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Aberrant methylation and expression of TNXB promotes chondrocyte apoptosis and extracellular matrix degradation in hemophilic arthropathy via AKT signaling

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Abstract

Background

Recurrent joint bleeding in patients with hemophilia frequently results in hemophilic arthropathy (HA). Drastic degradation of articular cartilage is a major characteristic of HA, but its pathological mechanisms has not yet been clarified. Here, we conducted a genome-wide DNA methylation study with the goal of identifying critical genes for HA cartilage degeneration.

Methods

DNA was isolated from human osteoarthritis (N = 5) and HA (N = 5) articular cartilages and analyzed using the Infinium Human Methylation 850 BeadChip array. Adeno-associated virus-mediated shRNA and siRNA were used to knock down Tenascin XB (TNXB) in chondrocytes and F8^{-/-} male mice, respectively. Then histopathological analysis, qPCR, Western blotting and immunofluorescence assays were conducted to detected chondrocyte homeostasis and HA progression.

Results

We found that Dnmt1 and Dnmt3a protein levels were increased in cartilages from HA patients compared with OA patients. Genome-wide DNA methylation analysis identified 1228 differentially methylated regions (DMRs) associated with HA. Functional enrichment analyses then revealed that DMR genes (DMGs) were related to extracellular matrix organization. Among these DMGs, TNXB protein expression was down-regulated in human and mouse HA cartilages. Further, the loss of *Tnxb* in F8^{-/-} mouse cartilage provides a disease-promoting role in HA by augmenting cartilage matrix degeneration and subchondral bone loss. *Tnxb* knockdown also promoted chondrocyte apoptosis and inhibited phosphorylation of AKT. Importantly, AKT agonist showed a chondroprotective effect following *Tnxb* knockdown.

Conclusions

Our study demonstrated that TNXB is a central mediator of cartilage matrix degradation following joint bleeding, which functions by regulating the activation of AKT. These mechanistic insights allow targeted development of potentially new strategies for cartilage protection in HA.

eLife assessment

This **important** study identifies the TNXB-AKT pathway as a potential mechanism underlying hemophilia-associated cartilage degeneration. The evidence supporting the conclusions is **convincing**, with murine and human patient evidence as well as genome-wide DNA methylation analysis. This paper would be of interest to cell biologists and biochemists working on the field of musculoskeletal disorders.

Introduction

Hemophilia is a X-linked bleeding disorder due to the deficiency of coagulation factor VIII (hemophilia A) and IX (hemophilia B). As a one of the most frequent rare diseases, hemophilia is a persistent, challenging condition for patients, physicians and society ^{1,2}. Joint bleeding (hemarthrosis), especially in knee joint, is the most common clinical manifestation in patients with severe hemophilia (i.e., plasma FVIII or FIX < 1 U/dl) ². Recent evidence also indicates that hemarthrosis may occur spontaneously in patients with moderate (plasma factor levels of 1–5 U/dl) or mild disease (plasma factor levels of >5 U/dl) ^{3,4}. Repeated episodes of hemarthrosis eventually result in hemophilic arthropathy (HA), a debilitating and irreversible condition. HA often starts at an early age and characterized by joint impairment, chronic pain, and reduced quality of life ^{5–10}.

Current efforts to prevent the development of HA are mainly focused on management of acute joint bleeding and optimizing prophylactic replacement therapy ^{2,11,12}. However, the widespread adoption of the modern hematological care has not conferred considerable protection against the development of HA; even in patients with hemophilia receiving intermediate- and high-dose prophylaxis, many still develop HA ¹³. Pathological changes of HA begin with the first joint bleedings, either breakthrough or sub-clinical, usually during the first years of life ^{4,14}.

They consist in progressive and irreversible alterations induced by the direct and indirect toxicity of the free blood in the joint space. The joint replacement surgery, such as Total Knee Arthroplasty (TKA), is considered an intervention for alleviating pain and restoring joint function in patients with end-stage HA. However, it is associated with a high incidence of complications, primarily attributed to septic loosening and recurrent postoperative bleeding¹³. Given no disease modifying therapy available to intervene in the HA perpetuating process, HA remains a persistent problem and challenge for hemophilia patients. Thus, to understand the pathogenesis of HA and subsequently explore possible targets for therapy is necessary.

One distinctive pathophysiological manifestation of HA is cartilage degeneration¹⁵. Cartilage is a rather inert tissue, consisting of matrix proteins and only one cell type, chondrocytes that are responsible for production of matrix synthesis and rely on synovial fluid for nutrients as cartilage lacks blood supply^{16,17}. Nevertheless, recurrent intra-articular bleeding creates a ‘toxic’ environment to cartilage, by inducing synovial inflammation, hemosiderin accumulation and excessive mechanical stress^{15,18}. As a consequence, dysregulation of metabolism and abnormal apoptosis occur in chondrocytes, ultimately resulting in deterioration of the cartilage matrix².

DNA methylation, a core epigenetic mechanism, is high dynamic and susceptible to cues from the environment^{19,20}. DNA methylation is the addition of a methyl group to the cytosine residue of DNA molecules, which is catalyzed by DNA methyl transferase (Dnmt) family of proteins that is comprised of three members: Dnmt1, Dnmt3a, and Dnmt3b. DNA methylation is mainly located in the gene regulatory region, usually repressing gene expression by blocking the transcriptional accessibility of regulatory genomic regions²¹. Recently, multiple studies confirmed the important role of abnormal DNA methylation in the pathogenesis of arthritis^{22,23}.

However, the epigenetic mechanisms underlying HA-related cartilage degradation have not been explored.

In this study, we conducted a genome-wide DNA methylation study with the goal of identifying differentially methylated genes (DMGs) and pathways for HA. Further, we knocked down *Tnxb*, a key DMG, in primary mouse chondrocytes and hemophilia A (F8^{-/-}) mice. The biological function and underlying mechanisms of TNXB in HA progression were also investigated *in vivo* and *in vitro*. Our findings provide a new mechanism for articular cartilage lesion in HA, which may lead to the discovery of novel therapeutic targets for the treatment of HA.

Materials and Methods Cartilage specimen collection

Osteoarthritis (OA) is often used as “disease” control to reveal the characteristics in HA^{24,25}, although the mechanistic and phenotypic are different between HA and OA. In this study, 5 HA and 5 OA knee cartilage specimens were collected from patients undergoing total knee replacement surgery. Knee cartilage tissues were dissected and then rapidly frozen in liquid nitrogen or fixed in 4% paraformaldehyde. All subjects were Chinese Han and their demographic characteristics were listed in Supplementary table 1. The human cartilage samples were obtained from Department of Orthopedic Surgery at The First Affiliated Hospital of Zhejiang Chinese Medical University. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University (2019-ZX-004-02).

Magnetic Resonance Image (MRI)

MRI examination: a GE 3.0 T Signa Excite superconducting MRI machine with a 32-channel body coil, a gradient field of 24 mT/m, and a creep rate of mT/(m.s) as well as a Siemens Verio 3.0 T superconducting magnetic resonance imaging machine with a 16-channel phased array coil were applied. An ultrashort echo time (UTE) pulse sequence (echo time 0.07 ms) was performed in the sagittal plane of the knee joint of the affected limb. Scanning parameters: layer thickness of 0.8

mm, field of view of 320 mm×320 mm, matrix of 160 mm×160 mm, intra-layer resolution of 0.6 mm×0.6 mm, and excitation of 2 times. Scanning software: NooPhase Wrap, Variable Bandwidth, Tailored RF.

Genome-wide DNA methylation profiling

Genomic DNA was prepared from cartilage specimens using QIAamp DNA Blood Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions and was stored at -80 °C until used. Then bisulfite treatment of genomic DNA was performed with the EZ DNA methylation kit (Zymo Research) according to manufacturer's procedure. Genome-wide DNA methylation patterns were evaluated by Infinium Human Methylation 850 K BeadChips (Illumina), which determine the methylation levels of 853,307 CpG sites. R/Bioconductor (version 3.3.3) package ChAMP was used to process the Illumina intensity data (IDAT) files from the chip.

DNA methylation level of each CpG site was described as a β value, ranging from zero (representing fully unmethylated) to one (representing fully methylated). Probes that had a detection P value of > 0.01 and those located on the X and Y chromosome were filtered away. We also removed SNP-related probes and all multi-hit probes.

BMIQ (Beta Mixture Quantile dilation) algorithm was used to correct for the Infinium type I and type II probe bias. Furthermore, the regression models were adjusted for age and sex. Differentially methylated probes (DMPs) at significance of $P < 0.05$ after the Benjamini & Hochberg correction for multiple testing. Differentially

Methylated Regions (DMRs) were identified by Bumphunter with default settings. Additionally, we annotated the DMR-related genes (DMGs), and then performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes (KEGG) pathway analysis by using the online tool DAVID (<http://david.abcc.ncifcrf.gov/>). The significant pathway/ GO term was identified with P value < 0.05 .

Mouse

The FVIII target gene knockout hemophilia (F8^{-/-}) mouse were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All mouse were kept in the Laboratory Animal Center of Zhejiang University of Chinese Medicine. The animals were placed in the controlled environmental condition with relatively constant air humidity and temperature as well as manual control of day and night replacement for 12h. The experiment was approved by the Experimental Animal Ethics Committee of Zhejiang University of Traditional Chinese Medicine (LZ12H27001).

The mouse experimental model of HA

The HA model is constructed according to the description of Hakobyan et al ²⁶, F8^{-/-} male mouse were induced with 3% isoflurane, and 1% isoflurane was maintained under anesthesia. The right knee joint capsule of mouse was pierced with a 30g needle for bleeding modeling, and the left knee of each animal was used as a control group. Mouse were sacrificed at 4 and 8 weeks after injury, and knee joints were collected for further experiments.

Intra-articular injection

F8^{-/-} male mouse were induced with 3% isoflurane, and 1% isoflurane was maintained under anesthesia. The right knee joint capsule of mouse was pierced with a 30g needle for bleeding modeling. The needle of the insulin syringe was sagittally inserted into the intercondylar area of the mouse right knee joint, and one dose of 10 μ L AAV-Tnxb (GenePharma, concentration: $1 \times 10^{10}/10\mu$ L) or AAV-NC was injected. After 4 weeks, mouse was sacrificed, respectively, and the right knee joints were collected for further experiments.

Micro-computed tomography (μ CT) analysis

Micro-computed tomography (μ CT) (Skyscan 1176, Bruker μ CT, Kontich, Belgium) was used to analyze the knee joints. The area between the proximal tibia growth plate and the tibial plateau was chosen as the region of interest. The parameters collected from μ CT were percent bone volume (BV/TV, %), Trabecular thickness (Tb.Th, mm), Trabecular number (Tb.N, 1/mm) and Trabecular Spacing (Tb.Sp, mm).

Histological analysis

The human cartilage samples were successively fixed in 4% paraformaldehyde for 5 days, decalcified with 14% EDTA solution for 3 months, and the mouse samples were successively fixed in 4% paraformaldehyde for 3 days, decalcified with 14% EDTA solution for 14 days. These samples subsequently embedded in paraffin. Then 3- μ m thick sections at the medial compartment of the joints were cut for Alcian blue hematoxylin/Orange G (ABH) or Toluidine Blue staining to analyze the gross cartilage structural changes. Histomorphometry analysis was performed through Osteomeasure software (Decatur, GA).

Immunohistochemistry

The immunohistochemistry was examined to observe the expressions of protein in cartilage. Briefly, the deparaffinized sections were soaked in 0.3% hydrogen peroxide to block the activity of endogenous peroxidase, then blocked with normal goat serum (diluted 1:20) for 20 min at room temperature. Subsequently, the primary antibodies were added and incubated overnight at 4° C. The next day, the sections were treated with secondary antibodies for 30 min and positive staining was visible by using diaminobenzidine solution (Invitrogen, MD, United States). Then counterstaining was performed with hematoxylin for 5 s. Anti-Col2a1 (Abcam, ab34712, 1:200), Anti-Mmp13 (Abcam, ab39012, 1:300), Anti-TNxB (Proteintech, 13595-1-AP, 1:50), Anti-Bax (Huabio, ET1603-34, 1:200), Anti-Bcl-2 (Huabio, ET1702-53, 1:200), Anti-p-Akt1 (Abclonal, AP0140, 1:200), Dnmt1 (Abcam, 1: 200), Dnmt3a (Abcam, 1: 200), Dnmt3b (Huabio, 1: 200) were used in this study.

Cell isolation and culture

Primary mouse chondrocytes were obtained from the femoral head of 2-week-old C57BL/6 J mouse purchased from the Experimental Animal Center of Zhejiang Chinese Medical University. Mouse were sacrificed and disinfected with 75% ethyl alcohol. Specimens were isolated and rinsed by Phosphate Buffer Saline (PBS) 3 times. Then the cartilage tissues were digested with 0.25% collagenase P at 37 °C for 4h. Chondrocytes were cultured in DMEM/F-12 medium containing 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin in 5% CO₂ at 37 °C for further experiment.

Tunel assay

To evaluate the apoptotic cells in the chondrocytes, we performed a Tunel assay according to the manufacturer's guideline (Beyotime, catalog C1088). Briefly, following deparaffinage and rehydration, sections were permeabilized with DNase-free Proteinase K (20 μ g/mL) for 15 minutes at 37°C. Subsequently, slides were treated with Tunel solution and incubated at 37°C for 1 hour in a dark environment and counterstained with DAPI (1:1000; Solarbio) for 10 minutes. Finally, TUNEL positive cells were detected by fluorescence microscope.

Transfection of siRNA-*Tnxb*

Chondrocytes were transiently transfected with siRNA targeting *Tnxb* (GenePharma) or negative control siRNA (scrambled; GenePharma) using XtremeGENE siRNA transfection reagent (Sigma) following the manufacturer's instructions. All experiments were performed 72 hours after

transfection, and the most effective single siRNA was used for further experiments. After transfection for 48 h, cells were treated with various concentrations of SC79 (10 μ M, Selleck) for 24 h.

Cell Immunofluorescence

The cells were fixed with 4% paraformaldehyde and blocked with goat serum for 1 hour. Then, cells were incubated with Col2a1 antibody (1:200; Abcam) and Mmp13 antibody (1:100; Abcam) overnight. The next day, the knee joints samples and Chondrocytes with goat anti-rabbit antibody (1:1000; Invitrogen) conjugated with Alexa Fluor 488 for 1 hour. Cells were stained with DAPI (1:1000; Solarbio).

Western blot analysis

Total proteins were extracted by RIPA buffer, and concentration of protein was determined by bicinchoninic acid (BCA, Beyotime, Shanghai, China). Proteins were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were then blocked with 5% skim milk for 1 h, and were incubated with primary antibodies (Col2a1, Abclonal, A1560, 1:1500), (Mmp13, Abclonal, A11755, 1:1000), (TNXB, Abclonal, A2535, 1:1000), (p-Akt1, Abclonal, AP0140, 1:1000), (Akt1, Huabio, ET1609-47, 1:1000), (Bcl-2, Huabio, ET1702-53, 1:1000), (Bax, Huabio, ET1603-34, 1:1000), (active-pro caspase-3, Huabio, ET1608-64, 1:1000), (Cleaved-Caspase9, Abclonal, A22672, 1:1000), (pSmad2, CST, 8828, 1:1000) overnight at 4°C. The membranes were washed 3 times, and then cultured with the secondary antibody for 1 h. The immunoreactivity was detected with the ECL substrate (Thermo Fisher Scientific, United States) on an Image Quant LAS 4000 (EG, United States). The grey value was calculated by the software of Image J. β -actin was used as an internal control in all western blot analysis.

Quantitative RT-PCR

TRIzol reagent (Invitrogen, United States) was used according to the manufacturer's protocol to extract total RNA from chondrocytes. The total RNA quantity and purity was evaluated by using NanoDrop 2000 (Thermo Fisher Scientific, United States). Reverse transcription was carried out with cDNA Synthesis Kit (Bmake, Beijing, China). The quantitative real-time-polymerase chain reaction (qPCR) was conducted with SYBR Premix Ex Taq™ II (Takara, Dalian, China), and performed on a QuantStudio™ 7 Flex Real-Time PCR System. All of the PCR reactions were repeated 3 times for each gene. The data were analyzed by the 2- $\Delta\Delta$ CT method using β -actin as the internal control. Supplementary table 2 showed the primer sequences of target genes used in the current study.

Treatment of DNA Methyltransferase inhibitors

To investigate whether DNA methylation affects the expression of TNXB, we treated primary mouse chondrocytes with RG108 or 5-Aza-dc (0, 1, 10, and 25 μ M) (RG-108, Beyotime, SD-1137; 5-Aza-2'-deoxycytidine, Beyotime, SD-1047) for 24 h. The cells were then collected for qPCR to detect the mRNA level of *Tnxb*.

Statistics

All experimental data of this subject were analyzed by GraphPad Prism software version 8.0, and the statistical results of measurement data were expressed as mean $\pm$ standard deviation. 2-tailed unpaired parametric Student's t test or nonparametric Mann-Whitney U test or one-way ANOVA with Tukey's multiple comparisons test was used for comparison between different groups. The difference was statistically significant with $P < 0.05$.

Results

Articular cartilages of HA patients show severe damage and aberrant elevations of Dnmt1 and Dnmt3a

In this study, we collected cartilage samples in tibial plateau from OA and HA patients undergo total knee replacement (TKR) surgery. Consistent with previous reports, the HA cartilage exhibited severe damage and significant hemosiderin deposition, compared with that of OA (**Figure 1A**). MRI analysis revealed the subchondral bone loss in HA patients (**Figure 1B**). ABH staining further assessed the cartilage degeneration, and detected a substantial sulfated glycosaminoglycan (sGAG) depletion in HA cartilages (**Figure 1C**, C and D). As expected, comparing to OA, HA cartilages showed prominent decrease in cartilage matrix protein Col2a1 and increase in expression of Mmp13, the primary enzymes responsible for cartilage degeneration (**Figure 1E**, E and F). Additionally, obvious increase in Dnmt1 and Dnmt3a protein levels was also detected in HA cartilages (**Figure 1G**, G and H), whereas no significant changes were found in Dnmt3b (Supplementary Figure 1). These data confirmed the severe damage in articular cartilage and subchondral bone of HA patients.

Genome-wide DNA methylation analysis reveals the epigenetic landscape of HA cartilage

To understand the methylation profile associated with HA cartilage degeneration, genome-wide DNA methylation alterations were examined in 10 subjects (5 OA cartilages and 5 HA cartilages) by using 850K ChIP. The methylation levels of whole genome-wide CpGs were in a classic bimodal distribution where most CpGs were either slightly or highly methylated (**Figure 2A**). Of note, global DNA methylation levels exhibited systematic differences between HA and OA, as illustrated by their separation pattern in principle component analysis (**Figure 2B**). Overall, 1288 significant differentially methylated regions (DMRs) ($P < 0.05$ after the Benjamini & Hochberg correction for multiple testing) were identified, with a mean fold change in methylation difference of 0.90 (**Figure 2C**, and Supplementary Table 3). Compared with the OA, 69.95% and 30.05% DMRs were hypermethylated and hypomethylated in HA, respectively (Supplementary Figure 2A). Given the specificity of DMR, we then determined whether the identified DMRs were preferentially colocalized with some specific genomic features. The large majority (93.49%) of these DMRs were located in gene promoters, 5'-UTRs, 3'-UTRs, and exons, with only 6.51% overlapping intergenic regions (Supplementary Figure 2B).

To determine the biological functions of these significant DMRs, GO and KEGG enrichment analysis were performed on DMR-related genes (DMGs). GO analysis revealed enrichment for terms largely related to extracellular matrix (ECM) organization, skeletal system development (**Figure 2D**, Supplementary Table 4). The top two KEGG pathways were ECM-receptor interaction (P value = 5.2×10^{-6}) and PI3K-Akt signaling pathway (P value = 5.3×10^{-4}) (**Figure 2E**, Supplementary Table 5). DMGs presented in these pathways have mostly been proved to play an important role in cartilage matrix homeostasis, such as *COL1A1*, *COL1A2*, *COMP* ^{27–29}. Among them, we found TNXB were significantly low expressed in human HA cartilage compared to OA cartilage by using IHC staining (**Figure 2F**, F and G). However, TNXB was not differentially expressed in OA and HA synovial membranes (Supplementary Figure 2, C and D).

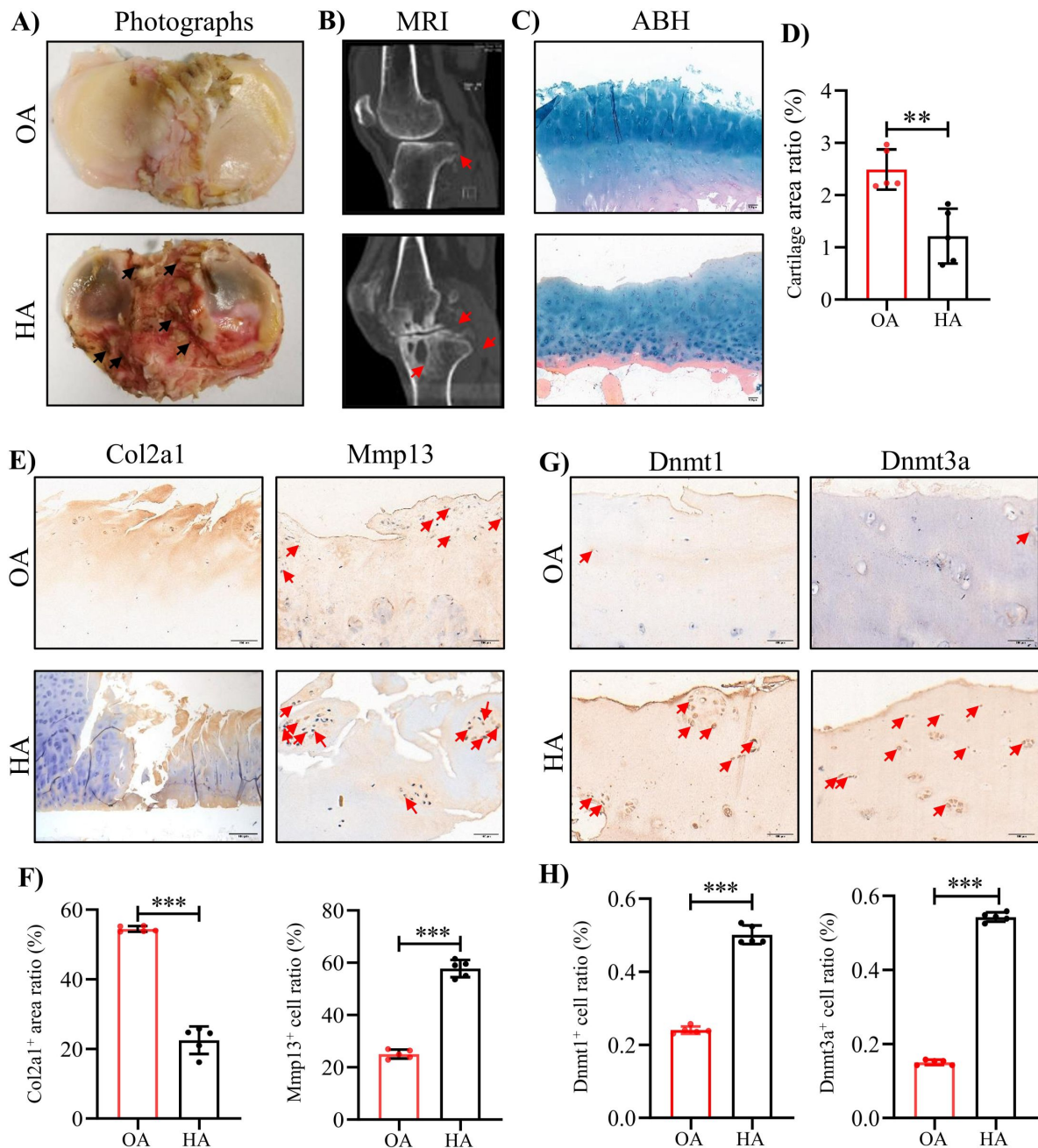


Figure 1.

The severe cartilage damage in human HA.

(A) Human cartilage samples in the tibial plateau were obtained from patients with OA and HA. Black arrow indicates hemorrhagic ferruginous deposits. (B) MRI imaging of the knee joint in patients with HA and OA. Red arrow indicates the wear area. (C) Representative images of ABH/OG staining for HA and OA cartilage. (D) The degree of cartilage degeneration was quantified according to the OARSI score. (E) Representative IHC staining of COL2A1 and MMP13. (F) Quantification of the proportion of COL2A1 and MMP13 positive regions in human cartilage. (G) Representative IHC staining of DNMT1 and DNMT3A. (H) Quantification of the proportion of DNMT1 and DNMT3A positive cells in human cartilage. Red arrows indicate positive cells. Scale bar: 100 μ m. Data were presented as means $\pm$ SD; n = 5 per group. And analyzed by 2-tailed unpaired parametric Student's t test, ** P < 0.01, *** P < 0.001.

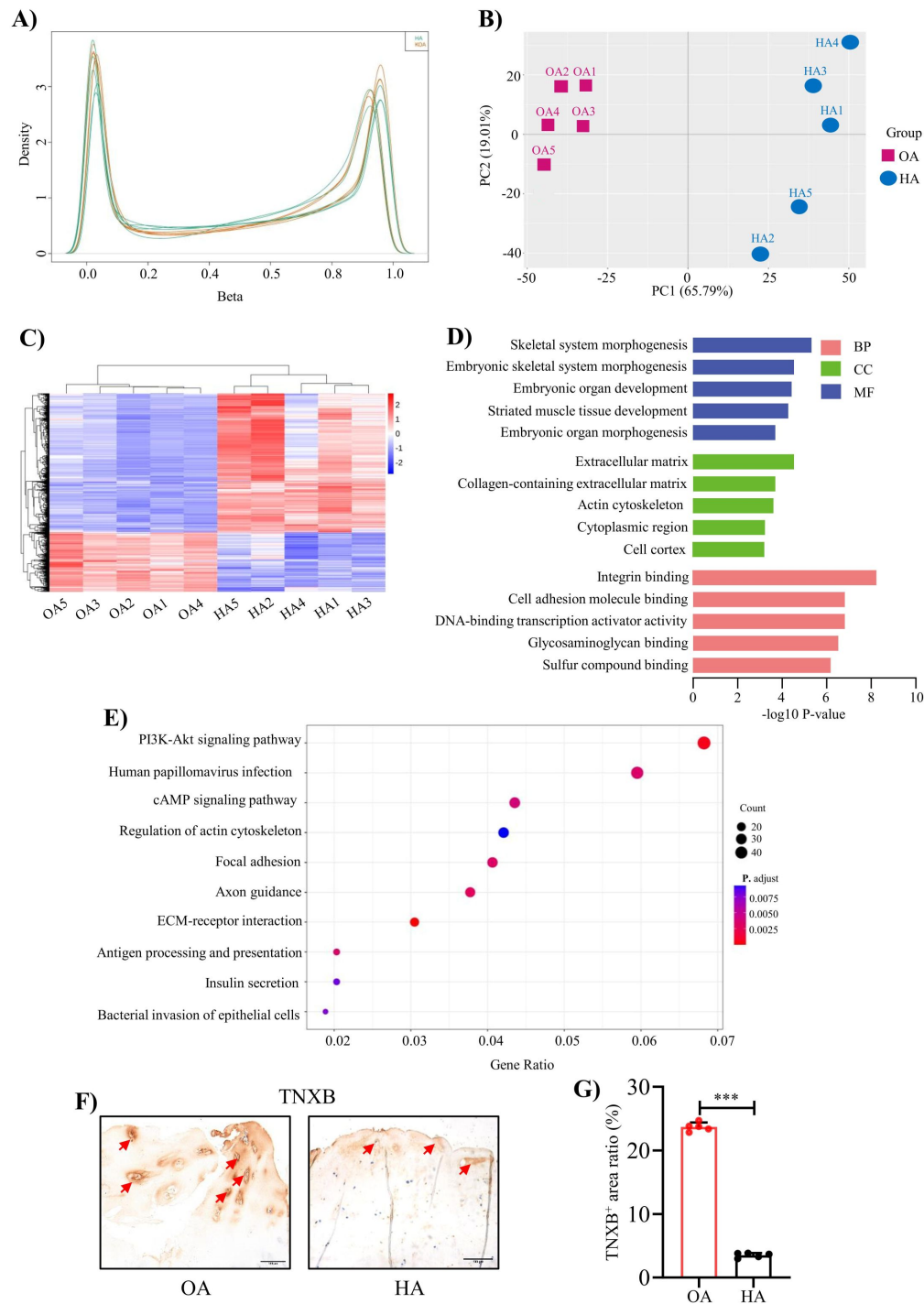


Figure 2.

Genome-wide DNA methylation profile in HA cartilage and biological functions of DMR-Related genes.

(A) Distribution of methylation level density of CpGs. Note: X = degree of methylation; Y = the CpG site density corresponding to the level of methylation. (B) Principal component analysis of DNA methylation data. (C) Heatmap shows the 700 significant DMRs between HA and OA. (D) Enriched GO terms for DMR-related genes. (E) KEGG enrichment analysis of DMR-related genes. (F) Representative IHC staining of TNXB. Red arrows indicate positive areas. Scale bar: 100 μ m. (G) Quantification of the proportion of TNXB positive regions in human cartilage. Data were presented as means $\pm$ SD; n = 5 per group. And analyzed by 2-tailed unpaired parametric Student's t test, ***P < 0.001.

Decreased expression of *Tnxb* is associated with cartilage degradation by joint bleeding in *F8^{-/-}* mice

To assess the potential relevance of TNXB in HA *in vivo*, we established a mouse model for HA, in which *F8^{-/-}* mice received a needle puncture injury to the knee joint^{30,31}. This needle puncture induced excessive bleeding and caused severe hemarthrosis (Supplementary Figure 3A). Analysis of cartilage tissue sections by Toluidine blue staining uncovered that joint bleeding remarkably promoted cartilage degeneration at 4 and 8 weeks after injury (Figure 3, A and B). IHC staining further demonstrated lower expression of *Col2a1* and increased expression of *Mmp13* (Figure 3, C-F). Next, micro-CT was used to assess the influence of hemarthrosis in osteogenesis. An obvious osteopenia was detected in subchondral bone at 4 and 8 weeks post injury (Supplementary Figure 3, B-E). As expected, the above histopathological changes in *F8^{-/-}* mice following needle puncture were similar with what we observed in Human HA. Meanwhile, decreased expression of *Tnxb* and increased expressions of *Dnmt1* and *Dnmt3a* were showed in articular cartilages 4 and 8 weeks following injury (Figure 3, G-L). Above results suggest that the suppression of TNXB in cartilage promotes the HA development.

TNXB knockdown impairs chondrocyte metabolism and aggravates the progression of HA

To verify the relationship between TNXB and methylation levels, we treated primary mouse chondrocytes with DNA Methyltransferase inhibitors RG108 or 5-Aza-dc, well-known to block DNA methylation^{32,33}. After 24 hours treatment, RG108 or 5-Aza-dc (10, 25 μ M) significantly up-regulated the mRNA level of *Tnxb*, indicating that the DNA methylation inhibits the expression of TNXB in chondrocytes (Supplementary Figure 4, A and B). To further identify the biological functions of TNXB in HA, we first knocked it down by specific siRNA in primary mouse chondrocytes. The efficiency of *Tnxb* knockdown was confirmed by results of qPCR and Western blot experiments (Figure 4, A-C). When compared with the siRNA-control group, we found that *Tnxb* knockdown led to a decrease in *Col2a1* mRNA expression and an increase in *Mmp13* mRNA expression (Figure 4, D and E). Western blot and immunofluorescence staining analysis also showed a downregulated expression of *Col2a1* protein and upregulated expression of *Mmp13* protein (Figure 4, E-H). Those observations revealed that TNXB positively regulated the metabolism of chondrocyte ECM.

Subsequently, we explored the effects of TNXB on HA cartilage degradation *in vivo*, using intra-articular injection of adeno-associated virus (AAV) carrying *Tnxb*-specific short hairpin RNA (shRNA) in *F8^{-/-}* mice. 4 weeks after injection, more severe cartilage degeneration was observed in *Tnxb*-KD mice, compared with vehicle group (Figure 4, I and J). In addition, *Tnxb*-KD mice showed decreased expression of *Col2a1* and increased expression of *Mmp13* in cartilages (Figure 4, K and L). The subchondral bone of *Tnxb*-KD mice was significantly less than vehicle group mice (Supplementary Figure 5, A-E). Collectively, the above results implied that TNXB knockdown in cartilage accelerated the HA development.

TNXB deficiency sensitizes chondrocytes to apoptosis by inhibiting AKT activation

Since blood exposure in joint cavity strongly causes chondrocyte apoptosis (Supplementary Figure 6, A and B), we next explored the functional mechanisms of TNXB in HA chondrocyte apoptosis. *Tnxb* knockdown induced the apoptosis in chondrocytes, as evidenced by an increased proportion of Tunel-positive cells (Figure 5, A and B). Meanwhile, protein expression levels of pro-apoptotic Bax and Cleaved-Caspase3 were significantly increased in *Tnxb*-KD chondrocytes, while anti-apoptotic Bcl-2 was decreased (Figure 5, C-F). Moreover, AAV-sh*Tnxb* injection also increased

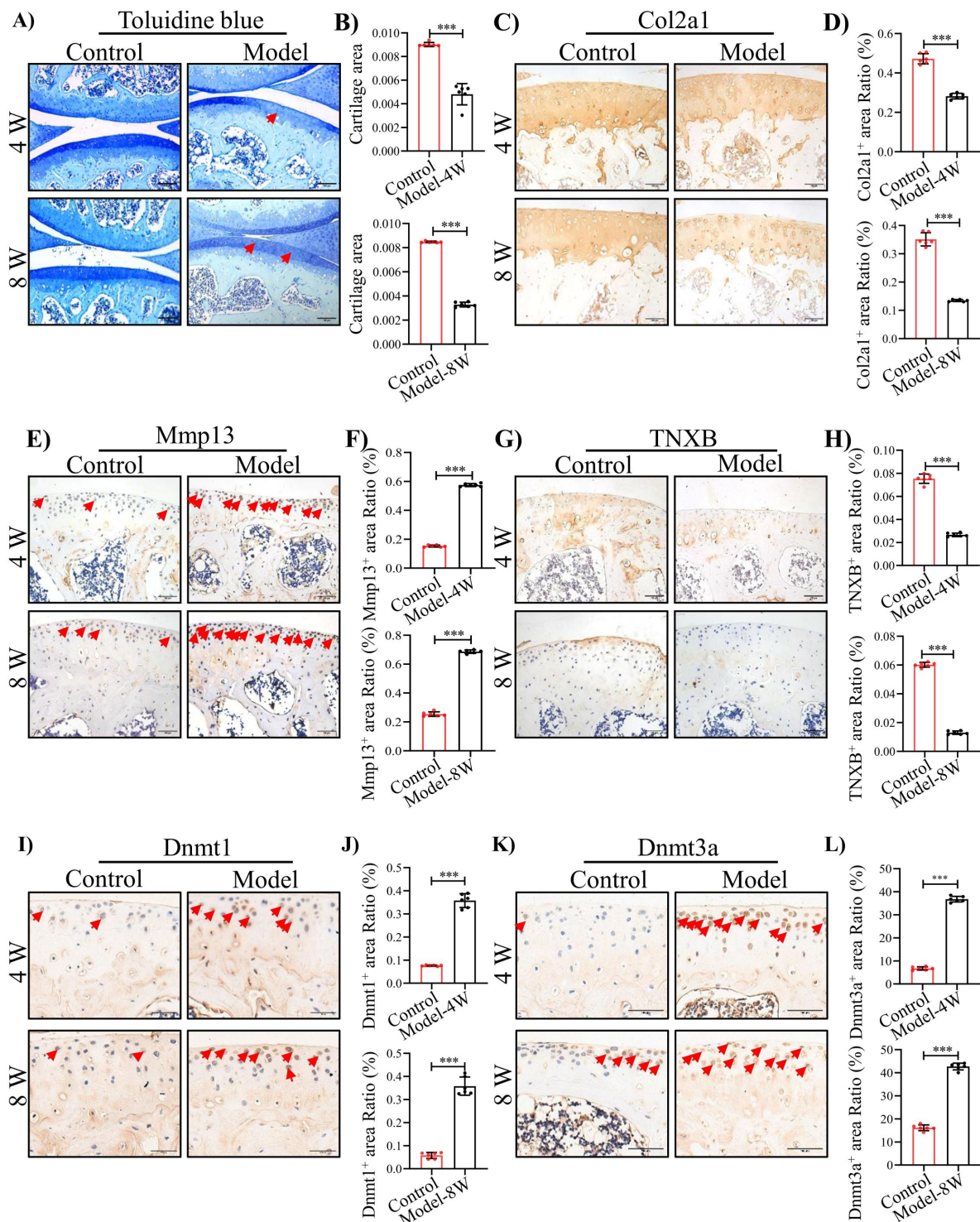


Figure 3.

TNXB expression is drastically reduced in HA mouse cartilages.

(A) Representative images and (B) Quantitative analysis of Toluidine blue staining for knee sections of $F8^{-/-}$ mice at 4 and 8 weeks following injury. Representative IHC staining of (C) Col2a1, (E) Mmp13, (G) Tnxb, (I) Dnmt1 and (K) Dnmt3a in $F8^{-/-}$ mice at 4 and 8 weeks post injury. Quantification of the proportion of (D) Col2a1, (F) Mmp13, (H) Tnxb, (J) Dnmt1 and (L) Dnmt3a positive regions. Scale bar: 100 μ m. Data were presented as means $\pm$ SD; n = 6 mice per group. And analyzed by 2-tailed unpaired parametric Student's t test, *** $P < 0.001$.

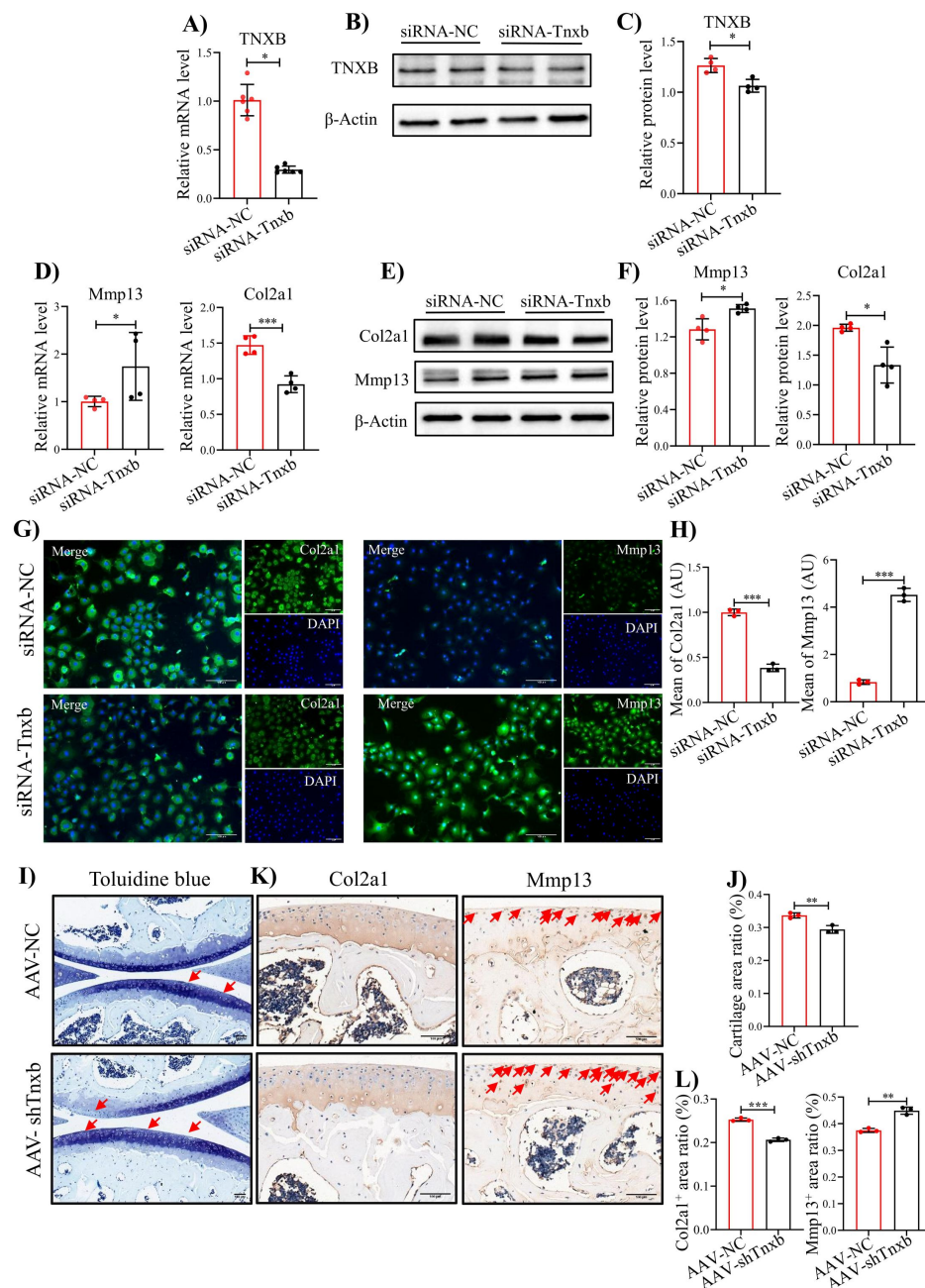


Figure 4.

knockdown of *Tnxb* in chondrocytes induces extracellular matrix degradation and accelerates the progression of HA.

(A) qPCR and (B) Western blotting analysis for *Tnxb* in mouse chondrocytes treated with siRNA-NC or siRNA-*Tnxb*. (C) Corresponding quantification analysis of *Tnxb* protein. Quantification of mRNA levels for (D) *Mmp13* and *Col2a1* in *Tnxb*-KD chondrocytes. (E) Western blot and (F) corresponding quantification analysis for *Mmp13* and *Col2a1* protein. (G) Representative immunofluorescence images of *Col2a1* and *Mmp13* expression in *Tnxb*-KD chondrocytes. (H) Quantification of *Col2a1* and *Mmp13* fluorescence intensity. Representative images of (I) Toluidine blue staining and (K) IHC staining of *Mmp13* and *Col2a1* for knee sections of F8^{-/-} mice at 4 weeks after Intra-articular injection of AAV-sh*Tnxb*. (J) Quantitative detection of the area of the tibial cartilage area. (L) Quantification of the proportion of *Col2a1* and *Mmp13* positive regions. Red arrow indicates the wear area. Scale bar: 100 μ m. Data were presented as means $\pm$ SD; $n \geq 3$ in each group. And analyzed by 2-tailed unpaired parametric Student's t test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the number of TUNEL-positive cells in articular cartilages in HA mice (**Figure 5**, G and H). IHC staining confirmed that Bcl-2 expression was significantly reduced in *Tnxb*-KD HA mice accompanied by enhanced expressions of Bax and Caspase-3 (**Figure 5**, I-L).

It is well known that the PI3K/Akt signaling pathway regulates apoptosis^{34–36}, and our KEGG analysis and IHC staining confirmed the involvement of this pathway in HA cartilage degeneration (**Figure 5**, M and N). We, therefore, analyzed PI3K/Akt pathway following *Tnxb* knockdown. In *Tnxb*-KD chondrocytes, p-Akt protein level decreased significantly, while Akt level remained unchanged (**Figure 6**, A and B). Next, we sought to investigate whether Akt activation could attenuate chondrocyte apoptosis induced by *Tnxb* knockdown. AKT agonist SC79 was used to treat *Tnxb*-KD chondrocytes, and then its efficiency was verified by western blotting (Supplementary Figure 5, C and D). 24 hours after SC79 treatment, the expressions of Bcl-2 and Col2a1 (**Figure 6**, A, B and F) were upregulated, while Bax, Mmp13 and Cleaved-Caspase9 expressions were markedly downregulated in *Tnxb*-KD chondrocytes (**Figure 6**, A, C, E and G). These findings indicate that *TNXB* knockdown induced chondrocyte apoptosis and ECM degradation through regulating AKT activation.

Discussion

HA, a common manifestation of hemophilia, is typically characterized by dramatic cartilage degradation⁶. Thus, the goal of our work was to provide a novel insight into pathogenic mechanisms underlying HA cartilage degeneration. **Figure 6** H summarized our findings. Genome-wide DNA methylation analysis revealed the DNA methylation changes in cartilages from hemophilia patients and identified *Tnxb* gene associated with HA. The decreased expression of *TNXB* protein was also confirmed in both human and mouse HA cartilages. Further, *Tnxb* knockdown promoted ECM catabolism and chondrocyte apoptosis, and eventually accelerated the development of HA in *F8^{-/-}* mice. Meanwhile, decreased *TNXB* expression inhibited the activation of AKT *in vivo* and *in vitro*. Notably, AKT agonist enhanced ECM synthesis and suppressed apoptosis in *Tnxb*-KD chondrocytes. From these findings, it could be hypothesized that abnormal methylation and decreased expression of *TNXB* are responsible for disruption of cartilage homeostasis in HA.

In hemophilia patients, recurrent joint bleeding creates a toxic environment including multifaceted processes such as hemosiderin deposition, inflammatory response and mechanical pressure overload^{37–40}. DNA methylation is a dynamic epigenetic modification in response to environment. Since there are fewer epigenetic studies on the pathological mechanisms of HA, we performed genome-wide DNA methylation analysis, we found 1288 significant DMRs associated with HA with 69.95% hypermethylated, which was also supported by a corresponding increase in Dnmt1 and Dnmt3a protein levels in HA cartilages. These results are in line with previous reports showing that aberrant elevations of Dnmt1 and Dnmt3a promote cartilage degeneration in destabilization of medial meniscus and aging models^{33,41}.

DNA methylation at gene promoter region is one of the important epigenetic mechanisms in the regulation of gene expression^{42,43}. The identified HA-related DMRs showed high proportion in transcriptional start sites. These data suggest that DNA methylation is a critical mediator in the pathology of HA. Nevertheless, due to the severe erosion of HA cartilage, the mRNA levels of DMR-related genes were not explored in this study. Besides, our sample size for DNA methylation sequencing is relatively small. Thus, further studies using additional more HA samples will be conducted to investigate the possible transcriptional alteration of identified genes as well as their potential roles in HA development.

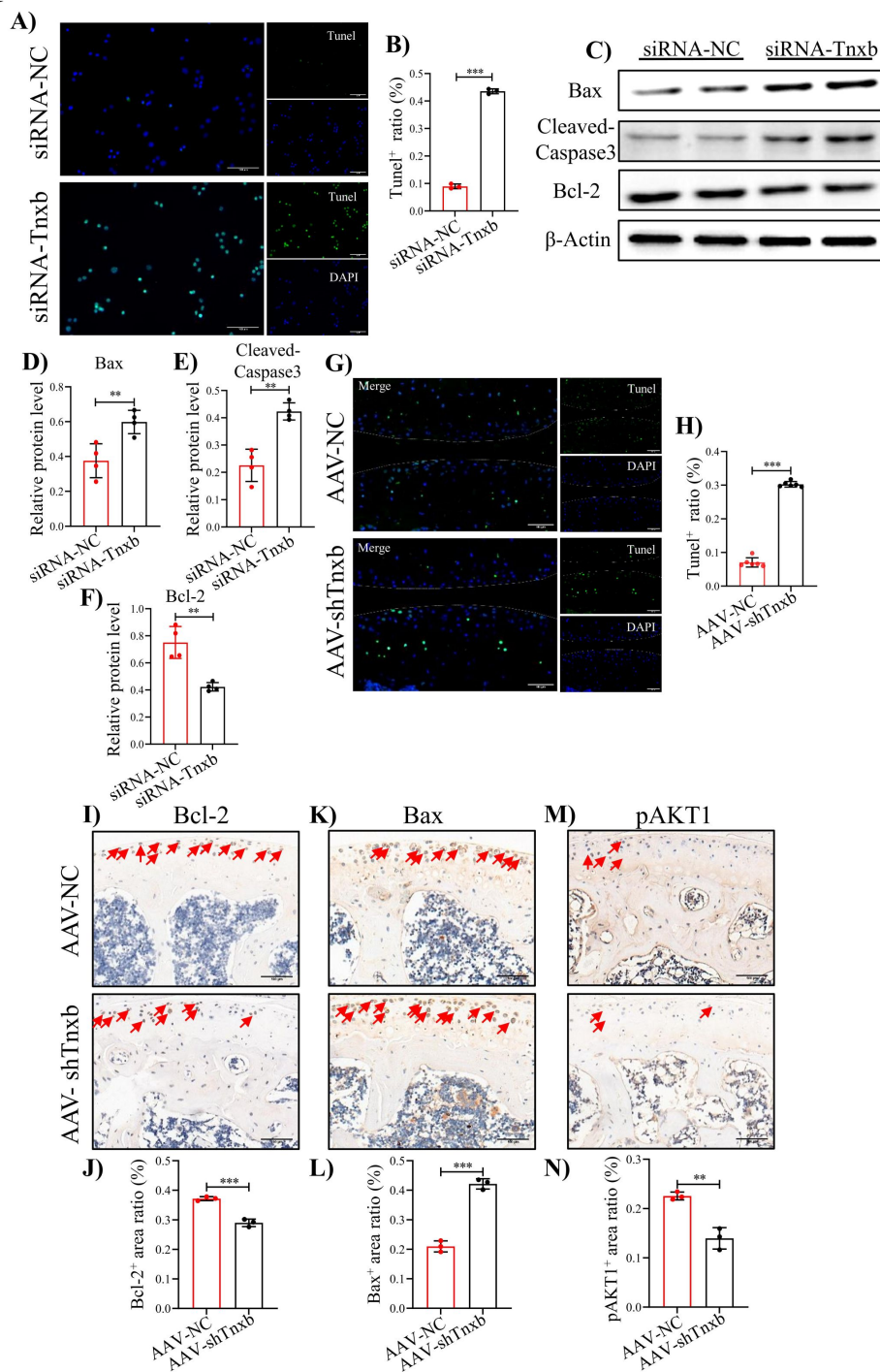


Figure 5.

Tnxb knockdown increases apoptosis in chondrocytes and HA cartilages.

(A) Representative images of TUNEL staining in *Tnxb*-KD chondrocytes **(B)** Quantification for TUNEL positive cells. **(C-F)** Western blot and corresponding quantification analysis for Bax, Cleaved-Caspase3, and Bcl-2. **(G)** TUNEL staining for apoptosis in articular cartilage from *Tnxb*-KD HA mice. **(H)** Quantification for TUNEL positive cells in articular cartilage. Representative IHC staining of Bax **(I)**, Bcl-2 **(K)**, and p-Akt1 **(M)** in articular cartilage from *Tnxb*-KD HA mice. Corresponding quantification of the proportion of **(J)** Bax, **(L)** Bcl-2 and **(N)** p-Akt1 positive regions. Red arrows indicate positive cells. Scale bar: 100 μ m. Data were presented as means $\pm$ SD; $n \geq 3$ in each group. And analyzed by 2-tailed unpaired parametric Student's t test, $**P < 0.01$, $***P < 0.001$.

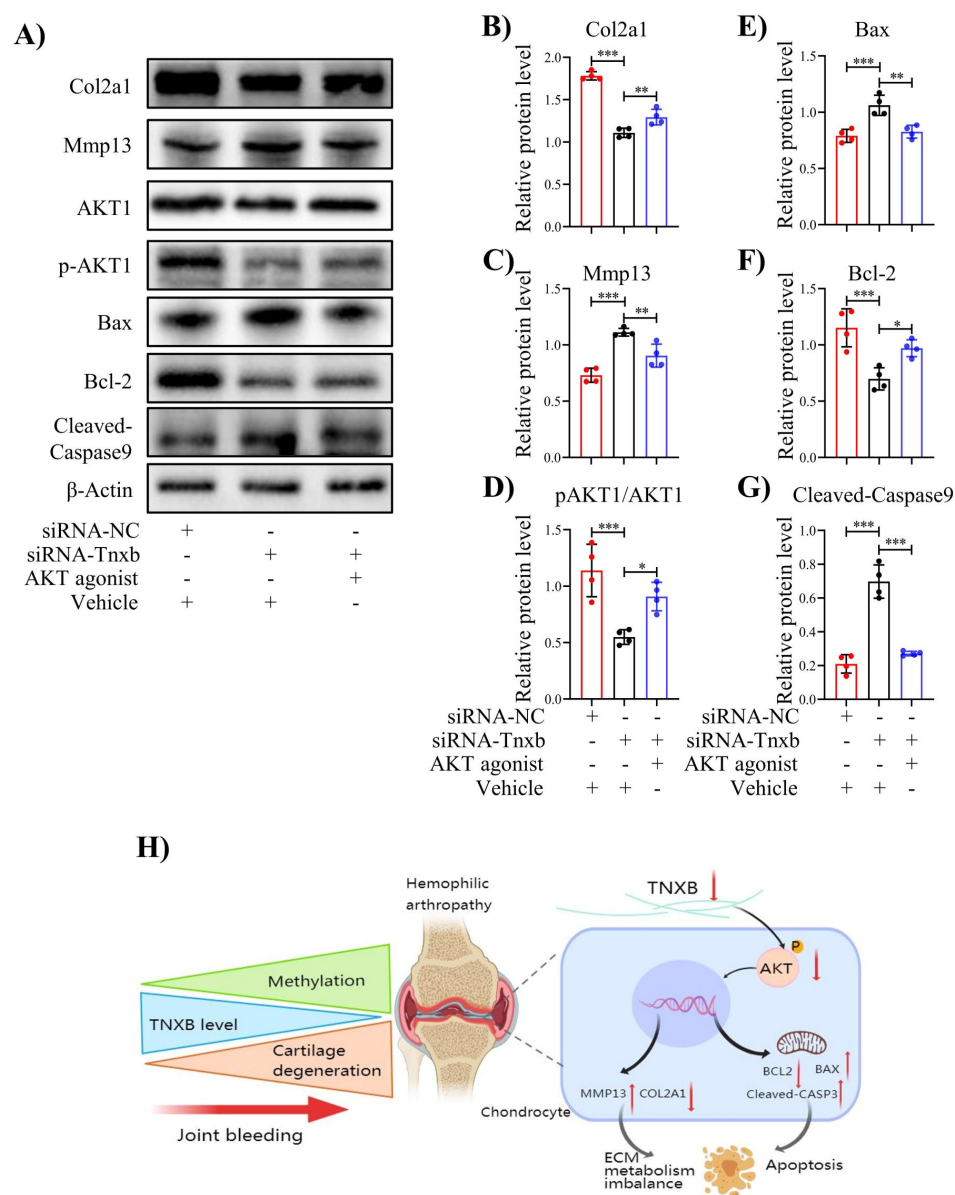


Figure 6.

Treatment with AKT agonist decreases apoptosis in TNXB-KD chondrocytes.

(A) Western blot analysis for Col2a1, Mmp13, Bax, Bcl-2, p-Akt1, Akt1 and Cleaved-Caspase9 in Tnxb-KD chondrocytes treated with SC79 (0 or 10 μ M) for 24 hours. **(B-G)** Corresponding quantification of these proteins. **(H)** Schematic of the role of TNXB in the pathogenesis of HA cartilage degeneration. Data were presented as means $\pm$ SD; n = 4 per group. And analyzed by 2-tailed unpaired parametric Student's t test or one-way ANOVA with Tukey's multiple comparisons test, * P < 0.05, ** P < 0.01, *** P < 0.001.

Furthermore, our enrichment analysis showed that DMR-related genes were significantly enriched in ECM organization and ECM-receptor interaction terms. It is consistent with previous reports that ECM acts as an epigenetic informational entity capable of transducing and integrating intracellular signals via ECM-receptor interaction ^{44,45,46}. DMR-related genes enriched in these terms, many are known to be critical compositions of cartilage matrix, such as *COMP*, *COL1A1* and *COL1A2* ^{27,28,29}. In Articular cartilage, ECM provides nutrients and mechanical support for chondrocytes ⁴⁷, and its age-related stiffening epigenetically regulates gene expression and compromises chondrocyte integrity ⁴¹. Above findings highlight that ECM-related genes are potential candidates for HA.

Tenascin-X (TNXB), an ECM glycoprotein, is the top differentially methylated gene in our DNA methylation analysis of HA cartilages. In addition, DNA methylation of TNXB has also been reported in whole blood from rheumatoid arthritis patients and in retinal pigment epithelium from patients with age-related macular degeneration ^{48,49}. Using IHC staining, we detected the downregulated expression of TNXB in human and mouse HA cartilages. TNXB belongs to tenascin family, whose members (TNC, TNR, TNXB, and TNW) share a similar domain pattern: an N-terminal oligomerization domain, a series of epidermal growth factor-like repeats, a variable number of fibronectin-type III (FNIII) repeats and a C-terminal fibrinogen-like domain ⁵⁰. Emerging evidence suggests the involvement of TNC in cartilage development and degeneration in arthritis ^{51,52}. Here, we prove for the first time a role of TNXB in cartilage homeostasis *in vivo* and *in vitro*. Specifically, *Tnxb* knockdown contributed to an increase in Mmp13 expression and decrease in Col2a1 expression in chondrocytes. In agreement with these findings, knockdown of *Tnxb* in cartilages of F8^{-/-} mice resulted in accelerated HA-like lesions. It has been previously reported that TNXB blocks the interaction between TGF- β and its receptor in endothelial cells. Since a potent role of TGF- β in chondrocyte differentiation, we examined the effect of TNXB on TGF- β signaling, but did not observe significant alterations in this signaling in *Tnxb*-KD chondrocytes (Supplementary Figure 7, A and B). The FNIII repeats domain of TNXB undergoes alternative splicing to interact with different ECM proteins and growth factors, and then affects ECM network formation and three-dimensional collagen matrix stiffness ⁵³. Thus, future study would benefit from exploring the effect of the FNIII repeats domain in HA development to further clarify the specific mechanisms of TNXB.

The process of HA development is accompanied by the chondrocyte apoptosis which in part promotes the ECM degeneration ^{15,54}. We also observed remarkably increased apoptosis following joint bleeding in F8^{-/-} mice. Interestingly, *Tnxb* knockdown could significantly induce apoptosis in chondrocytes *in vitro*, and even aggravate apoptosis in mouse HA cartilage, by enhancing expression of markers associated pro-apoptosis (Bax, C-Caspase3) and suppressing anti-apoptotic proteins (Bcl-2). In TNXB family, TNC was reported to inhibit human chondrosarcoma cell apoptosis by activation of AKT ⁵⁵. PI3K signaling pathway regulated AKT phosphorylation to suppress apoptosis ^{56,57}. Our KEGG analysis of DMGs showed the potential association of PI3K/AKT signaling with HA. We then detected much lower phosphorylation level of AKT in *Tnxb*-KD chondrocytes and HA models. These results are in line with previous report showing that the expression of p-AKT1 was down-regulated in the articular cartilage of patients with HA compared with that in patients with OA ⁵⁸. Notably, we further demonstrated that AKT agonist effectively restored the abnormal Mmp13 expression and apoptosis induced by *Tnxb* knockdown. Recent studies have shown that AKT signaling affected cartilage metabolism by regulating Mmp13 expression ^{59,60}. These findings indicate that AKT1 activation mediates the effect of TNXB on HA chondrocytes apoptosis and ECM catabolism. Thus, therapeutic effect of AKT agonist on HA development deserves further study.

Conclusions

Conclusively, our study provides the first comprehensive methylation profile in the progression of knee HA, and shows that abnormal expression of TNXB is important in HA cartilage degeneration by suppressing the activation of AKT1. These findings provide a new insight into mechanisms responsible for HA disease and suggest TNXB as a potentially novel therapeutic target for HA treatment.

List of abbreviations

- HA: hemophilic arthropathy
- DMRs: differentially methylated regions
- DMGs: DMR genes
- TNXB: Tenascin XB
- DNMT: DNA methyl transferase
- ABH: Alcian blue hematoxylin/Orange G
- ECM: extracellular matrix
- AAV: adeno-associated virus.

Study approval

In the current study, the human cartilage samples were obtained from Department of Orthopedic Surgery at The First Affiliated Hospital of Zhejiang Chinese Medical University. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University (2019-ZX-004-02). And all the animal operating procedures in this study were approved by the Experimental Animal Ethics Committee of Zhejiang Chinese Medical University (LZ12H27001).

Data Availability Statement

The data used to provide support for the results of this study can be obtained from the corresponding authors.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

All authors declare that they have no conflict of interest.

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Author contributions

J.C., Q.Z., D.C., P.T., and H.J. conceptualized the study. Q.Z. conducted the *in vivo* experiments. J.C., X.W. and R.X. collected the human samples and performed the *in vitro* assay. W.W., Y.H. and Q.S. analyzed the data. J.C. wrote the original draft and W.Y., P.W., D.C., P.T., and H.J. revised the manuscript critically for important intellectual content. D.C., P.T. and H.J. supervised this study. All the authors read and approved the final version of this manuscript.

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Editors

Reviewing Editor

Jie Shen

Washington University School of Medicine, Saint Louis, United States of America

Senior Editor

Tony Yuen

Icahn School of Medicine at Mount Sinai, New York, United States of America

Reviewer #1 (Public Review):

Summary:

Chen and colleagues first compared the cartilage tissues collected from OA and HA patients using histology and immunostaining. Then, a genome-wide DNA methylation analysis was performed, which informed the changes of a novel gene, TNXB. IHC confirmed that TNXB has a lower expression level in HA cartilage than OA. Next, the authors demonstrated that TNXB levels were reduced in HA animal model, and intraarticular injection of AAV carrying TNXB siRNA induced cartilage degradation and promoted chondrocyte apoptosis. Based on KEGG enrichment, histopathological analysis, and western blot, the authors also showed the relationship between TNXB and AKT phosphorylation. Lastly, AKT agonist, specifically SC79 in this study, was shown to partially rescue the changes of in vitro-cultured chondrocytes induced by Tnxb knock-down. Overall, this is an interesting study and provided sufficient data to support their conclusion.

Strengths:

- (1) Both human and mouse samples were examined.
- (2) The HA model was used.
- (3) genome-wide DNA methylation analysis was performed.

Weaknesses:

- (1) In some experiments, the selection of the control groups was not ideal.
- (2) More details on analyzing methods and information on replicates need to be included.
- (3) Discussion can be improved by comparing findings to other relevant studies.
- (4) The use of transgenic mice with conditional Tnxb depletion can further define the physiological roles of Tnxb.

<https://doi.org/10.7554/eLife.93087.2.sa1>

Reviewer #2 (Public Review):

Summary:

This manuscript mainly studied the biological effect of tenascin XB (TNXB) on hemophilic arthropathy (HA) progression. Using bioinformatic and histopathological approaches, the authors identified the novel candidate gene TNXB for HA. Next, authors showed that TNXB knockdown lead to chondrocyte apoptosis, matrix degeneration and subchondral bone loss in vivo/vitro. Furthermore, AKT agonist promoted extracellular matrix synthesis and prevented apoptosis in TNXB knockdown chondrocytes.

Strengths:

In general, this study significantly advances our understanding of HA pathogenesis. The authors utilize comprehensive experimental strategies to demonstrate the role of TNXB in cartilage degeneration associated with HA. The results are clearly presented, and the conclusions appear appropriate.

Weaknesses:

Additional clarification is required regarding the gender of the F8^{-/-} mouse in the study. Is the mouse male or female?

<https://doi.org/10.7554/eLife.93087.2.sa0>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

Chen and colleagues first compared the cartilage tissues collected from OA and HA patients using histology and immunostaining. Then, a genome-wide DNA methylation analysis was performed, which informed the changes of a novel gene, TNXB. IHC confirmed that TNXB has a lower expression level in HA cartilage than OA. Next, the authors demonstrated that TNXB levels were reduced in the HA animal model, and intraarticular injection of AAV carrying TNXB siRNA induced cartilage degradation and promoted chondrocyte apoptosis. Based on KEGG enrichment, histopathological analysis, and western blot, the authors also showed the relationship between TNXB and AKT phosphorylation. Lastly, AKT agonist, specifically SC79 in this study, was shown to partially rescue the changes of in vitro-cultured chondrocytes induced by Tnxb knock-down. Overall, this is an interesting study and provided sufficient data to support their conclusion.

Strengths:

- (1) Both human and mouse samples were examined.*
- (2) The HA model was used.*
- (3) Genome-wide DNA methylation analysis was performed.*

Weaknesses:

- (1) In some experiments, the selection of the control groups was not ideal.*

Thank you for comments. The reviewer raised the concerns about using human OA cartilage as control, instead of health cartilage. This is an important detail we didn't describe in the previous version. We have added our explanation in revised Methods.

- (2) More details on analyzing methods and information on replicates need to be included.*

We greatly appreciate your careful review and helpful suggestions. We have added detailed information to our revised draft.

(3) Discussion can be improved by comparing findings to other relevant studies.

Thank the reviewer very much for the opportunity to improve our manuscript. We have improved discussions as reviewer suggested in Recommendation 13.

(4) The use of transgenic mice with conditional *Tnxb* depletion can further define the physiological roles of *Tnxb*.

Thanks for this valuable comment. We understand that conditional *Tnxb*-KO mice is much helpful for the study of biological roles of *Tnxb*, and it will be constructed and used in our future studies.

Recommendations For the Authors:

(1) Please add more information about HA such as incidence to highlight the importance of the study.

We greatly appreciate your careful review and helpful suggestions. We have provided more information about the importance of HA study in revised Introduction. Please see lines 90-93 and 103-112.

(2) Please justify the use of OA cartilage, instead of normal tissues, as the control.

Thanks for your suggestion. We certainly would have liked to use healthy cartilage as control, but we were extremely difficult to obtain enough control samples from healthy individuals. Despite the mechanistic and phenotypic differences between HA and OA, OA is often used as “disease” control to reveal the characteristics in HA 1,2. Thus, we measured cartilage degeneration and DNA methylation difference in HA and OA patients. We have provided the statement and evidence in revised manuscript. Please see lines 144-145.

(3) Please provide details of how to calculate the Cartilage wear area ratio in Figure 1D, and measure the positive staining area in Figure 1F.

We apologize for the issue you pointed out. Here, we provide detailed information for how positively stained areas are calculated. Specifically, in Figure 1D, we obtained the cartilage area ratio by calculating the ratio of blue cartilage staining area to the whole tissue area by using image J software. In Figure 1F, the area of positive staining was determined upon secondary antibody treatment and color development using DAB chromogen (brown stain). We then obtained the positive staining area ratio by calculating the ratio of positive staining area to the whole cartilage area by using image J software.

(4) Please label the location of hemorrhagic ferruginous deposits in Figure 1.

Thank you for your valuable suggestion. We have used black arrows to indicate hemorrhagic ferruginous deposits in revised Figure 1A.

(5) Please define the meaning of “n” in all figure legends, such as technical or biological replicates.

Thanks for your suggestion. We have defined the meaning of “n” in all figure legends in revised manuscript.

(6) In Figure 3, please increase the font size of B, D, F, H, and J. The same applies to other figures.

Thank you for your valuable suggestion. We have increased the font size of figures in our revised manuscript.

(7) Line 327, "(Figure 1, F and G)" should be Figure 2F, G.

Thanks for your reminding. We have corrected it in the revision. Please see lines 347.

(8) Reduced *TNXB* levels in human HA cartilage are one of the major findings in this study. Currently, only semi-quantitative IHC was used to draw the conclusion. A second method, such as real-time PCR or western blot, is required.

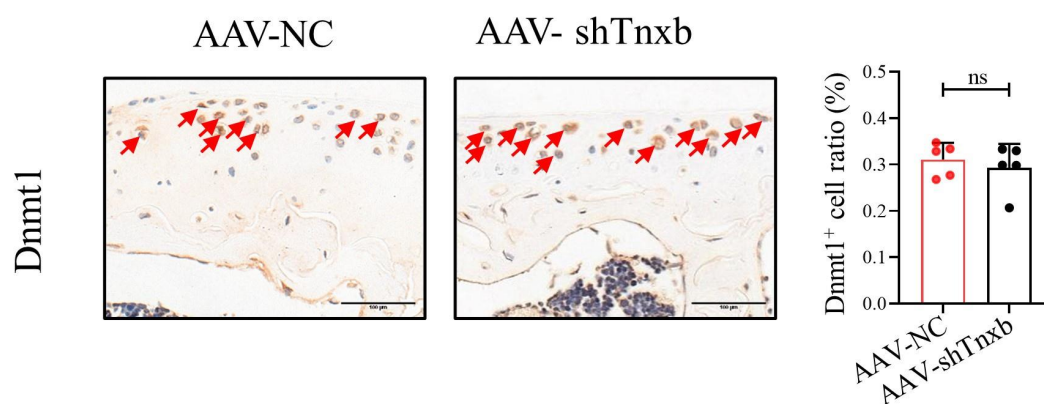
Thanks for your suggestion. We feel very sorry that we did not have enough samples of human HA cartilages for qPCR and WB experiments, due to severe erosion of the HA cartilage. We have pointed out this limitation in revised drafts. Please see lines 445-448.

(9) Figure 3 shows that reduced *Tnxb* was accompanied by the increased *Dnmt1*. In addition, this study is about methylation. Have the authors tested the change of *Dnmt1* levels when *Tnxb* was knocked down?

Thanks for your suggestion. According to the reviewer's suggestion, we have tested the expression of *Dnmt1* in *Tnxb*-KD chondrocytes, and no significant alteration was observed. Please see the following Figure.

Author response image 1.

Figure Legend: Representative IHC staining of *Dnmt1* in articular cartilage from *Tnxb*-KD HA mice. Corresponding quantification of the proportion of *Dnmt1* positive regions. Red arrows indicate positive cells. Scale bar: 100 μ m. Data were presented as means $\pm$ SD; n = 5 in each group. ns = no significance by unpaired Student's t test.



(10) Also, is there a causal relationship between *Tnxb* levels and the distribution of methylation levels? Any related study was performed?

Following the valuable suggestion of the reviewer, we used two well-known DNA methyltransferase inhibitors (RG108 or 5-Aza-dc) 3 to examine whether DNA methylation

regulates transcriptional expression of TNXB. We found that both inhibitors significantly up-regulated Tnxb mRNA level. We have added this result to the revised Supplementary Figure 4 and draft (lines 292-296 and 369-374).

(11) In Figure 6, what was the control of "AKT agnost" group?

Thank you for your suggestion. We feel sorry for our negligence and we have added the vehicle group as a control for AKT agonists in Figure 6 in our revised manuscript.

(12) Previous studies have reported the involvement of TNXB in TGF- β signaling. Have the authors examined the effect of TNXB on TGF- β signaling in chondrocytes?

Thank you for your suggestion. Here, we examined the expression of TGF- β signaling in Tnxb-KD chondrocyte and no significant changes were observed. We have discussed this result in revised draft (lines 475-479). We have added this result to the revised Supplementary Figure 7.

(13) Discussion can be improved. For example, have previous studies reported the association between TNXB and methylation in other cells/tissues? In addition to apoptosis, are there other potential mechanisms underlying the protective role of TNXB in chondrocytes?

Thank you for your valuable comments. Previous studies have shown the different DNA methylation of TNXB in whole blood from rheumatoid arthritis patients and in retinal pigment epithelium from patients with age-related macular degeneration 4,5. Herein, we were the first to report the association between DNA methylation of TNXB and HA cartilage degeneration. As for TNXB, there are limited public studies regarding physiological function of TNXB, among which mostly report the effect of TNXB on extracellular matrix organization 6,7. In our work, we found that TNXB regulated the phosphorylation of AKT. Since previous reports showed AKT controlled the expression of Mmp13 8, we thought that TNXB might regulated the chondrocyte extracellular matrix organization, in addition to its function on apoptosis. We have discussed these in revised manuscript (lines 462-464, and 495-501).

(14) The manuscript writing needs to be improved. Typos and grammar issues were noted.

Thanks. We have modified and polished our language and we hope the revised version could be acceptable for you.

Reviewer #2 (Public Review):

Summary:

This manuscript mainly studied the biological effect of tenascin XB (TNXB) on hemophilic arthropathy (HA) progression. Using bioinformatic and histopathological approaches, the authors identified the novel candidate gene TNXB for HA. Next, the authors showed that TNXB knockdown leads to chondrocyte apoptosis, matrix degeneration, and subchondral bone loss in vivo/vitro. Furthermore, AKT agonists promoted extracellular matrix synthesis and prevented apoptosis in TNXB knockdown chondrocytes.

Strengths:

In general, this study significantly advances our understanding of HA pathogenesis. The authors utilize comprehensive experimental strategies to demonstrate the role of TNXB in

cartilage degeneration associated with HA. The results are clearly presented, and the conclusions appear appropriate.

Weaknesses:

Additional clarification is required regarding the gender of the F8^{-/-} mouse in the study. Is the mouse male or female?

We feel sorry that we did not provide enough information about the gender of the F8^{-/-} mouse in the previous draft. Here, we used male F8^{-/-} mice as the study subjects for our experiments. Hemophilia A is predominantly seen in males because of the X chromosome linkage 9.

Recommendations For The Authors:

Some issues need to be addressed in the manuscript:

(1) During the progression of HA, in addition to cartilage degeneration, synovial hypertrophy and inflammation are also significant symptoms. How is the expression of TNXB in HA synovium?

Thank you for your valuable comments. According to the reviewer's suggestion, we tested the expression of TNXB in the synovium, and there was no statistically significant difference in the expression level of TNXB in the synovium (Supplementary Figure. 2) Please see lines 347-349.

(2) Lines 183-188. The methods of virus infection should be more detailed. What was the concentration of the AAVs injected? And how many doses were administrated?

Thank you for your suggestion. We have added an explanation of virus infection and injected doses in revised methods section (lines 205-206).

(3) Line 197-198. Could the author double-check the decalcification time for human cartilage samples? Is it for 3 months? Or for 3 weeks?

Thank you for your suggestion. We have reconfirmed the decalcification of human cartilage samples for 3 months.

(4) Line 343-344 "Above results suggest that TNXB might be protective against HA and its cartilage suppression is closely related to HA development." The conclusion is inappropriate, please revise it.

Thanks for your suggestion. We have revised this conclusion into "Above results suggest that the suppression of TNXB in cartilage promotes the HA development". Please see lines 365-366.

(5) Line 326-327, the IHC staining for human samples is shown in Figure 2, not Figure 1. Please double check and revise it.

Thanks for your reminding. We feel sorry for our negligence and we have corrected it in the revision.

(6) For Figure 1B, it shows the MRI images of knee joints. However, the method section lacks details regarding the MRI imaging scan and analysis. Could the author include this information in the method section?

Thank you for your valuable comments. We have added the method of MRI imaging scan and analysis in revised Methods. Please see lines 154-163.

(7) In Figure 5, The statistical result of Bcl-2 is inconsistent with its Western blot band. Please check.

Thanks for your reminding. We have modified it in the revision.

(8) Please read through the text carefully to check for language problems. For example, in Line 68 "Our" not "our".

Thanks for your reminding. In revision, we have corrected it. Please see Line 68.

Reviewer #3 (Public Review):

Summary:

The manuscript by Dr. Chen et al. investigates the genes that are differentially methylated and associated with cartilage degeneration in hemophilia patients. The study demonstrates the functional mechanisms of the TNXB gene in chondrocytes and F8-/- mice. The authors first showed significant DNA methylation differences between hemophilic arthritis (HA) and osteoarthritis through genome-wide DNA methylation analysis. Subsequently, they showed a decreased expression of the differentially methylated TNXB gene in cartilage from HA patients and mice. By knocking down TNXB in vivo and in vitro, the results indicated that TNXB regulates extracellular matrix homeostasis and apoptosis by modulating p-AKT. The findings are novel and interesting, and the study presents valuable information in blood-induced arthritis research.

Strengths:

The authors adopted a comprehensive approach by combining genome-wide DNA methylation analysis, in vivo and in vitro experiments using human and mouse samples to illustrate the molecular mechanisms involved in HA progression, which is crucial for developing targeted therapeutic strategies. The study identifies Tenascin XB (TNXB) as a central mediator in cartilage matrix degradation. It provides mechanistic insights into how TNXB influences cartilage matrix degradation by regulating the activation of AKT. It opens avenues for future research and potential therapeutic interventions using AKT agonists for cartilage protection in hemophilic arthropathy. The conclusions drawn from the study are clear and directly tied to the findings.

Weaknesses:

(1) The study utilizes a small sample size (N=5 for both osteoarthritis and hemophilic arthropathy). A larger sample size would enhance the generalizability and statistical power of the findings.

Thank you for pointing out this deficiency. Indeed, our sample size is relatively small, although the overall sample size was sufficient for statistical analyses. And we have added this limitation in discussion in revised manuscript. Please see line 445-448. Considering the small sample size, we subsequently performed functional validation study for TNXB, one of the most significant genes, and demonstrated that TNXB exerted critical impacts on chondrocytes apoptosis in HA pathogenesis in vivo and in vitro.

(2) The use of an animal model (F8-/- mouse) to investigate the role of TNXB may not fully capture the complexity of human hemophilic arthropathy. Differences in the biology

between species may affect the translatability of the findings to human patients.

Thank you for your valuable comments. We recognize that biological differences between species can affect the clinical translation of research findings. In our work, we sequenced human cartilage samples to obtain the differentially methylated gene-TNXB. Meanwhile, we demonstrated that protein expression of TNXB protein was significantly down-regulated in HA human cartilage and F8-/- transgenic mouse cartilage. The F8-/- transgenic mouse serves as a well-accepted model for the study of hemophilia, which is phenotypically similar to that of human patients suffering from the disease and spontaneously bleeds into the joints and soft tissues. Besides, this model mouse has been widely used in the study of hemophilia and hemophilic arthritis 9-11.

(3) The study primarily focuses on TNXB as a central mediator, but it might overlook other potentially relevant factors contributing to cartilage degradation in hemophilic arthropathy. A more holistic exploration of genetic and molecular factors could provide a broader understanding of the condition.

Thanks for your suggestion. Since our human sample size is relatively small, we should interpret differentially methylated genes cautiously. Therefore, we mainly focused on the most top significant gene TNXB for functional study. In our further study, we will expand the sample size to more comprehensively explore the molecular mechanisms of HA.

Recommendations For The Authors:

The following are my suggestions:

(1) Why do the authors choose to concentrate on the knee joint in the introduction when hemophilia, characterized by a deficiency in clotting factor F8, is recognized as a systemic disease?

Thank you for your valuable comments. Although hemophilia a systemic disease, approximately 80%-90% of bleeding episodes in patients with hemophilia occur within the musculoskeletal system, especially in the knee joint 12.

(2) While Figure 1 illustrates distinct expressions of Dnmt1 and Dnmt3a, only Dnmt1 results are presented in HA mice models in Figure 3. To address this, it is suggested that the expression of Dnmt3a be explored in animal models.

Thank you for your suggestion. According to the reviewer's suggestion, we examined the expression of Dnmt3a in mouse articular cartilage, and the expression level of Dnmt3a was significantly up-regulated in both the 4W and 8W model groups compared with the control group (Figure 3). Please see line 364.

(3) In Figure 3, the sample size for Dnmt1 is smaller than the other indicators; therefore, supplementing the sample count is recommended.

Thanks for your reminding. We have corrected it in the revision.

(4) Regarding Figure 4G, a few apoptotic cells were observed in the AAV NC group. It is advised that this figure be reviewed for accuracy.

Thanks for your suggestion. In Figure 5D, the AAV-NC group is the case of needle-injected with AAV. Therefore, it is normal for apoptotic cells to appear in the cartilage layer.

(5) *The authors concluded that TNXB plays a role in apoptosis and AKT signaling. Providing expression data for Caspase9 would be valuable to strengthen this assertion, as PI3K/AKT signaling directly influences its activation during apoptosis.*

Thank you for your comments. We have examined the expression of Cleaved-Caspase9 protein, and found that knockdown of TNXB resulted in upregulation of Cleaved-Caspase9 protein expression, which was reversed by addition of SC79. This result has added in revised Figure 6 and manuscript. Please see line 414.

(6) *Quantitative analysis of the differences between the two groups in Supplemental Figures is necessary.*

Thank you for your suggestion. We have added the quantitative analysis of the differences between the two groups in Supplemental Figures.

(7) *With three major isoforms (homologs) of AKT in mammals-AKT1, 2, and 3 - why did the authors specifically focus on AKT1?*

Thank you for your comments. Based on the results of the KEGG enrichment analysis of differential methylated genes, we investigated the role of PI3K/AKT pathway in apoptosis of HA chondrocytes. AKT is universally acknowledged as a core factor in the PI3K/AKT pathway that plays critical roles in various cellular activities such as cell proliferation, cell differentiation, cell apoptosis, metabolism and so on 13,14. More notably, several studies demonstrated that in AKT family, Akt1 primarily was involved in regulation of chondrocyte survival and proteoglycan synthesis 15. Therefore, we detected phosphorylation of AKT1 in HA cartilages and TNXB-KD chondrocytes, and found that TNXB regulation chondrocytes ECM and apoptosis by AKT1. Reference:

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