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Activation of β-catenin in *Col2*-expressing chondrocytes leads to osteoarthritis-like defects in hip joint

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Abstract

Although osteoarthritis (OA) in the hip joint is a common and debilitating degenerative disease, the precise molecular mechanisms underlying its pathological process remains unclear. This study sets out to investigate whether β-catenin plays a critical role in hip OA pathogenesis. Here, we showed overexpressed β-catenin protein in human OA cartilage tissues. Then, we analyzed *β-cat(ex3)*^{Col2ER} mice, in which *β-catenin* gene was conditionally activated in femoral head chondrocytes. At 2 months of age, *β-cat(ex3)*^{Col2ER} mice already showed a phenotype of severe cartilage degeneration in the femoral head. More changes observed in *β-cat(ex3)*^{Col2ER} mice with age included subchondral sclerosis and osteophyte formation along joint margins, resembling a hip OA phenotype in humans. In addition, cartilage degradation and chondrocyte apoptosis as the results of *β-catenin* activation possibly contributed to this hip OA-like phenotype. Overall our findings provide direct evidence about the importance of β-catenin in hip OA pathogenesis.

KEYWORDS
cartilage degeneration, β-catenin, hip joint, osteoarthritis, apoptosis

*Xia and Wang have contributed equally to this work.

1 | INTRODUCTION

Hip osteoarthritis (OA) is a joint disease that is highly prevalent and affects up to a quarter of people over a lifetime (Murphy et al., 2010). Symptoms associated with hip OA include pain, stiffness, and joint dysfunction, which can seriously deteriorate the quality of patients' life (Eitzen, Fernandes, Nordsletten, & Risberg, 2012; Hurwitz, Hulet, Andriacchi, Rosenberg, & Galante, 1997; Nakamura et al., 2013; Rutherford, Moreside, & Wong, 2015). Currently, hip joint replacement surgery is the final therapeutic option, as no effective treatment can prevent or reverse the progression of hip OA (Berenbaum, 2011; Hawker, Mian, Bednis, & Stanaitis, 2011). Our knowledge about hip OA is derived mostly from clinical studies (Baird et al., 2018; Tolk et al., 2018) and many aspects of hip OA, especially the potential molecular mechanisms, still need largely under-researched.

Cartilage degeneration or damage, a prominent feature of OA, is thought to be a critical pathological event accelerating the progression of OA (Glasson et al., 2005). Cartilage degeneration is a complex and irreversible pathological process characterized by the large loss of collagen and proteoglycan, cartilage fibrillation, and chondrocyte clustering as well as osteophyte formation along joint margins (Xu et al., 2003). In addition, apoptosis and poor self-renewability of chondrocytes play an important role in the initiation and development of cartilage degeneration (Correa & Lietman, 2017). Canonical β-catenin signaling has shown an unparalleled function in regulating cartilage development and maintenance, while its dysfunctions can cause severe cartilage defects (Usami, Gunawardena, Iwamoto, & Enomoto-Iwamoto, 2016; Yuan et al., 2016; Zhou, Wang, Hamilton, & Chen, 2017). Findings from our previous studies have demonstrated that activation of β-catenin causes various forms of arthritis, such as OA in knee joint and temporomandibular joint (TMJ), rheumatoid arthritis, and spondyloarthritis (Heiland et al., 2012; Hui et al., 2018; Xie, Zhou, Li, Hui, & Chen, 2016; Zhu et al., 2009). It is our goal to comprehensively understand the functions of β-catenin in the pathogenesis of arthritis. However, whether β-catenin plays a pivotal role in hip OA pathogenesis remains unclear.

Despite a similar pathological progression between hip OA and OA in other joints (knee joint, facet joint, and TMJ), the anatomical structure and risk factors differ across different joints (Hosseininia, Lindberg, & Dahlberg, 2013). For instance, meniscus injury, obesity, and muscle weakness are commonly regarded as risk factors for knee joint OA (Felson et al., 2000; Roos et al., 1998), whereas hip OA patients frequently undergo acetabular dysplasia and abnormal loading (Jacobsen & Sonne-Holm, 2005; Thorstensson, Petersson, Jacobsson, Boegård, & Roos, 2004). Therefore, it is still necessary to examine whether similar molecular mechanisms is shared among different joints.

In the present study, we analyzed OA samples from hip arthroplasty patients and found that the levels of β-catenin protein were highly upregulated in OA cartilage. Then, *β-catenin* conditional activation mice were generated by administration of tamoxifen into *β-cat(ex3)*^{Col2ER} mice at 2 weeks of age. Interestingly, *β-cat(ex3)*^{Col2ER} mice presented an OA-like phenotype in the hip joint at adult age, including articular cartilage degeneration, subchondral sclerosis and osteophyte formation. Activation of *β-catenin* could induce cartilage matrix degradation and chondrocyte apoptosis that possibly contributed to the OA-like defects observed in *β-cat(ex3)*^{Col2ER} mice.

2 | MATERIALS AND METHODS

2.1 | Animals

Col2-CreER^{T2} mice and *β-catenin(ex3)*$^{flox/flox}$ mice were generated in professor Chen's Lab (Rush University, Chicago, IL; Chen et al., 2007; Zhu, Chen, Lichtler, O'Keefe, & Chen, 2008). *β-catenin(ex3)*$^{flox/flox}$ mice bred with *Col2-CreER*^{T2} mice to generate *Col2-CreER*^{T2}*;β-catenin (ex3)*$^{flox/flox}$ [*β-cat(ex3)*^{Col2ER}] transgenic mice. Mice of 2-week old were administered with tamoxifen (Sigma, St. Louis, MO) for 5 consecutive days (ip, 1 mg/10 g body weight). Animal protocols were conducted in accordance with the guidelines published by Zhejiang Chinese Medical University (LZ12H27001). studies with human hip joint tissues were approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang Chinese Medical University (2018-KL-005).

2.2 | *Cre*-recombination efficiency

To evaluate *Co12-Cre* recombination efficiency in the femoral head, we generated *Col2-CreER*^{T2}*;ROSA*^{tdTomato} mice by breeding *Col2-CreER*^{T2} mice with *Rosa26-loxp-stop-loxp-tdTomato* [*ROSA*^{tdTomato}] mice. Femoral head samples from 1-month-old mice were harvested and processed for frozen sections. Ten-micrometer-thick sections were stained with 4′,6-diamidino-2-phenylindole and then analyzed using a fluorescence microscope (Zeiss AxioScope A1; Zeiss, Ltd., Oberkochen, Germany).

2.3 | Microcomputed tomography (μCT) analysis

Femoral head samples were performed with μCT analysis before histological processing. Briefly, samples were scanned from proximal to distal for 400 slices using a μCT (Skyscan 1176, Bruker, Kontich, Belgium) at a resolution of 10 μm. We then reconstructed three-dimensional structure of femoral head samples using NRecon Software (Bruker). Fifty slices of the subchondral bone of each sample were selected for morphometric analysis.

2.4 | Histology and histomorphometry

Femoral head tissues were processed for paraffin section as previously described (Wang et al., 2018). Three-micrometer-thick sections were stained with pathological staining for morphologic analysis. Furthermore, these histology data were analyzed and recorded by two researchers in accordance with the scoring system suggested by Osteoarthritis Research Society International (OARSI; Glasson, Chambers, Van Den Berg, & Little, 2010).

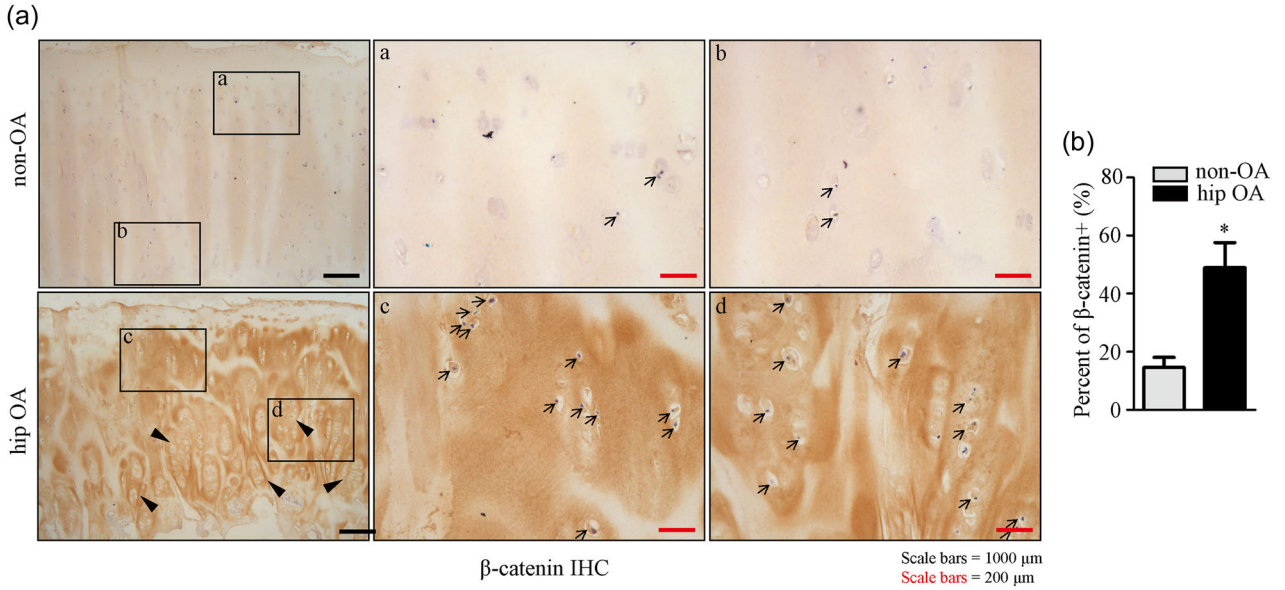

FIGURE 1 β-Catenin levels were increased in cartilage tissues from patients with hip OA. (a) Non-OA samples from trauma patients ($n = 10$) and OA cartilage from hip arthroplasty patients ($n = 15$) were harvested and processed for β-catenin immunostaining. Chondrocytes in hip OA cartilage are arranged in groups or clusters (black arrowheads). β-Catenin protein was highly expressed in articular cartilage from hip OA patients but was hardly expressed in non-OA samples (boxed areas a–d, black arrows). (b) Quantitative analysis of β-catenin proteins showed the same results. IHC: immunohistochemistry; OA: osteoarthritis [Color figure can be viewed at wileyonlinelibrary.com]

2.5 | Immunohistochemistry (IHC)

IHC assay was applied to detect the expressions of β-catenin, type II collagen (Col-II), matrix metalloproteinase 13 (MMP13), aggrecan neoepitope (ACAN) in articular cartilage. Sections were incubated in citrate buffer (0.01 M, pH 6.0, Cat. no. C1010; Solarbio, Beijing, China) at 60°C for 4 hr or Pepsinum (Cat. no. ZLI-9013; ZSGB Biotechnology, Beijing, China) at 37°C for 30 min as antigen retrieval. After that, sections were incubated with primary antibodies of Col-II (diluted 1:1,000, ab34712; Abcam, Cambridge, UK), MMP13 (diluted 1:200, ab39012; Abcam), aggrecan (diluted 1:200, ab76956; Abcam), ACAN (diluted 1:200, NB100-74350; Novus, Oakville, Canada) and β-catenin (diluted 1:500, ab32572; Abcam) overnight at 4°C. Second goat anti-mouse/rabbit antibody (diluted 1:1,000) was added for 20 min, and diaminobenzidine solution was used for detecting positive staining while hematoxylin for counterstaining. A total of four randomly selected fields from at least three different tissue sections were selected for quantification of positive staining. The mean optical density defining as the ratio of integrated optical density to the corresponding cavity area and the rate of the positive cells were evaluated using Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD).

2.6 | Cell apoptosis assays

Chondrocyte apoptosis was evaluated by TUNEL staining. The protocol was performed according to the instructions of the Staining Kit (Roche, IN).

2.7 | Statistical analysis

Statistical significance was determined by unpaired Student's t test. $^*p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Increased β-catenin expression in human hip OA cartilage samples

In this study, OA cartilage samples from hip OA patients undergoing arthroplasty surgery ($n = 15$) and non-OA cartilage tissues from trauma patients ($n = 10$) were analyzed immunohistochemically for β-catenin expression. The results revealed that articular chondrocytes in hip OA samples were aggregated to form clusters (Figure 1a, black arrowheads) and this phenomenon is a characteristic feature for osteoarthritic cartilage (Kim & Blanco, 2007). β-Catenin protein was highly expressed in the osteoarthritic cartilage (Figure 1a, black arrows and Figure 1b), indicating the involvement of β-catenin signaling in hip OA pathogenesis.

3.2 | High *Cre*-recombination efficiency and *β-catenin* activation in *β-cat(ex3)*^{Col2ER} mice

We then analyzed *β-cat(ex3)*^{Col2ER} mice. For evaluation of target efficiency in hip joint in this transgenic mice, *Col2-CreER*^{T2};*ROSA*^{tdTomato} mice were administrated with tamoxifen at 2 weeks of age and sacrificed at 1 month of age (ip, 1 mg/10 g body weight, 5 consecutive days). Analysis of histologic sections using fluorescence microscopy and hematoxylin and eosin (H&E) staining showed that *Cre*-recombination

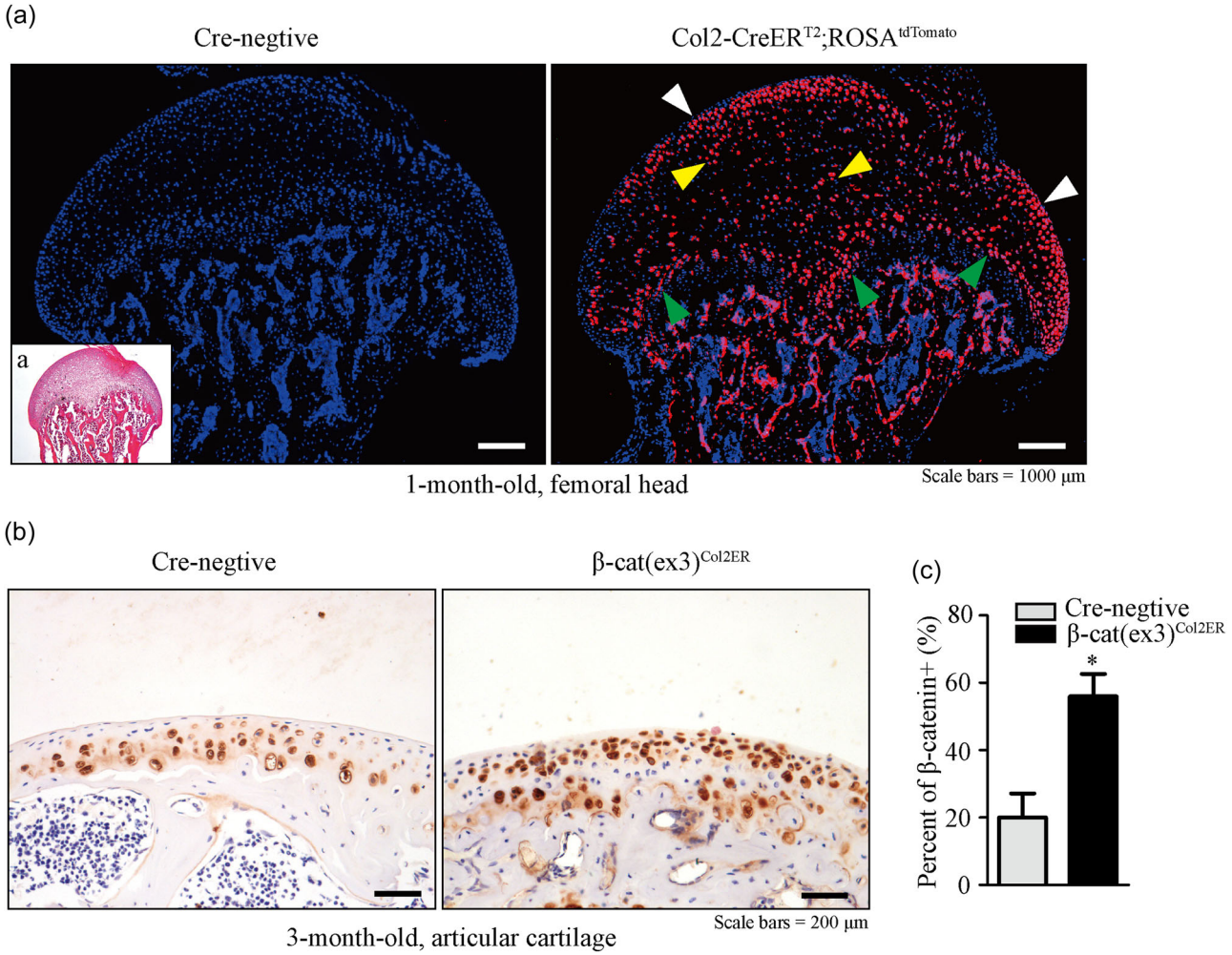

FIGURE 2 *Col2-CreER* directed *Cre*-recombination in femoral head chondrocytes. (a) *Col2-CreER^{T2};ROSA^{tdTomato}* mice were generated by breeding *Col2-CreER^{T2}* transgenic mice with *ROSA^{tdTomato}* mice. Femoral head samples were harvested from 1-month-old mice after they were injected with tamoxifen at the age of 2-weeks old for 5 consecutive days. High *Cre*-recombination efficiency was observed in the femoral head chondrocytes in *Col2-CreER^{T2};ROSA^{tdTomato}* mice, including superficial, middle, and deep layers of femoral head chondrocytes (white, yellow, and green arrowheads). Red: tdTomato^{+} cells; blue: nuclear staining by DAPI; a: H&E staining of an adjacent section. (b,c) IHC staining showed that β-catenin protein was significantly increased in femoral head chondrocytes in 3-month-old *β-cat(ex3)^{Col2ER}* mice. [Color figure can be viewed at wileyonlinelibrary.com]

expressed efficiently in growth plate chondrocytes (Figure 2a, green arrowheads) and articular chondrocytes (Figure 2a, white arrowheads), partially in middle layers of hypertrophic chondrocytes (Figure 2a, yellow arrowheads). Results of IHC assay showed increased β-catenin expression in femoral head chondrocytes in *β-cat(ex3)^{Col2ER}* mice (Figure 2b,c) suggesting that this *β-catenin* conditional activation mouse model were successfully established.

3.3 | Conditional activation of *β-catenin* caused OA-like defects in hip joint

In this study, we then throughly analyzed the morphologic changes of femoral head tissues from *β-cat(ex3)^{Col2ER}* mice. The chondrocytes in 2-month-old Cre-negative mice were arranged orderly in three layers: round and small articular chondrocytes in the superficial layer; hypertrophic and bigger second ossification center chondrocytes in the middle layer; quadrate and columnar growth plate chondrocytes in the deeper layer (Figure 3a). Chondrocytes in second ossification center and growth plate were gradually replaced by bone and bone marrow tissues with the progression of endochondral ossification in femoral head (Figure 3b,c). However, compared to Cre-negative mice, *β-cat(ex3)^{Col2ER}* mice showed a delayed endochondral bone development that abundant immature chondrocytes persistently existed in the second ossification center and growth plate (Figure 3b,c, green arrowheads). In addition, *β-cat(ex3)^{Col2ER}* mice at 2 months of age already exhibited some early signs of hip OA, such as vast loss of cartilage collagen stained with less Alcian blue and decreased articular chondrocyte numbers (Figure 3a, boxed areas a,b). At 3 and 6 months of age, besides visibly reduced Alcian blue staining (Figure 3b,c, boxed areas c–f), we also observed rough articular surface (Figure 3b,c, black arrowheads) and subchondral

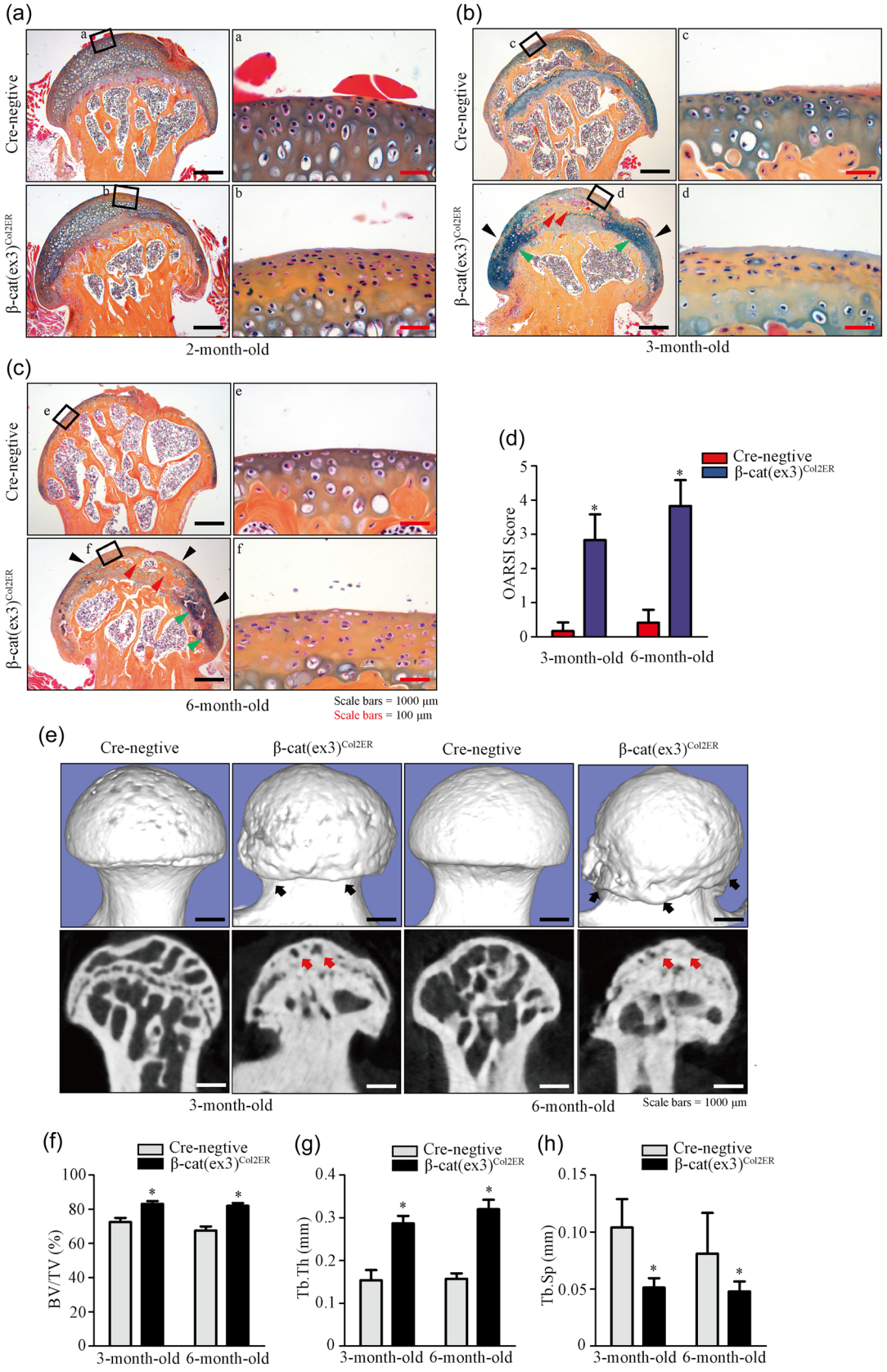

FIGURE 3 Continued.

sclerosis (Figure 3b,c, red arrowheads) in *β-cat(ex3)*^{Col2ER} mice. In contrast, superficial articular cartilage was well preserved in Cre-negative mice until the age of 6 months, which stained positive with Alcian blue (Figure 3a–c, boxed areas a–f). Furthermore, these morphologic changes were estimated using OARSI scoring system and the results showed higher scores for cartilage degeneration in *β-cat(ex3)*^{Col2ER} mice (Figure 3d). Also, *β-cat(ex3)*^{Col2ER} mice presented typical μCT features of OA including remarkable osteophyte formation along margins of the femoral head (Figure 3e, black arrows) and increased bone mass of subchondral bone (Figure 3e, red arrows). The sclerotic changes of subchondral bone were further confirmed by the μCT parameters (Figure 3f–h).

3.4 | Alterations of matrix degradation enzymes in *β-cat(ex3)*^{Col2ER} mice

Then, we explored the underlying molecules involved in the hip OA-like phenotype. MMP13 can degrade cartilage collagens including Col-I and Col-II (Tuckermann, Pittois, Partridge, Merregaert, & Angel, 2000). ACAN, a degradation fragment of aggrecan, specifically reflect the activity of Adamts5 during OA development (Olszewski, McDonnell, Stevens, Visco, & Moore, 1996). To test the changes of these collagenase and aggrecanases, we performed IHC assays on femoral head tissues from *β-cat(ex3)*^{Col2ER} mice of 3-month old. The results showed that MMP13 and ACAN expressions were highly increased in articular cartilage in *β-cat(ex3)*^{Col2ER} mice (Figure 4b,d,f,h). In addition, we revealed significant downregulation of Col-II and aggrecan proteins in *β-cat(ex3)*^{Col2ER} mice (Figure 4a,c,e,g), which was consistent with the results of Alcian blue staining. These findings suggested that *β-catenin* activation induced cartilage degeneration is very likely due to the alteration of collagenase and aggrecanases activities.

3.5 | Changes of chondrocyte apoptosis in *β-cat(ex3)*^{Col2ER} mice

Chondrocytes solely reside in articular cartilage and their apoptosis may accelerate the progression of OA (Hwang & Kim, 2015). TUNEL staining was applied for evaluation of chondrocyte apoptosis and the results showed that *β-cat(ex3)*^{Col2ER} mice had a higher level of apoptotic chondrocytes in articular cartilage (Figure 4i,j) indicating that the hip OA-like phenotype induced by *β-catenin* activation was partly caused by the changes of chondrocyte apoptosis.

4 | DISCUSSION

Ex vivo studies with human arthritis samples and studies with mutant rodent have suggested that β-catenin signaling plays a critical role in the development of various forms of arthritis. However, its role in hip OA pathogenesis remains unclear. Here, we showed a significant increase of β-catenin expression in OA cartilage from hip replacement patients. Furthermore, we analyzed *β-cat(ex3)*^{Col2ER} mice and revealed that activation of *β-catenin* in femoral head chondrocytes could result in degenerative defects in articular cartilage resembling a hip OA phenotype in humans. In this mouse model, we also found significant increased matrix degradation enzymes and articular chondrocyte apoptosis that may contribute to this hip OA-like phenotype.

In this study, we comprehensively studied morphological changes of the hip joint in *β-cat(ex3)*^{Col2ER} mice. In detail, 2-month-old *β-cat(ex3)*^{Col2ER} mice already presented significant degradation of articular cartilage in the developing femoral head. With the growth of femoral head, more characteristic changes of hip OA including subchondral sclerosis and osteophyte formation appeared in this model mouse. The above findings suggested a critical role of β-catenin in hip OA pathogenesis. In humans, hip joint continues to develop during postnatal life and gradually matures at childhood. For example, the triradiate cartilage mineralized to form acetabulum with age, do not fully ossify until ages 15–18 (Ponseti, 1978) and the epiphysis cartilage of femoral head is completely ossified at ages 16.8 (Parvaresh, Upasani, Bomar, & Pennock, 2018). Mice undergo a maturation process of hip joint similar to human beings (Ford, Nowlan, Thomopoulos, & Killian, 2017). In this study, we found that growth plate chondrocytes and chondrocytes in the secondary ossification center of the femoral head were gradually replaced by bone and bone marrow tissues during the endochondral ossification process. However, compared with Cre-negative mice, *β-cat(ex3)*^{Col2ER} mice showed a delayed development and maturation of these chondrocytes, indicating that an appropriate level of β-catenin is required for the maintenance of cartilage homeostasis.

Furthermore, we determined the underlying molecular mechanisms causing this hip OA-like changes presented in *β-cat(ex3)*^{Col2ER} mice. MMP13 and Adamts5, the primary enzymes, can degrade the collagen network and proteoglycan that are the major components of the cartilage matrix (Glasson et al., 2005; Neuhold et al., 2001).

FIGURE 3 *β-catenin* conditional activation mice show an osteoarthritis (OA)-like phenotype in hip joint. (a–c) Femoral head samples were dissected from 2-, 3- and 6-month-old mice and Alcian blue/hemotoxylin, and Orange G staining were performed. *β-cat(ex3)*^{Col2ER} mice displayed typical signs of hip OA-like phenotype, including extensive degeneration of articular cartilage (a–f, boxed areas), rough articular surface (black arrowheads) and subchondral sclerosis (red arrowheads). In addition, *β-cat(ex3)*^{Col2ER} mice also showed a delayed formation of the chondrocytes in second ossification center and growth plate (green arrowheads). (d) OARSI scoring revealed severe cartilage destruction in 3- and 6-month-old *β-cat(ex3)*^{Col2ER} mice. (e) μCT images showed early osteophyte formation (black arrows) and increased subchondral bone mass (red arrows) in 3- and 6-month-old *β-cat(ex3)*^{Col2ER} mice. (f–h) Quantitative analysis showed microstructural parameters, such as bone volume (f) and trabecular thickness (g) were decreased in *β-cat(ex3)*^{Col2ER} mice. Conversely, trabecular separation (h) was significantly increased. BV: bone volume; μCT: microcomputed tomography; OARSI: Osteoarthritis Research Society International; Tb.Sp: trabecular separation; Tb.Th: trabecular thickness; TV: total volume [Color figure can be viewed at wileyonlinelibrary.com]

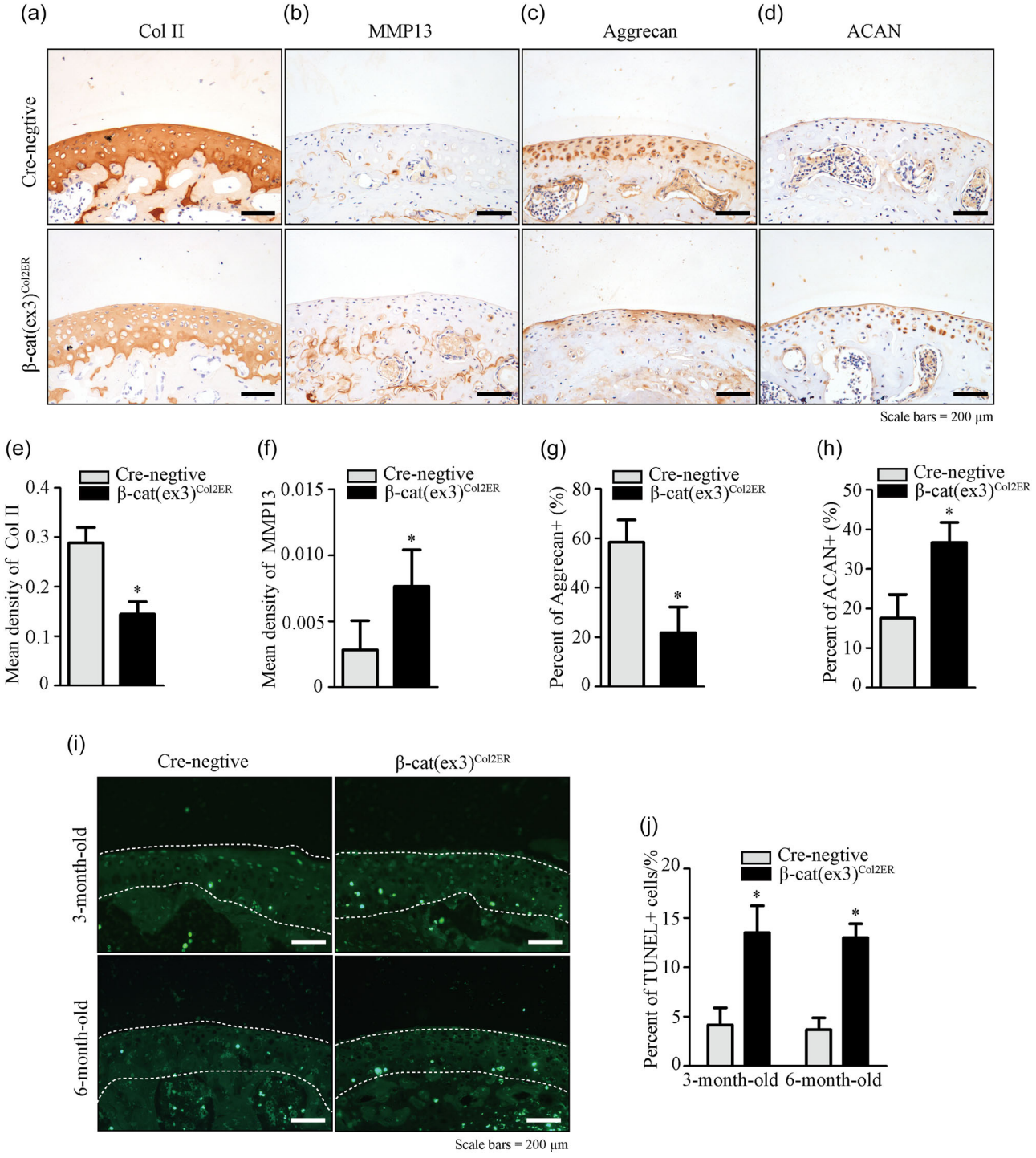

FIGURE 4 Alterations of matrix protein expression and cell apoptosis in *β-cat(ex3)*^{Col2ER} mice. (a–d) The immunostaining for Col-II, MMP13, aggrecan and aggrecan neoepitope (ACAN) were performed on femoral head tissues from 3-month-old *β-cat(ex3)*^{Col2ER} mice and Cre-negative littermates. Representative images showed decreased expression of Col-II and Aggrecan proteins but increased expression of MMP13 and ACAN proteins in *β-cat(ex3)*^{Col2ER} mice. (e–h) Quantitative analysis of these proteins showed the same results. (i,j) Results of TUNEL staining demonstrated increased numbers of apoptotic chondrocytes in femoral head cartilage of *β-cat(ex3)*^{Col2ER} mice. Bright green spots: TUNEL positive cells. Col-II: collagen type 2; MMP13: matrix metalloproteinase 13; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling [Color figure can be viewed at wileyonlinelibrary.com]

β-catenin is thought to be an upstream regulator of MMP13 and Adamts5 during this matrix-degrading process (Hui et al., 2018). Results of IHC analysis have revealed that MMP13 and ACAN expression were highly increased in articular cartilage as *β-catenin* was conditionally activated in the femoral head chondrocytes. ACAN is a degradation fragment of aggrecan that can specifically reflect Adamts5 activities during OA progression (Olszewski et al., 1996). In addition, the decreased expression of Col-II and aggrecan proteins further confirmed MMP13- and Adamts5-induced cartilage degradation in $\beta\text{-}cat(ex3)^{Col2ER}$ mice. At the cellular level, the numbers of apoptotic articular chondrocytes were highly increased in $\beta\text{-}cat(ex3)^{Col2ER}$ mice. Several lines of evidence have revealed that there is an inseparable correlation between chondrocyte apoptosis and cartilage matrix damage (Hwang & Kim, 2015). Chondrocytes, the solely living cells in cartilage, secrete and synthesize the extracellular matrix. In return, the cartilage matrix not only provides structural and biochemical support but also transports nutrients and oxygen for the resident chondrocytes. However, it is still unclear whether chondrocyte apoptosis is a trigger for cartilage degradation or a by-product of cartilage damage. Possibly, a vicious cycle forms between chondrocyte apoptosis and cartilage matrix loss, with the progression of one aggravating the other, eventually resulting in the hip OA-like defects presented in $\beta\text{-}cat(ex3)^{Col2ER}$ mice.

In summary, our data demonstrated the critical role of β-catenin in the regulation of chondrocyte development, maturation, and apoptosis. Dysfunction of β-catenin in the femoral head chondrocytes could lead to cartilage degeneration resembling a human hip OA phenotype.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

AUTHOR CONTRIBUTIONS

Study conception and design: D. C., P. T., and H. J. Acquisition of data: C. X., P. W., L. F., Q. G., Z. Z., R. D., P. Z., Z. S., and R. X. Analysis and interpretation of data: L. Z., C. L., J. Y., L. X., and J. S. Drafting the article or revising it critically for important intellectual content: C. X., D. C., and H. J. Final approval of the version of the article to be published: D. C. and H. J.

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