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Suppression of Osteoarthritis progression by post-natal Induction of Nkx3.2

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ABSTRACT

Osteoarthritis (OA) is an incurable joint disease affecting 240 million elderly population, and major unmet medical needs exist for better therapeutic options for OA. During skeletal development, Nkx3.2 has been shown to promote chondrocyte differentiation and survival, but to suppress cartilage hypertrophy and blood vessel invasion. Here we show that Nkx3.2 plays a key role in osteoarthritis (OA) pathogenesis. Marked reduction of Nkx3.2 expression was observed in three different murine OA models. Consistent with these findings, analyses of surgery-induced and age-driven OA models revealed that cartilage-specific post-natal induction of Nkx3.2 can suppress OA progression in mice. These results suggest that Nkx3.2 may serve as a promising target for OA drug development.

1. Introduction

Osteoarthritis (OA), a disease characterized by degeneration of joints, is the most common chronic form of arthritis in humans and is a leading cause of locomotive disability in elderly population. Currently, major therapeutics used for OA patients are limited to palliative anti-inflammatory drugs, pain alleviating opioids, and immune suppressive medications [1,2]. Thus, up to the present time, a disease-modifying OA drug (DMOAD), which can meaningfully modulate OA pathogenesis, is yet to be developed.

Endochondral ossification, which involves chondrocyte differentiation, proliferation, and hypertrophic calcification, is essential for bone development [3]. During bone development, Nkx3.2 (Bapx1) expression is robust in pro-chondrocyte and proliferating chondrocytes, while its expression is down-regulated in fully matured chondrocytes [4,5]. In agreement with these findings, it has been demonstrated that Nkx3.2 can function for chondrogenic differentiation [4–6] and chondrocyte viability [7,8], whereas it inhibits chondrocyte hypertrophy [9–12] and suppresses vascularization into cartilage [13].

Considering cellular properties, early-stage non-hypertrophic chondrocytes in growth plate are comparable to non-calcified articular chondrocytes in joint. For example, both cell types express high levels of type II collagen and aggrecan and maintain viability, in that preserve quantity and quality of cartilage; however, these two cell types are destined to undergo different cell-fate programs afterwards. During endochondral bone development, growth plate chondrocytes are subject to hypertrophic calcification and cell death, which, in turn, prompt mature bone tissue formation. On the other hand, articular chondrocytes restrain hypertrophic maturation in order to maintain steady production of extracellular matrices (ECMs) necessary for protection of articular cartilage from mechanical stresses. In this context, it is of significance to note that increased cell death and elevated hypertrophy towards the terminal stages of growth plate cartilage maturation

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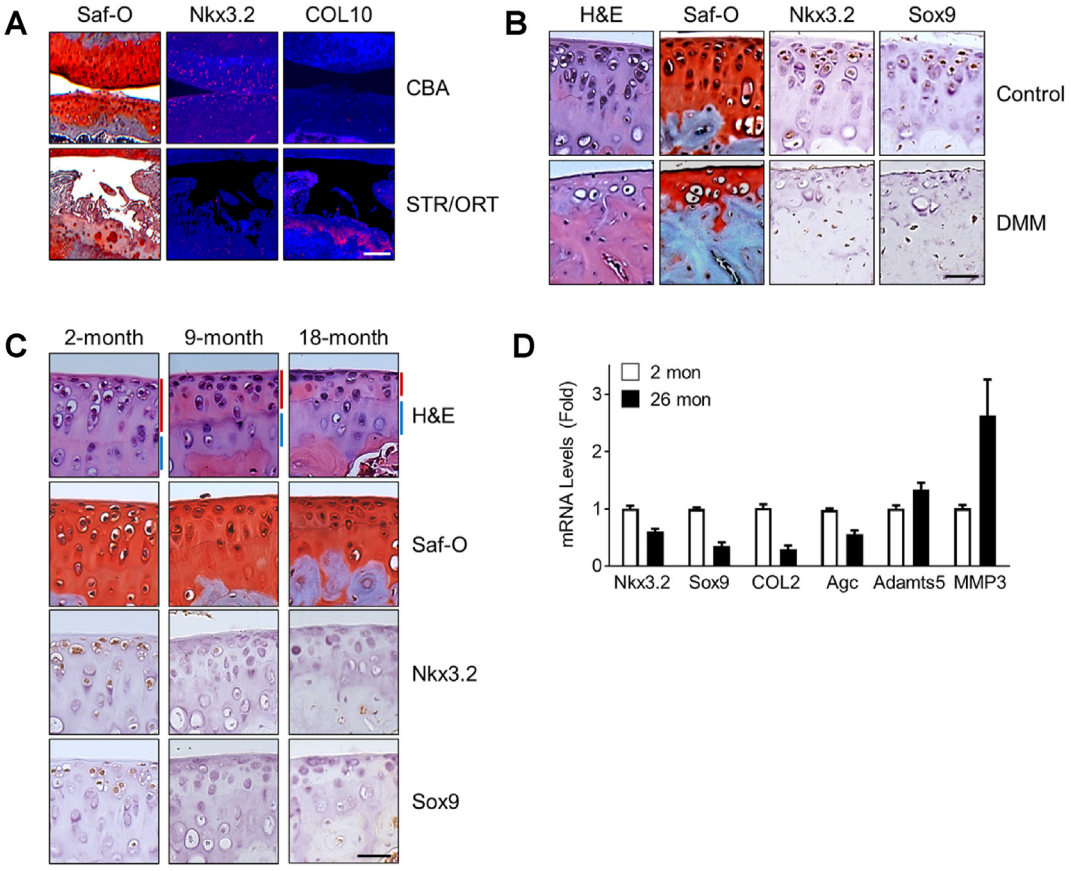

Fig. 1. Decreased expression of Nkx3.2 in OA
(A) Saf-O staining and IHC of Nkx3.2, and COL10 from knee joint of 1-year-old CBA or STR/ORT. Scale bar: 40 μm. (B-C) H&E, Saf-O, IHC (for Nkx3.2, Sox9) of knee joint of 12-week post-DMM mice (B; scale bar: 20 μm), or knee joint of 2, 9, and 18 -month-old mice (C; scale bar: 20 μm). (D) RT-qPCR analyses of knee joints of 2 and 26 -month-old mice. GAPDH served as a reference gene and compared fold changes relative to 2-month-old for indicated genes. Values are mean ± SEM (n = 6/group).

resemble osteoarthritic phenotypes of articular cartilage [14,15]. Given these parallels, a factor (e.g., Nkx3.2) supporting chondrocyte differentiation and survival, while inhibiting cartilage hypertrophy during skeletal development may serve as an OA suppressor in post-developmental stage.

It has been suggested that Nkx3.2 may play a role in OA pathogenesis in association with hypertrophic maturation of articular chondrocyte [16]. However, it has yet to be examined whether Nkx3.2 can function as an OA suppressor in vivo. It has been shown that embryonic overexpression of Nkx3.2 in chondrocytes results in skeletal dwarfism by delaying cartilage hypertrophy during endochondral bone development. Thus, to avoid complications in interpreting the phenotypes for OA regulation by Nkx3.2, we employed a transgenic approach using Aggrecan-Tetracycline-Cre (ATC) approach [17], permitting cartilage-specific induction of Nkx3.2 in skeletally mature mice. In this work, by analyzing ATC-dependent Nkx3.2 transgenic mice, we demonstrate that postnatal expression of Nkx3.2 can modify disease severity in both surgery-induced and age-driven OA models.

2. Materials and methods

2.1. Animal husbandry

All animal experiments were performed in accordance with Korean Ministry of Food and Drug Safety (MFDS) guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei Laboratory Animal Research Center (YLARC) (Permit #: 2009-0033). All mice were housed in individually ventilated micro isolation cages in the specific pathogen-free facility of the Yonsei Laboratory Animal Research Center (TLARC) and Korea Mouse Phenotyping Center (KMPC). All animals were maintained on a 12 h light/12 h dark cycle with access to food and water ad libitum. All procedures were conducted during the light phase of the cycle.

2.2. Mice

STR/OrtOlaHsd and control CBA/CaOlaHsd mice were purchased from Harlan Laboratories. STR/Ort and CBA mice were analyzed at 12-month-old. For DMM-OA models, we used skeletally mature 12-week-old male mice, and the DMM surgery was carried out as previously described [18]. Transgenic mice were exposed for 2 weeks to either with 2 mg/ml doxycycline (Sigma D3447) dissolved in filter-sterilized 5 % sucrose water or with sucrose alone. Mice were housed individually to prevent cross-contamination.

2.3. RT-qPCR

Total RNA was isolated from mouse knee joint or HAC using Trizol (Thermo Fisher), according to manufacturer's instructions. The concentration and purity of the RNA samples were measured with a NanoDrop (Thermo Fisher). TOPscript™ cDNA synthesis kit (Enzynomics) was used for cDNA synthesis. Quantitative PCRs were

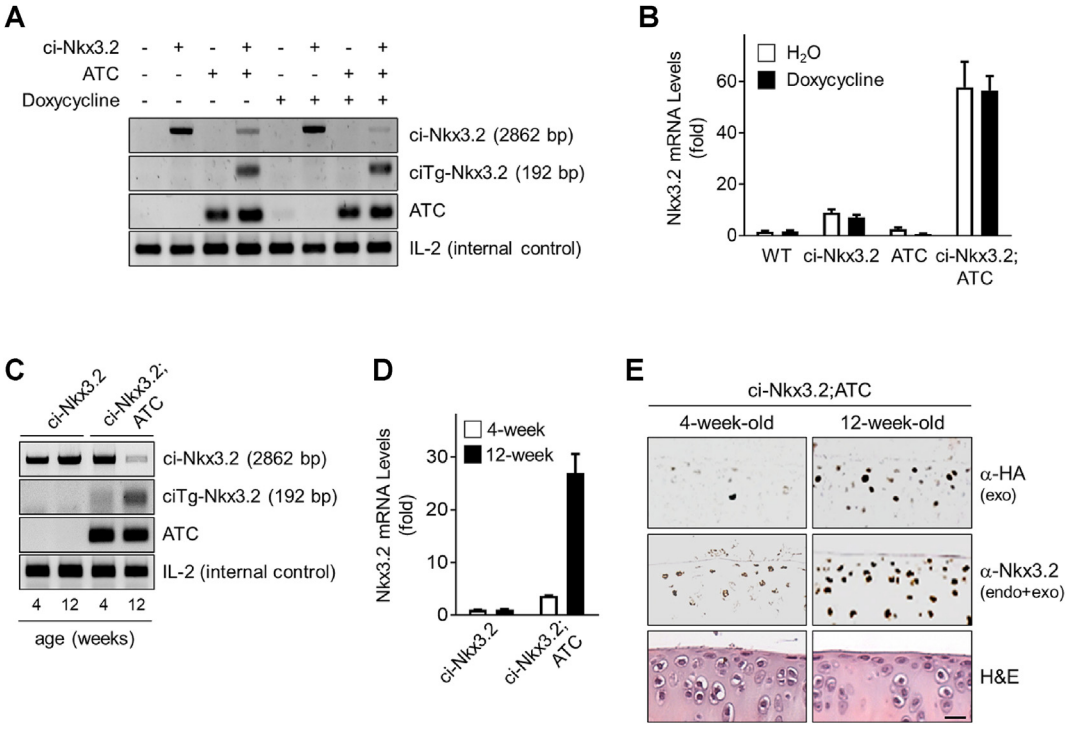

Fig. 2. Generation and characterization of ci-Nkx3.2; ATC mice
(A) PCR genotyping of WT, ATC, ci-Nkx3.2, and ci-Nkx3.2; ATC. As summarized in Fig. S2, ci-Nkx3.2 (top panel) and ciTg-Nkx3.2 (second panel) represent transgenic Nkx3.2 allele before and after cre-mediated recombination, respectively; IL-2 was used as an internal control. (B) RT-qPCR analysis for Nkx3.2 expression in knee joint of 12-week-old mice in the absence or presence of Doxycycline administration. Each value was normalized with GAPDH and compared fold changes relative to wild type (WT). (n = 5/group). (C–D) PCR genotyping (C), RT-qPCR analysis for Nkx3.2 mRNA expression (D) in the absence of Doxycycline. Each value was normalized with GAPDH and compared fold changes relative to 4-week-old ci-Nkx3.2. Values are reported as mean ± SEM. (E) IHC staining for exogenous Nkx3.2 protein using anti-HA, endogenous and exogenous Nkx3.2 protein using anti-Nkx3.2 antibodies, and H&E staining for articular cartilage morphology. Scale bar: 10 μm.

performed using SYBR1 Premix Ex Taq (Tli RNaseH Plus; TaKaRa). PCRs were performed with the following oligonucleotides consisting of targeted gene sequences.

Mouse GAPDH forward: 5′-ATACGGCTACAGCAACAGGG-3’
Mouse GAPDH reverse: 5′-GCCTCTCTTGCTCAGTGTCC-3’
Mouse Nkx3.2 forward: 5′-GTCGCTACAAGACCAAACGC-3’
Mouse Nkx3.2 reverse: 5′-GCACCTTTACGGCCACTTTC-3’
Mouse type II Collagen forward: 5′-TCATCTTGCCGCATCTGTGT-3’
Mouse type II Collagen reverse: 5′-GTTGAGGCAGTCTGGGTCTT-3’
Mouse Aggrecan forward: 5′-CCGCATCACCCAAACTGCAT-3’
Mouse Aggrecan reverse: 5′-AGGTACCACGGGGTTACAGT-3’
Mouse Sox9 forward: 5′-AACTTCTGTGGGAGCGACAA-3’
Mouse Sox9 reverse: 5′-GGGAGGGAAAACAGAGAACGA-3’
Mouse ADAMTS5 forward: 5′-GGCCTACCCAAAGCACAGAA-3’
Mouse ADAMTS5 reverse: 5′-ACGGTACCATCATTCACCAAGT-3’
Mouse MMP3 forward: 5′-GTTCTGGGCTATACGAGGGC-3’
Mouse MMP3 reverse: 5′-TTCTTCACGGTTGCAGGGAG-3’

2.4. Molecular and histological analyses

PCR genotyping, histopathology and immunohistochemistry (IHC) were performed as previously described [7,9,13]. Transgenic mouse generation and genotyping strategy is summarized in Fig. S1 and Fig. S2, respectively, and PCR primers are listed in Table S1. The severity of OA was evaluated using the Osteoarthritis Research Society International (OARSI) scoring system [19]. The osteophyte maturity was graded on a scale of 0–3 (0 = Normal, 1 = Mild, 2 = Moderate, 3 = Severe). Antibodies used for immunohistochemistry were as follows: Anti-Nkx3.2 (abcam, ab83288), anti-Sox9 (Santa Cruz, sc-20095), anti-MMP13 (abcam, ab-39012), anti-HA (Cell Signaling, 3724), anti-C1,2C (IBEX Pharmaceuticals, IBEX 50–1035), anti-Aggrecan Neoepitope NOVUS, NB100-74350), anti-collagen X (Cosmo Bio), Dako EnVision®+ System-HRP kit (Agilent Dako) or Cy3-conjugated secondary antibody (Jackson ImmunoResearch).

2.5. Micro-CT analysis

Micro-CT analyses of the distal part of the femur and proximal part of the tibia in mice were performed with a desktop micro-computer tomography scanner (SkyScan 1276, Bruker-microCT). The samples were scanned through a 0.5-mm-thick filter using a 57 μA, 70 kV and an exposure time of 1140 msec. Micro-CT images from each sample were reconstructed with the NRecon application.

2.6. Statistical analyses

Statistical analysis was performed by using Graphpad Prism software (Graphpad Prism, version 8). Tests were two-sided, Mann-Whitney for non-parametric and unpaired *t*-test for parametric values where applicable. $*P < 0.05$, $**P < 0.01$ were considered statistically significant. Individual tests used and specific *P* values were indicated in the figure legends.

3. Results

3.1. Decreased Nkx3.2 expression in osteoarthritic conditions

To examine whether Nkx3.2 expression can be associated with

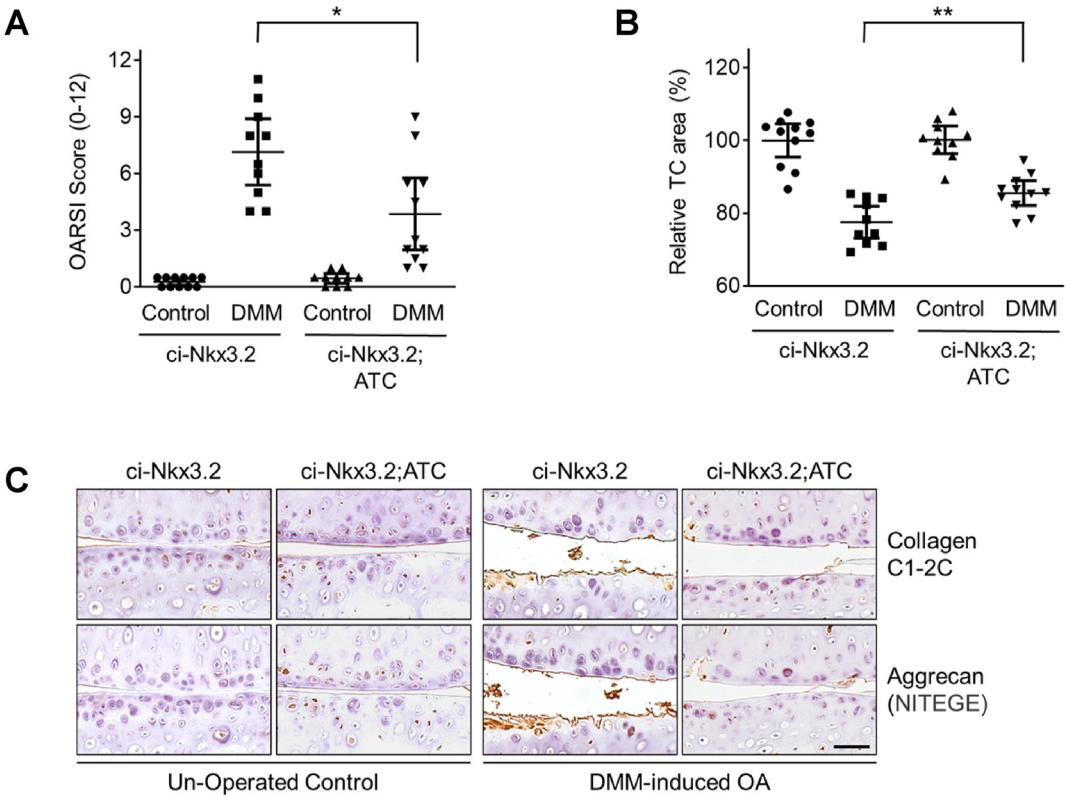

Fig. 3. Suppression of DMM-induced OA in ci-Nkx3.2; ATC mice
Mice with ci-Nkx3.2 allele only (n = 10) and both ci-Nkx3.2 and ATC alleles (n = 11) were evaluated at 8-week post-DMM for cartilage destruction. (A) Comparison of OARSI scoring (*$P = 0.0199$) and (B) total cartilage (TC) area (**$P = 0.0048$) were evaluated in sagittal sections of knee joints. Values are mean ± 95 % CI. (C) IHC analyses of ECM fragments of collagen (C1−2C) and aggrecan (NITEGE). Scale bar: 20 μm.

OA pathogenesis, we examined the STR/ORT mouse, which has been shown to develop joint degeneration comparable to human OA phenotypes [20]. Sagittal sections of knee joints obtained from 12-month-old CBA (control) or STR/ORT (OA-prone) mice were analyzed. As expected, Safranin-O staining revealed that the conditions of articular cartilage of STR/ORT mice are highly destructive, compared to CBA controls (Fig. 1A). Along with these, we observed that Nkx3.2 expression is remarkably diminished, whereas type X collagen (COL10), a well-established marker for cartilage hypertrophy, expression was notably elevated in STR/ORT mice (Fig. 1A). These results indicate that levels of Nkx3.2 expression can be negatively associated with OA.

To further confirm the correlation between Nkx3.2 and OA, we next examined the levels of Nkx3.2 expression in a surgery-induced OA model employing the DMM (Destabilization of the Medial Meniscus) operation. In the murine DMM-OA model we generated, apparent cartilage destruction was routinely observed, and expression of Nkx3.2 and Sox9 was found to be decreased, indicating that reduction in Nkx3.2 expression can be linked to OA progression in a surgery-induced OA model (Fig. 1B).

Next, the knee joints of 2, 9, 18 -month-old normal C57BL6 mice were analyzed to assess Nkx3.2 expression. It has been acknowledged that the joint cartilage tissue can be divided into two distinct domains; the non-calcified articular cartilage (AC), located above the tidemark (TM), functions to alleviate frictions and endure weight-bearing, whereas the calcified cartilage (CC) is the fibrous tissue below TM mediating the attachment between AC and subchondral bone [19,21,22]. Thus, preservation of AC would be critically associated with OA pathogenesis; AC and CC tissues are indicated with red and blue lines, respectively, in top H&E panels of Fig. 1C. As expected, the thickness of AC, compared to CC, was more noticeably reduced with aging, and this AC decrease was accompanied by diminished Safranin-O staining and declined expression of Nkx3.2 and Sox9 (Fig. 1C). In agreement with these, RT-qPCR analyses showed the expression of chondro-protective genes (e.g., Nkx3.2, Sox9, COL2, Agc) was decreased with aging, whereas the expression of cartilage catabolism promoting genes (e.g., ADAMTS5 and MMP3) was increased (Fig. 1D). These results indicate that reduced Nkx3.2 expression can be associated with joint degeneration by aging.

3.2. Post-natal induction of Nkx3.2 in skeletally mature mice by using ci-Nkx3.2; ATC

It has been shown that embryonic overexpression of Nkx3.2 affects skeletal development by inhibiting cartilage hypertrophy. Thus, in order to address a role of Nkx3.2 in OA pathogenesis, leaving skeletal development unaltered, we crossed ci-Nkx3.2 (a transgenic with Cre-inducible HA-tagged Nkx3.2 allele) mice [9] and ATC mice [23] to generate a transgenic line permitting ectopic post-natal induction of Nkx3.2 after skeletal maturation. Schematic diagram for Cre-inducible transgenic model is shown in Fig. S1. Since it has been reported that ATC-mediated allele recombination becomes tetracycline-independent after birth, we examined whether post-natal transgene induction can occur in a tetracycline-independent and ATC-dependent manner in ci-Nkx3.2; ATC mice. In agreement with the previous findings [23], transgenic allele recombination (Fig. 2A) and transgenic mRNA expression (Fig. 2B) effectively occurred in 12-week-old ci-Nkx3.2; ATC in the absence of tetracycline (doxycycline) administration. Further, we also

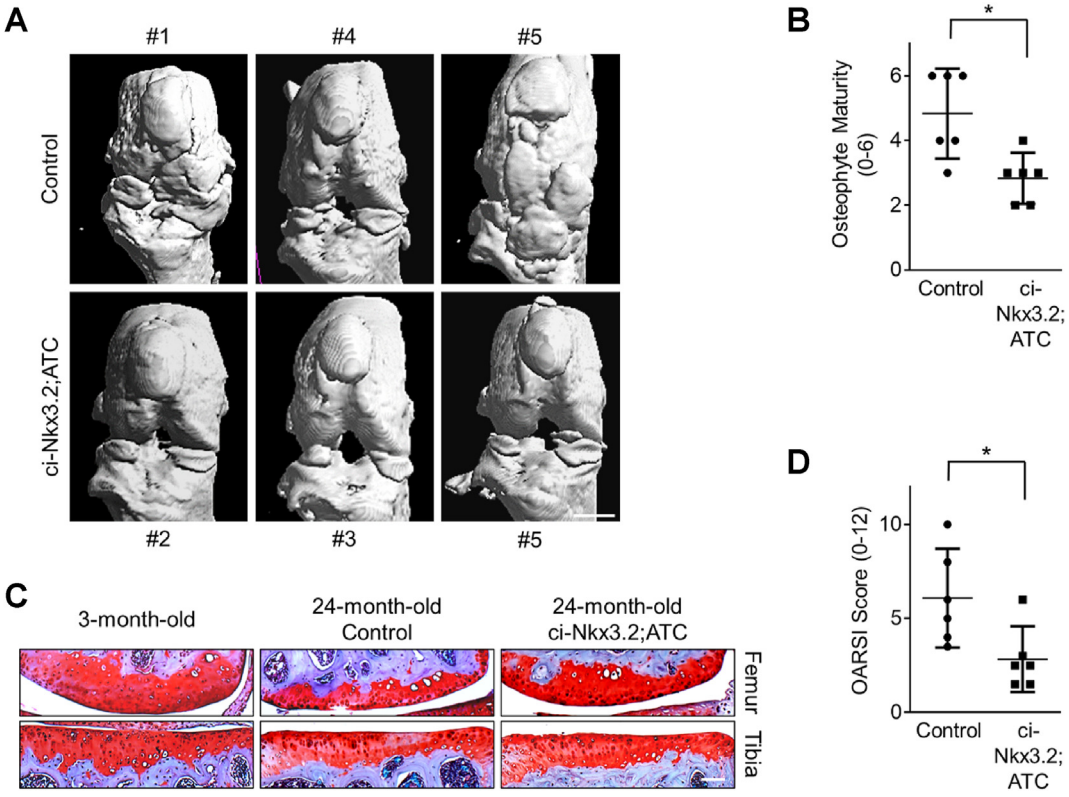

Fig. 4. Amelioration of age-driven OA in ci-Nkx3.2; ATC mice
(A) Representative micro-CT images of distal femoral and proximal tibial metaphysis. Scale bar: 1 mm. (B) Osteophyte maturity comparison for distal femoral and proximal tibial metaphysis with micro-CT scan (*$P = 0.0281$). (C) Representative Saf-O staining images for knee joint to compare in 3-month-old control and 24-month-old control or ci-Nkx3.2; ATC; scale bar 40 μm. (D) OARSI scoring comparison for cartilage damage (*$P = 0.0195$). Values are expressed as mean ± 95 % CI (n = 6 per group).

confirmed that transgene allele recombination (Fig. 2C), mRNA expression (Fig. 2D) and protein expression (top panels in Fig. 2E) in ci-Nkx3.2; ATC can be detected at 12-week-old, but not at 4-week-old. Consequently, overall (i.e., endogenous plus exogenous) Nkx3.2 protein levels in ci-Nkx3.2; ATC were significantly elevated at 12-week-old (middle panels in Fig. 2E). Consistent with these, as shown in X-ray radiographs (Fig. S3), ci-Nkx3.2 and ci-Nkx3.2; ATC were comparable in gross skeleton morphology and size, indicating skeletal development is not affected by post-natal induction of Nkx3.2.

3.3. Suppression of DMM-induced OA in ci-Nkx3.2; ATC mice

To assess a role of Nkx3.2 in OA, ci-Nkx3.2 and ci-Nkx3.2; ATC mice at 12-week-old age were subjected to DMM surgery to induce OA. Subsequently, the knee joint tissues were harvested at 8-week post-surgery, and the OA severity was evaluated by histopathology. First, we conducted semi-quantitative OARSI scoring by evaluating Saf-O staining images. The scoring was expressed as summed scores ranging 0–6 from femur and tibia, respectively, and the average OARSI score for the DMM-operated ci-Nkx3.2 (n = 10) and ci-Nkx3.2; ATC (n = 11) were 7.2 and 3.9, respectively (Table S2, Fig. 3A). Representative Safranin-O stained cartilage images were shown in Fig. S4. Second, total cartilage (TC) area was estimated in the same sized ROI (region of interest) within central weight bearing domain of the sagittal sections as delineated with yellow lines in Fig. S5; the average TC area for the DMM-operated ci-Nkx3.2 and ci-Nkx3.2; ATC was 77.5 and 85.6, respectively (Table S3, Fig. 3B). Finally, the appearance of cartilage matrix degradation markers, such as collagen C1–2C and aggrecan neo-epitope [24], in superficial zone was manifest in DMM-operated ci-Nkx3.2, but not in ci-Nkx3.2; ATC (Fig. 3C).

3.4. Alleviation of age-driven OA in ci-Nkx3.2; ATC mice

As we have observed the activity of Nkx3.2 in surgery-induced OA models as in Fig. 3, we next asked whether joint degeneration driven by aging can be modified by Nkx3.2. Since osteophyte maturation is typically observed in age-driven OA, micro-CT imaging [25,26] was employed to examine knee joints of 24-month-old control and ci-Nkx3.2; ATC mice. Distinct topographical changes in the knee joints were visualized by 3-dimensional reconstruction of the serially-stacked micro-CT images (Fig. 4A). While superficial bone thickening, roughed patches, and distinct bone protrusions (e.g., osteophytes) were obvious in control mice (top panels), the appearance of osteoarthritic bony structures was minimal in ci-Nkx3.2; ATC (bottom panels). Then, based on these micro-CT images, the osteophyte maturity was graded on a 3-point scale, from femur and tibia, respectively (i.e., total 6-point per joint); osteophyte maturity score for control and ci-Nkx3.2; ATC were 4.8 and 2.8, respectively (Table S4, Fig. 4B). Next, histopathology analyses based on Saf-O staining were performed. In agreement with the micro-CT results, age-driven cartilage destruction was significantly attenuated in ci-Nkx3.2; ATC (Fig. 4C). Then, OA severity was quantitatively assessed. The average OARSI score for control and ci-Nkx3.2; ATC were 6.1 and 2.8, respectively (Table S5, Fig. 4D).

4. Discussion

While OA is a complex disease associated with a broad range of joint defects, many preceding attempts for OA drug development have relied on anti-inflammatory rationales. However, apart from inflammatory responses, disruptions in homeostatic control of chondrocyte differentiation, survival and hypertrophy would be crucial during the onset and early progression of OA.

Nkx3.2 was initially identified as a pro-chondrogenic factor promoting cartilage development [4–6], and subsequent studies have shown that Nkx3.2 can regulate chondrocyte viability, chondrocyte hypertrophy, and blood vessel invasion [7–13]. Considering these multi-faceted activities of Nkx3.2 and its logical links with OA pathogenesis, it is conceivable that enhancing Nkx3.2 expression and function may suppress OA. In support of this hypothesis, we found that Nkx3.2 expression is markedly reduced in three different rodent OA models, and post-natal ectopic expression of Nkx3.2 effectively suppress OA progression in mice caused by surgical operations as well as natural aging. These results suggest that Nkx3.2 may be a promising target for OA drug development.

It is of significance that osteophyte maturation can be restrained by Nkx3.2. It has been noted that osteophyte formation is closely associated with the inflammatory signaling accompanied by angiogenic micro-environment; briefly, initial joint inflammation induces angiogenic factors such as HIF-1α, which gives rise to a series of events including chronic inflammation, aberrant cartilage hypertrophy, and ectopic bone formation and osteophyte maturation [27–30]. Along with these observations, Nkx3.2 has been shown to inhibit angiogenesis by inducing lysosomal degradation of HIF-1α, which, in turn, delays chondrocyte hypertrophy [9,10,12,13]. Thus, based on these lines of information, Nkx3.2 may well position to negatively regulate osteophyte maturation by being capable of inhibiting cartilage hypertrophy as well as angiogenesis.

Our current results well align with previous studies. First, Ihh, a well-known regulator of chondrocyte hypertrophy [31], is up-regulated under OA conditions and aggravates OA severity [32–34]. Interestingly, we have previously found Ihh signaling, triggered by chondrocyte hypertrophy, induces Nkx3.2 protein degradation [12]. Thus, it is plausible to interpret that elevated Ihh signaling under OA environment triggers Nkx3.2 protein degradation, which, in turn, enhance OA progression.

RelA p65 plays a role in supporting chondrocyte survival as well as in potentiating cartilage catabolism, implicating its bi-phasic role in OA pathogenesis [7,8,35–38]. Given remarkable differentials in Nkx3.2 expression under healthy and OA conditions, the following hypothesis is conceivable. High levels of Nkx3.2 expression may selectively permit RelA activation for cell survival via nuclear degradation of IκB [7,8] without triggering RelA-dependent cartilage destruction pathways, which requires cytoplasmic degradation of IκB [36,37]. On the other hand, pro-survival function of RelA may be disabled by the absence of Nkx3.2, while RelA-induced cartilage destruction pathways are potentiated by inflammatory cytokines. Thus, it is quite intriguing to postulate that different levels of Nkx3.2 expression may dictate the context-dependent NF-κB signaling between healthy and OA environment.

In this work, we show that Nkx3.2 can effectively suppress OA progression by functioning to protect articular cartilage, and inhibit osteophyte maturation, and these results suggest that Nkx3.2 may serve as a therapeutic target for OA treatment.

Author contributions

Study conception and design. MP, DUJ, BJK, SI, BHC, BHM, DWK. Acquisition of data. HKO, MP, SWC, DUJ, BJK, SI, JL. Technical support for micro-CT. YC, JHK. Aged mouse support. JKS. Analysis and interpretation of data. HKO, MP, SWC, DUJ, BJK, SI, JHK, DWK. DWK takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data availability statement

All data associated with this study are present in the paper or the supplementary materials.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.07.074.

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