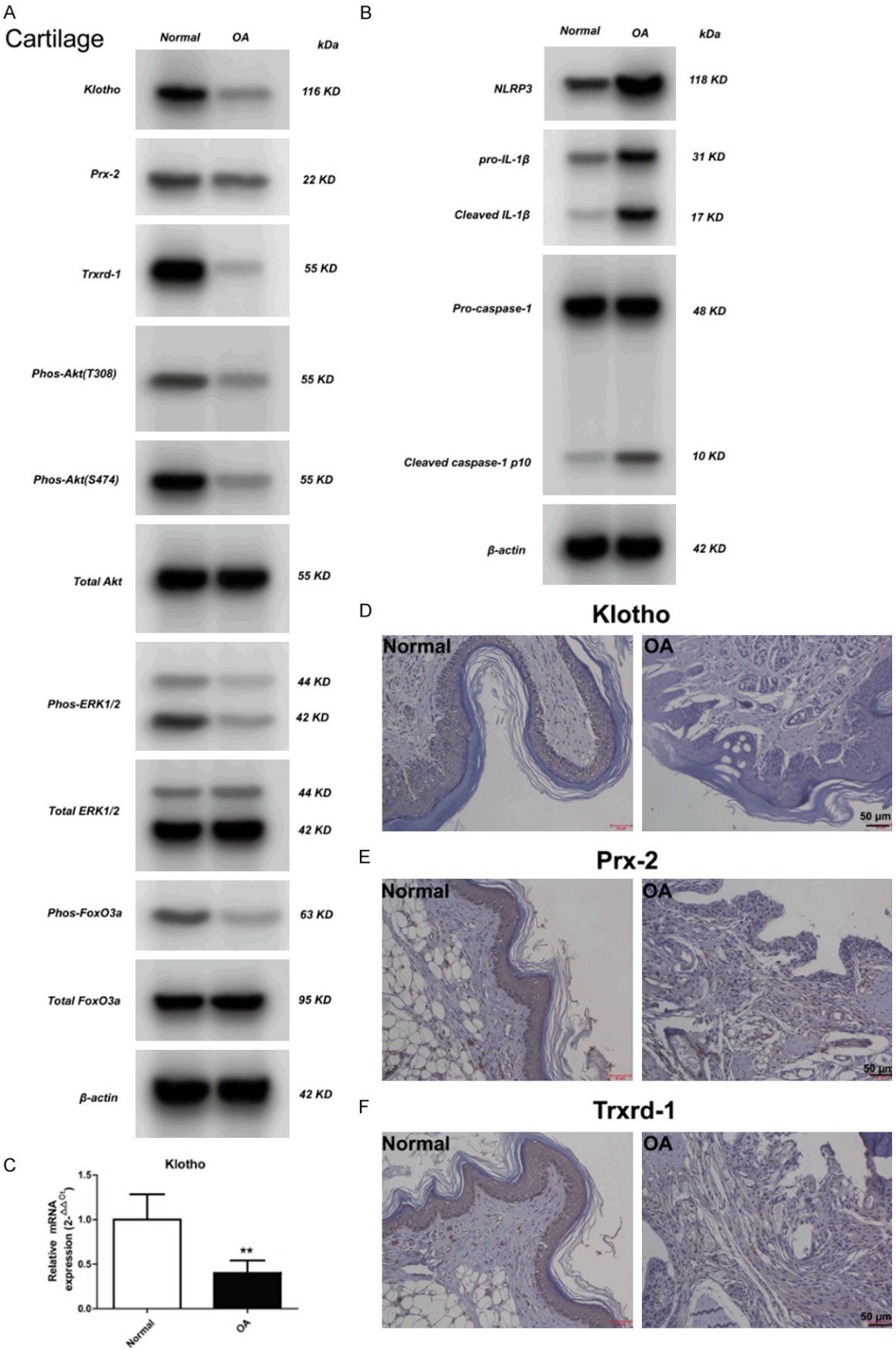
A
Cartilage
Normal
OA
kDa
Klotho
116 KD
Prx-2
22 KD
Trxrd-1
55 KD
Phos-Akt(T308)
55 KD
Phos-Akt(S474)
55 KD
Total Akt
55 KD
Phos-ERK1/2
44 KD
42 KD
Total ERK1/2
44 KD
42 KD
Phos-FoxO3a
63 KD
Total FoxO3a
95 KD
β-actin
42 KD
B
Normal
OA
kDa
NLRP3
118 KD
pro-IL-1β
31 KD
Cleaved IL-1β
17 KD
Pro-caspase-1
48 KD
Cleaved caspase-1 p10
10 KD
β-actin
42 KD
C
Klotho
Relative mRNA expression (2-ΔΔCt)
1.5
1.0
0.5
0.0
**
Normal
OA
D
Klotho
Normal
OA
50 μm
E
Prx-2
Normal
OA
50 μm
F
Trxrd-1
Normal
OA
50 μm

Figure 2. Multiple factors associated with the pathogenesis of OA stimulated the Kltoho expression in chondrocytes. (A) At 12 week after ACLT, the relative expression levels of Klotho, Prx-2, Trxrd-1, p-Akt (T308), p-Akt (S473), Akt, ERK1/2, p-ERK1/2, p-FoxO3a and FoxO3a in OA mice were quantified by using densitometry analysis and normalized to β-action. (B) The expression of NLRP3/caspase-1/interleukin-1β (IL-1β) axis, the relative expression levels of IL-1 analysis and NLRP3, caspase-1 p20 and pro-caspase-1 were quantified by using densitometry analysis and normalized to β-actin. (C) QRT-PCR analysis for Klotho mRNA expression in cartilages, (D-F) representative photomicrographs of immunohistochemical staining for Klotho, Prx-2 and Trxrd-1. The brown represented the expressions of Klotho, Prx-2 and Trxrd-1 in cartilages. Results were expressed using the data from 3 independent experiments. **P*<0.05, ***P*<0.01.

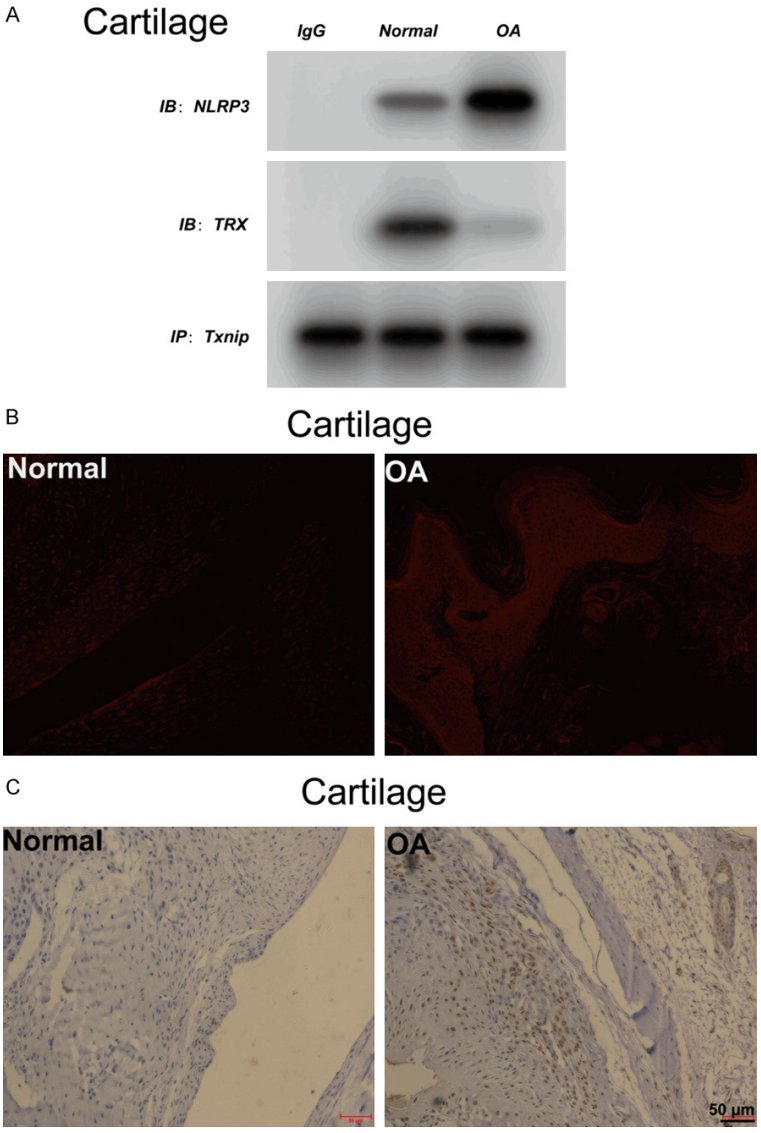

Figure 3. ROS production, TXNIP-NLRP3 binding and chondrocyte apoptosis were induced in the cartilages of OA mice. A. Co-immunoprecipitation study demonstrated the *in vivo* TXNIP-NLRP3 binding and TXNIP-Trx binding in cartilages. B. The cellular ROS levels were assessed by using the ROS-sensitive fluorophore CM-H2DCFDA (DCF-DA). C. Representative images for TUNEL-positive cells. Scale bar, 50 μm.

Prx-2 and Trxrd-1 also decreased in the knee cartilages of OA mice (**Figure 2E** and **2F**).

In this present study, medial meniscus was cut off to establish the knee joint model of OA mice, thereby examining Klotho expression. After surgery, the Klotho expression in knee cartilages was observed in different ways, and the results showed that the expression level of Klotho in OA mice significantly decreased (**Figure 2C**). Meanwhile, the immunostaining intensity of Klotho in the knee cartilages of OA mice also decreased (**Figure 2D**). Furthermore, the expressions of Trx/Prx family members and NLRP3/caspase-1/IL-1β axis in OA mice also largely decreased (**Figure 3A** and **3C**).

Consistently, IL-1 release and caspase-1 activity significantly increased in OA mice (**Figure 2B**), which indicates that NLRP3 inflammasome was formed and activated because IL-1β and caspase-1 were the downstream products of this inflammasome.

Accordingly, **Figure 2A** demonstrated the phosphorylation of 2 essential sites (Thr308 and Ser473) in Akt phosphorylation and the decrease of ERK in OA mice.

The ROS production, TXNIP-NLRP3 binding and chondrocyte apoptosis induced in the cartilages of OA mice

ROS production in OA mice significantly increased compared with that in the control mice

(**Figure 3B**). This production was triggered by almost NLRP3 agonists via the ROS-sensitive TXNIP protein, resulting in the activation of NLRP3 inflammasome. As presented in **Figure 3A**, the aggregation of TXNIP with NLRP3 increased. *In vivo*, the recruitment of TXNIP to NLRP3 inflammasome was observed in this present study, implying the aggregation of TXNIP with NLRP3 inflammasome complex. Additionally, according to the chondrocyte apoptosis detected by TUNEL assay, increased positive cells were observed in OA groups relative to the control group (**Figure 3C**).

CTS reduced the expressions of Klotho and Trx/Prx family members but promoted the NLRP3/caspase-1/IL-1β axis expression in chondrocytes

To further examine the expression of Klotho in chondrocytes and mimic the cartilages stimulated with the *in vivo* stress, an *in vitro* model of chondrocytes exposed to CTS was established. After 48 hours of treatments, Klotho protein levels in chondrocytes cells were significantly down-regulated by CTS (0.25, 0.5 and 1 HZ) in a strength-dependent manner (**Figure 4A** and **4C**). Furthermore, the expressions of Trx/Prx family members also reduced after CTS treatment (**Figure 4A**). On the contrary, NLRP3/caspase-1/IL-1β axis expression in chondrocytes increased after CTS treatment (**Figure 4B**).

CTS promoted ROS production and TXNIP-NLRP3 binding in chondrocytes

As shown in **Figure 5A** and **5B**, ROS production distinctly increased after CTS treatment. Meanwhile, the aggregation of TXNIP with NLRP3 also increased after CTS treatment (**Figure 5C**).

The in vitro overexpression of Klotho alleviated the CTS-induced OA formation

To investigate the effects of Klotho overexpressing on the CTS-induced OA formation, the chondrocytes were firstly transfected with Klotho-overexpressed lentivirus vectors and negative control lentivirus vectors, respectively. The results indicated that after CTS treatment, the expressions of Trx/Prx family members in the chondrocytes transfected with Klotho-overexpressed lentivirus vectors obviously decreased compared with those with empty vectors (**Figure 6A**).

Figure 6B demonstrated that after CTS treatment, the NLRP3/caspase-1/IL-1β axis expression distinctly decreased in the chondrocytes cells transfected with Klotho-overexpressed lentivirus vectors than that with empty vectors. And the TXNIP-NLRP3 binding release was also blocked by the overexpression of Klotho (**Figure 6C**). Hence, the exposure of chondrocytes to increasing CTS may lead to the increase of ROS accumulation, whereas the ROS production was significantly down-regulated by Klotho pretreatment (**Figure 6D** and **6E**). Taken together, these results suggest that the overexpression of Klotho plays a protective role in the CTS-treated chondrocytes.

Discussion

Growing evidences showed that the down-regulation of Klotho could exacerbate the inflammation and oxidative stress in many diseases [18-20]. However, whether Klotho is involved in the pathogenesis of OA is now poorly understood. Current studies demonstrated that the Klotho expression in OA models significantly decreased [8-10]. Additionally, a genome-wide gene array analysis in the chondrocytes from OA patients was performed, and it revealed that the expression of ZIP8/MMP13 and NOS2 catabolic remodeling axis was suppressed by the reduced incubation with recombinant Klotho [7]. Klotho expression in chronically IL-1β-treated chondrocytes also decreased in OA mice.

In our study, the expressions of Trx/Prx family members decreased in OA models with CTS treatment. Further experiment discovered that several members of Trx/Prx system could be induced by Klotho, in which Prxs were a newly characterized ethnic group of peroxide-scavenging enzymes. In addition, Prxs, Trx and Trx reductase belong to an increasingly significant antioxidant enzymatic system. A previous study indicated that the expressions of Prx-3 and Prx-2 altered in OA [21]. And John A. Collins *et al*. found that Prx hyperoxidation was very high in the chondrocytes of older adults, which inhibited peroxidase function and consequently resulted in an increase in intracellular ROS levels [7]. Therefore, our results further supported the regulatory relationship between Klotho and Prx-2.

According to a previous study, Akt and ERK played a vital role in the Klotho-induced up-

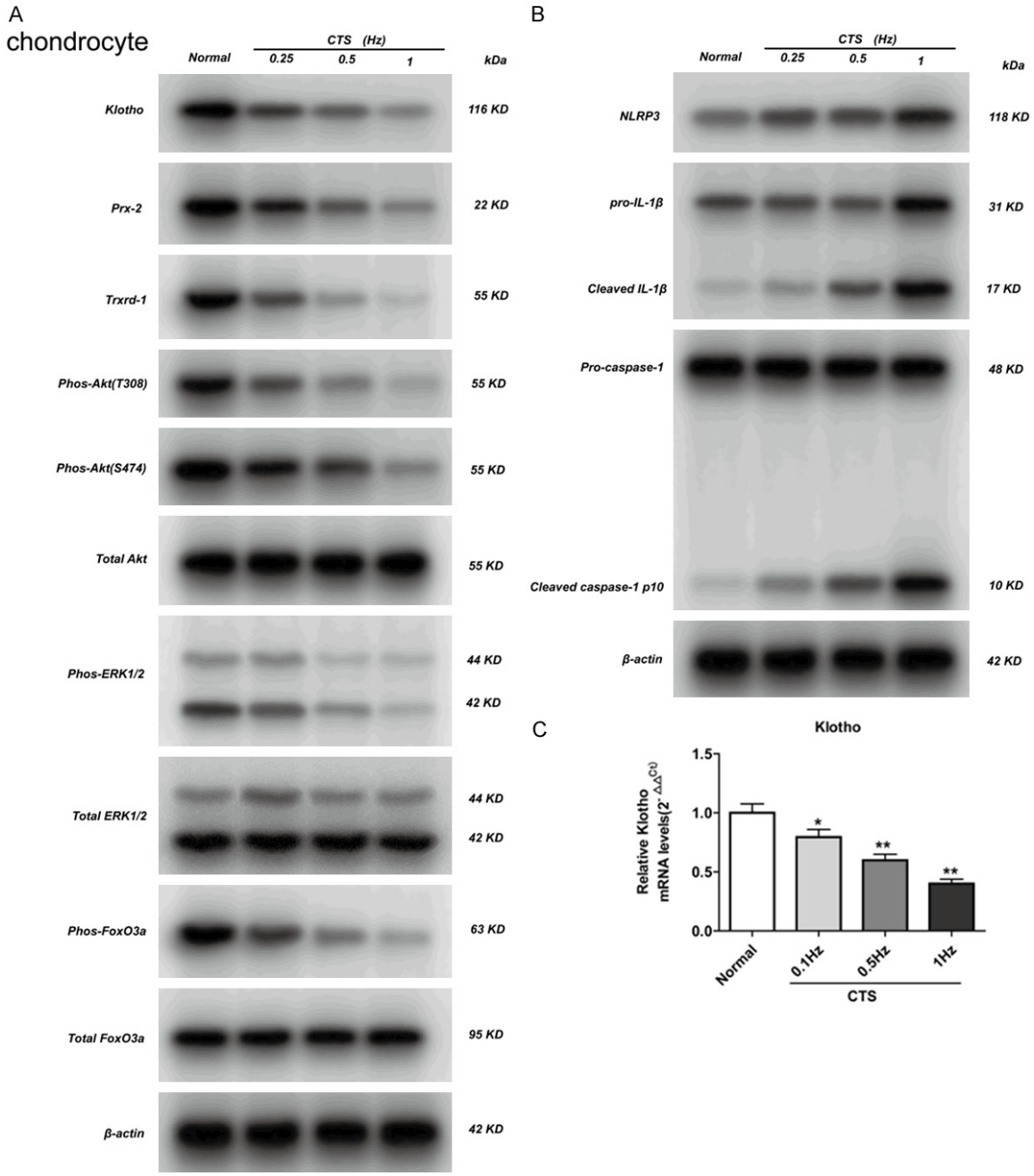

Figure 4. After the treatment of CTS (0.25 to 1 HZ) for 48 h, the expressions of Klotho, Trx/Prx family members and NLRP3/caspase-1/IL-1c axis decreased. A. Western blot analyses for the expressions of Klotho and Trx/Prx family members (Prx-2, Trxrd-1, p-AktT308, p-Akt, Akt, ERK1/2, p-ERK1/2, p-FoxO3a and FoxO3a) in chondrocytes after 48 hours of CTS (0.25 to 1 HZ) treatments. B. Western blot analysis for the expression of NLRP3/caspase-1/IL-1β axis (IL-1-β/pro-IL-1-β, NLRP3, caspase-1 p20 and pro-caspase-1) in chondrocytes after 48 hours of tensile (0.25 to 1 HZ) treatments. C. QRT-PCR analysis for Klotho mRNA expression in chondrocytes after 48 hours of tensile (0.25 to 1 HZ) transfections. Results were expressed using the data from 3 independent experiments. **P*<0.05, ***P*<0.01.

regulation of Prx-2 [11]. In order to investigate the upstream events mediating Klotho-induced Prx-2 and the subsequent cartilages rescue from death, this work attempted to explore the Akt- and ERK-mediated signaling pathways. Our results showed that both kinases were phosphorylated in OA models and chondrocytes following CTS treatment. More importantly, Klotho

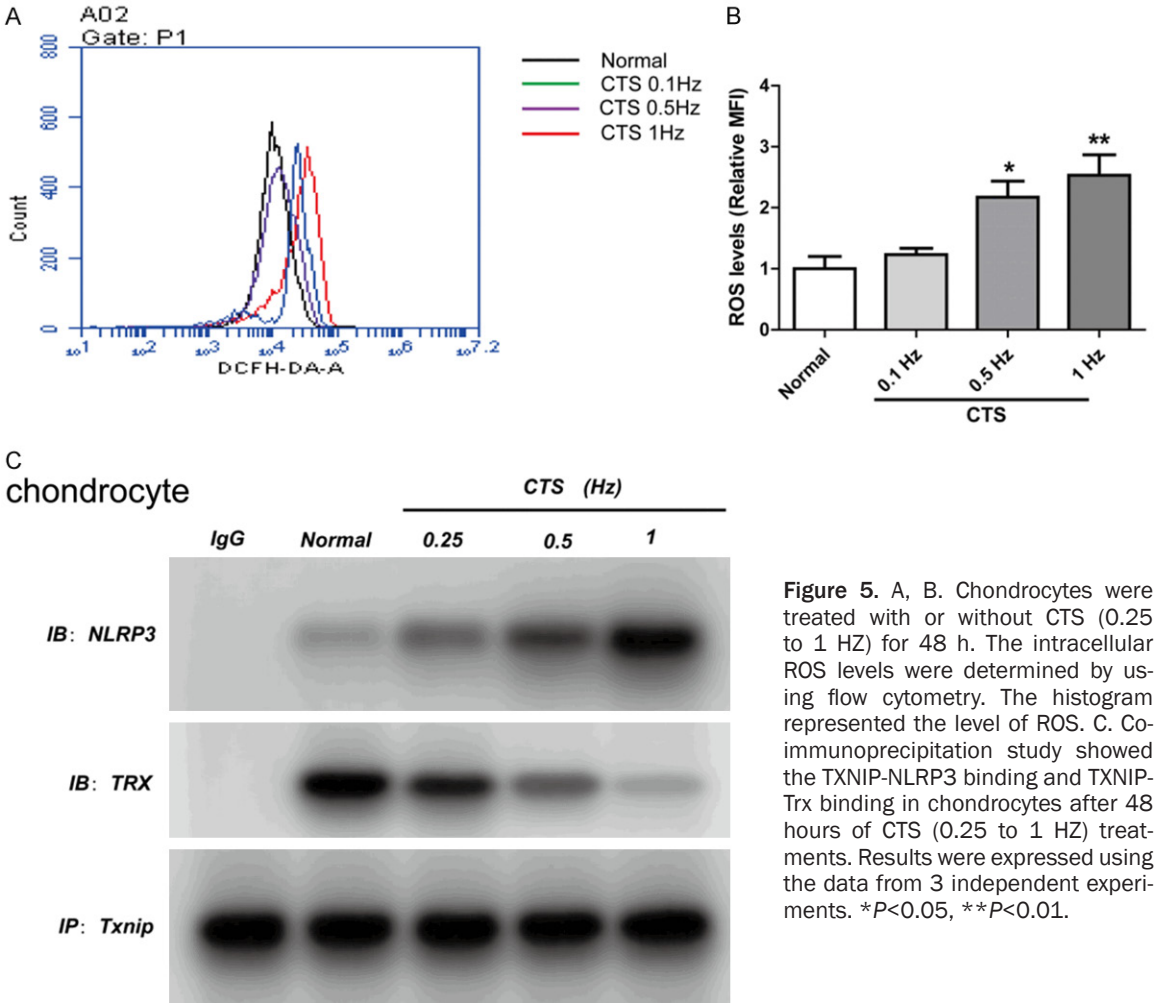

Figure 5. A, B. Chondrocytes were treated with or without CTS (0.25 to 1 HZ) for 48 h. The intracellular ROS levels were determined by using flow cytometry. The histogram represented the level of ROS. C. Co-immunoprecipitation study showed the TXNIP-NLRP3 binding and TXNIP-Trx binding in chondrocytes after 48 hours of CTS (0.25 to 1 HZ) treatments. Results were expressed using the data from 3 independent experiments. **P*<0.05, ***P*<0.01.

activated 2 essential sites (Ser473 and Thr308) of ERK and Akt phosphorylation, which further confirmed that Akt and ERK played an important role in the Klotho-induced up-regulation of Prx-2.

FoxO transcription factor is one of the targets lying the downstream of Akt signaling pathway, and has been already proved to be involved in the protection against apoptotic injury [22]. Recent studies have shown that Klotho can activate FoxO transcription factor, and thus alleviating oxidative stress [19, 23, 24]. Moreover, the suppression for Akt-mediated phosphorylation could also activate FoxO3a, which has been identified as a primary factor to mediate the chondrocyte death responding to glucocorticoids [25]. In this present study, FoxO phosphorylation was also found to be activated by the overexpression of Klotho. This suppression for FoxO3a phosphorylation against the apoptotic process was realized through a number of mechanisms, such as cytochrome release and the modulation of mitochondrial membrane depolarization, which has been already confirmed in primary hippocampal neurons [22] and lung adenocarcinoma cells [26]. Moreover, the Trx-interacting protein (Txnip) was proved to be also a target gene for the subsequent inactivation of FoxO [27].

Trx is another important antioxidant, and its capability to clear ROS is suppressed by its binding with Txnip [28]. In human chondrocytes, when the Txnip increased under the H_2O_2-induced cartilage damage, Txnip would decrease at the late OA stage [29, 30]. The role of Txnip as a link between the increased pro-inflammatory gene expressions and cellular ROS levels is beyond the transcriptional factor level. In addition, Txnip has a direct protein-protein interaction with NLRP3 inflammasome, which means that pro-inflammatory cytokines IL-18 and IL-1β are matured and released through this interaction. However, the underlying mechanism of TXNIP directly activating NLRP3

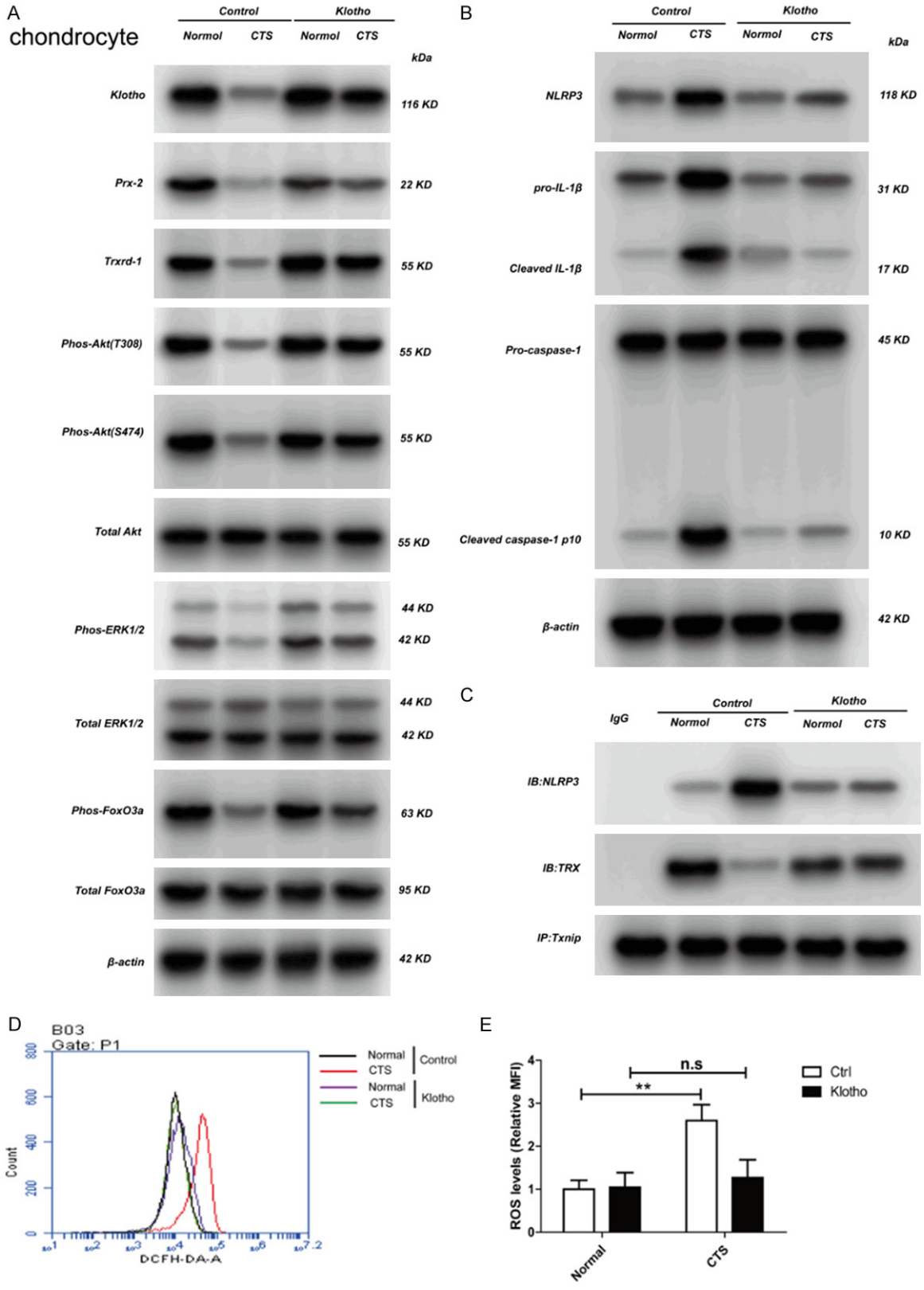

Figure 6. Klotho suppressed the phosphorylations of Trx/Prx family members and NLRP3/caspase-1/IL-1β axis and DNA binding activity in the CTS-induced chondrocytes. Chondrocytes transfected with empty vectors and Klotho-overexpressed lentivirus vectors, respectively, and were inoculated with CTS for 48 h. A. Western blot analysis was used to detect the expressions of Prx-2, Trxrd-1 and phosphorylated Akt, ERK1/2 and FoxO3a. B. Western blot

analysis was used to detect the expressions of NLRP3, cleaved IL-1β, pro-IL-1β, caspase-1 p20 and pro-caspase-1. C. Co-immunoprecipitation study showed the TXNIP-NLRP3 binding and TXNIP-Trx binding expressions in chondrocytes. D, E. The ROS level was determined. Results were expressed using the data from 3 independent experiments. *P<0.05, **P<0.01.

inflammasome is not well understood. Zhou *et al*. [31] investigated this activation in a ROS-dependent manner, and the results demonstrated that the lack of Klotho broke the balance of antioxidants and increased the cellular ROS levels in OA models, resulting in the dissociation of Txnip from Trx, further leading to the strong binding with NLRP3, as shown in our study.

The increased ROS could result in oxidative damage, which is a mechanism of ROS inducing age-related disease. Immunohistochemical studies on the articular cartilages of mice, humans, and non-human primates demonstrated that the oxidative damage marker nitrotyrosine was existed in aged and OA cartilages [13-17].

According to previous literatures, there were 3 models of NLRP3 activations including the generation of ROS, the lysosomal damage induced by crystalline or particulate structures, and K^+ efflux and the gradual recruitment of pannexin-1 membrane pore [12, 32, 33]. This present study indicated that the expressions of IL-1β, caspase-1 and NLRP3 were up-regulated in OA models and the CTS-induced chondrocytes.

NLRP3 inflammasome is also a molecular platform, which means that it can function as the NLRP3 scaffold, the effector molecule IL-1 NLRP3 scaffold, the effector molecule [34]. Expression of inactive IL-1 to can be induced by proinflammatory stimuli, but inflammasome and caspase-1 can regulate the activity, maturation, and secretion [32, 35]. The inflammation-related signaling pathways are mediated by caspase-1, meanwhile the activity of caspase-1 can be controlled by NLRP3 inflammasome. Afterwards, the expressions of proinflammatory factors including IL-1β would be up-regulated. As previously described, for the determinations of pathogenic microorganisms and sterile stressors, inflammasomes are vital signaling platforms [36]. In our study, a significant increase in NLRP3/caspase-1/IL-1 axis was observed in OA mice.

Previous studies on the dysfunction of NLRP3 inflammasome and the final breakdown product IL-1β clarified that these factors were crucial for the pathogenesis of some diseases, such as fibromyalgia [39], gastric tumors [38], chronic kidney disease [37], and coronary artery disease [40]. Additionally, several studies on osteoarticular diseases demonstrated that caspase-1 was a substantial factor to cause OA, which could also additionally accelerate the destruction of articular cartilages [41-43]. However, few studies that underline the relationship between NLRP3/caspase-1/IL-1β axis and Klotho change are reported to date. Therefore, our study for the first time proposes a positive relationship between these 2 factors.

The oxidative stress-mediated cartilage damage also affects OA to a large extent. Our study demonstrated the protective role Klotho played in cartilages and its ability to counteract the chondrocyte loss associated with OA. Our results also revealed that Klotho could protect cartilages via Trx/Prx anti-oxidative system and NLRP3/caspase-1/IL-1β axis, which is significant for redox balance in cartilages. Furthermore, our study also suggested that Klotho might be a good target for the development of new drugs used for the treatment of OA.

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Disclosure of conflict of interest

None.

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