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MMP-10 Deficiency Effects Differentiation and Death of Chondrocytes Associated with Endochondral Osteogenesis in an Endemic Osteoarthritis

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Abstract

Objective. The objective of this study was to determine the matrix metalloproteinase-10 (MMP-10) expression pattern and to assess how it contributes to endochondral osteogenesis in Kashin-Beck disease (KBD). *Design.* The cartilages of KBD patients, Sprague-Dawley rats fed with selenium (Se)-deficient diet and/or T-2 toxin, and ATDC5 cells were used in this study. ATDC5 cells were induced into hypertrophic chondrocytes using a 1% insulin-transferrin-selenium (ITS) culture medium for 21 days. The expressions of MMP-10 in the cartilages were visualized by immunohistochemistry. The messenger RNA (mRNA) and protein expression levels were determined by real-time polymerase chain reaction (RT-PCR) and Western blotting. MMP-10 short hairpin RNA (shRNA) was transfected into hypertrophic chondrocytes to knock down the gene expression of MMP-10. Meanwhile, the cell death of MMP-10-knockdown chondrocyte was detected using flow cytometry. *Results.* The expression of MMP-10 was decreased in the growth plates of children with KBD. A decreased expression of MMP-10 also was observed in the growth plates of rats fed with an Se-deficient diet and/or T-2 toxin exposure. The mRNA and protein expression levels of MMP-10 increased during the chondrogenic differentiation of ATDC5 cells. MMP-10 knockdown in hypertrophic chondrocytes significantly decreased the gene and protein expression of collagen type II (Col II), Col X, Runx2, and MMP-13. Besides, the percentage of cell apoptosis was significantly increased after MMP-10 knockdown in hypertrophic chondrocytes. *Conclusion.* MMP-10 deficiency disrupts chondrocyte terminal differentiation and induces the chondrocyte's death, which impairs endochondral osteogenesis in the pathogenesis of KBD.

Keywords

matrix metalloproteinase-10, Kashin-Beck disease, chondrocyte differentiation, cell apoptosis

Introduction

Kashin-Beck disease (KBD) is an endemic degenerative osteoarthropathy in humans. The main lesions of KBD involve the degradation of the growth plate and articular cartilage, further causing joint deformity, bone shortening, and even growth retardation in children.[1] The typical pathological feature of KBD is the disorder of endochondral ossification, necrosis and excessive death of chondrocytes, abnormal differentiation of chondrocytes, and matrix degradation.[2] Among these, abnormal differentiation of chondrocytes and matrix degradation are the basis of the cartilage injury in KBD. The chondrocyte differentiation process continuously occurs in the postnatal growth plate that drives rapid skeletal growth during the development of the organism.[3,4] The abnormal chondrocyte differentiation can affect joint and bone development and other physiological processes, resulting in the deformity of joint and bone and KBD occurrence. Therefore, finding out the molecular

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mechanisms that drive and regulate the abnormal differentiation of chondrocytes is a prerequisite for understanding the pathogenesis of KBD.

The abnormal differentiation of chondrocytes in KBD presents dedifferentiation and terminal differentiation disturbance of chondrocytes, which is characterized by the reduced synthesis of type II collagen and the increased expression of type I, III, and X collagen.[5,6] The differentiation programs of chondrocytes can be regulated by many factors, in which matrix metalloproteinases (MMPs) are particularly an important class.[7-9] MMP family includes more than 25 proteolytic enzymes in mammals, and it is classified into several subclasses, such as collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), and stromelysins (MMP-3, MMP-10, and MMP-11) according to their enzyme-substrate specificity.[10] The roles of most MMP members in maintaining and improving the physiological function of chondrocytes have been revealed. For example, MMP-14 is implicated in multiple steps of the differentiation process, acting to regulate homeostasis of the osteogenic differentiation.[11] MMP-14 distributes in all zones of the growth plate and has an essential effect on the differentiation of human mesenchymal stem cells (MSCs) to osteoblasts.[12] MMP-13 is required for chondrocyte differentiation, and MMP-13 loss leads to a breakdown in primary human articular chondrocyte differentiation by altering the expression of multiple regulatory factors.[13,14] MMP-10, an MMP with high homology to MMP-3, was strongly expressed in hypertrophic chondrocytes that remodel the extracellular matrix (ECM) and participate in physiological processes such as bone growth and wound healing.[15,16] Besides, several studies indicate that the MMP-10 expression is upregulated in arthritis and osteoarthritis, and this upregulation represents an important mechanism contributing to cartilage degradation.[17,18] However, the expression pattern and regulatory role of MMP-10 in cartilage or chondrocytes of KBD, especially its role in chondrogenic terminal differentiation, have not yet been extensively studied.

Trichothecene, T-2 toxin, and selenium (Se) deficiency in the environment were proposed as the most important risk factors for KBD.[19,20] In previous studies, rats administered T-2 toxin and fed with an Se-deficiency diet successfully presented similar pathological features with KBD, such as chondrocyte death in the cartilage deep zone, chondrocyte-focus necroses, and decreased expression of Col2α1, glutathione peroxidase (Gpx) 1, and Gpx4 in cartilage tissues.[21-23] These findings supported the notion that T-2 toxin contamination and Se deficiency are the main etiologies of KBD, and this could be an effective animal model to explore the mechanism of KBD cartilage damage.

We sought to explore MMP-10 expression in cartilages from both KBD patients and rat models, and then identify its role in regulating the process of chondrocyte differentiation by developing MMP-10 gene knockdown chondrocytes. In this study, we hypothesized that MMP-10 may affect and regulate chondrocyte differentiation and cell death, which ultimately caused the pathological damage of cartilage in KBD.

Methods

Patient Tissue Samples

Child KBD cartilage samples were obtained from finger joints of 5 KBD patients who lived in diseased areas and died from accidents or other diseases such as bacillary dysentery and acute diarrhea. The normal cartilage samples were from 5 normal children from non-KBD areas who had died from clinical problems such as traffic accidents or amputations that resulted from trauma, with no history of osteoarthritis or other inflammatory joint diseases. The age of KBD patients was 3 to 7 years, while the age of normal children was 3 to 12 years. KBD patients were all diagnosed according to the national diagnosis criteria for KBD in China (diagnostic code GB16395-1996) with documented x-ray diagnosis. The cartilage samples were collected after obtaining the patient's or guardian's informed consent, and the ethical approval for the acquisition of these patient samples was provided by the Human and Ethical Committee for Medical Research at School of Medicine in Xi'an Jiaotong University (NO.3063058).

Experimental Animal Samples

All male Sprague-Dawley (SD) rats were 1 month old and weighed between 60 and 80 g to mimic the age of 5- to 13-year-old children who are most susceptible to KBD. In all, 24 SD rats were randomly classified into the control group and low-Se group, and fed with a normal diet and low-Se diet for 4 weeks, respectively. After the low-Se rat model was confirmed by blood Se levels and serum glutathione peroxidase activity levels, the rats were divided into 4 groups that were treated with a normal diet, Se-deficient diet, normal diet plus T-2 toxin, and Se-deficient diet plus T-2 toxin for 4 weeks, respectively. The concentration of T-2 toxin was 200 ng/g body weight (BW)/day (d) and it was administered by the intragastric route. T-2 toxin was provided by Professors Yang Jinsheng and Peng Shuangqin (Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences).

Immunohistochemical Localization of MMP-10

Paraffin-embedded cartilage sections were first deparaffinized in xylene and rehydrated in graded ethanol series. After antigen unmasking and blocking, the sections were incubated overnight at 4 °C with MMP-10 primary

antibodies (Abcam, Cambridge, UK) and visualized using alkaline phosphatase–labeled secondary antibodies. Nuclei were counterstained with hematoxylin. The stained sections were observed by a microscope (Olympus Corporation, Tokyo, Japan) and photographed by a digital camera (Canon Corporation, Tokyo, Japan). The positive and negative stained cells in the whole of the three articular cartilage zones from each image were labeled and counted using Image J software (NIH). Six randomly chosen fields in each zone were counted at 400x magnification. The positive rate of the cells was then calculated.

Cell Culture and Chondrogenic Differentiation Induction

The chondroblast cell line ATDC5 was obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). The ATDC5 cells were plated in 12-well plates with a density of 1×10^4 cells per well and cultured with Dulbecco's modified Eagle's medium/F-12 medium (DMEM/F12 1:1; Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (Gibco, Australia), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 at 37 °C. After attachment for 24 hours, ATDC5 cells were induced by the medium containing 1% insulin-transferrin-selenium (ITS; BD Biosciences, Franklin Lakes, NJ). The medium was changed every other day for 7, 14, or 21 days, respectively.

Transfection of MMP-10 Gene for ATDC5 Chondrocytes

MMP-10 in ATDC5 chondrocytes was knocked down by transfecting MMP-10 short hairpin RNA (shRNA) using a kit (Santa Cruz, Dallas, TX). The MMP-10 small interfering RNA (siRNA) sequences were 5′-CAGUAGUCCUGUUGUCAAAtt-3′ (sense) and 5′-UUUGACAACAGGACUACUGtt-3′ (antisense). MMP-10 shRNA plasmids were transfected into ATDC5 chondrocytes according to the manufacturer's instruction on the 14th day and 21st day after ITS induction, respectively. Meanwhile, shRNA plasmid-A was used as a negative control (shN, sc-108060; Santa Cruz Biotechnology, Dallas, TX). Then, for cell culture 2 days later, real-time polymerase chain reaction (RT-PCR) and Western blot were performed to detect the expression of chondrocyte differentiation–related genes.

RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) following the manufacturer's instructions. First-strand complementary DNAs (cDNAs) were synthesized using the RevertAidTM first-strand synthesis kit (Fermentas, Burlington, ON, Canada) and then used as templates for RT-PCR. A threshold cycle for each PCR amplification was subjected to 40 cycles of denaturation at 95 °C for 5 minutes, annealing at 95 °C for 15 seconds, elongation at 60 °C for 30 seconds, and finally followed by a melting curve analysis. Primers for the following genes were used: MMP-10 (forward: 5′-GCAGCCCATGAACTTGGCCACT-3′; reverse: 5′-AGGGACCGGCTCCATACAGGG-3′); MMP-13 (forward: 5′-GATGACCTGTCTGAGGAAGACC-3′; reverse: 5′-GCATTTCTCGGAGCCTGTCAAC-3′); Col X (forward: 5′-CTCCTACCACGTGCATGTGAA-3′; reverse: 5′-ACTCCCTGAAGCCTGATCCA-3′); Col II (forward: 5′-CCAGCTGACCTCGCCACTGA-3′; reverse: 5′-GGGTCCAGGCGCACCCTTTT-3′); Runx2 (forward: 5′-GGTTGTAGCCCTCGGAGAGG-3′; reverse: 5′-GCCATGACGGTAACCACAGTC-3′); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5′-TGAGGCCGGTGCTGAGTATGTCGT-3′; reverse: 5′-GGTCCTTTTCACCAGCAAGC-3′). GAPDH was used to normalize expression levels. The results were analyzed using the comparative ΔΔCT method ($2^{(-\Delta\Delta CT)}$) for the relative quantification of gene expression.

Western Blot

The cells were lysed in radio immunoprecipitation assay (RIPA) buffer (Heart, Xi'an, China) with protease inhibitor and phosphatase inhibitor. Total protein concentrations of cell lysates were determined using the bicinchoninic acid (BCA) protein assay (Heart). A total of 30 μg protein was separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Then membranes were blocked at room temperature for 2 hours in Tris-buffered saline with 0.5% Tween20 and 10% nonfat dry milk and incubated overnight at 4 °C with primary antibodies, anti-mouse MMP-10 (1:800; Abcam), Col X (1:1,000; Abcam), Col2a1 (1:1,000; Proteintech, Wuhan, China), Runx2 (1:1,000; Abcam), MMP-13 (1:1,000; Abcam), and GAPDH (1:2,000; BOSTER, Wuhan, China) antibodies separately. After washing with TBS-T, the membranes were incubated for 1 hour with the appropriate secondary antibody. Immunoreactivity was visualized using the ECL system (Amersham Biosciences, Piscataway, NJ). Signals were quantified using Image J software.

Cell Death and Apoptosis Assay

Cell apoptosis was detected by Annexin-V/propidium iodine (PI) staining kit (Becton Dickinson, San Jose, CA) following the manufacturer's instruction. The main steps are summarized as follows: wash cells twice with cold phosphate-buffered saline (PBS) and then resuspend cells in 1x binding buffer at a concentration of 1×10^6 cells/ml,

then transfer 100 µl of the cell suspension (1×10^5 cells) to a 5-ml culture tube with 5 µl of FITC Annexin V and 5 µl of PI, gently vortex the cell suspension and incubate at room temperature for 15 minute in the dark, and then dilute it with 400 µl of 1x binding buffer. Samples were analyzed using the flow cytometer (EMD Millipore, Darmstadt, Germany). The percentages of Annexin-V-positive and PI-positive were evaluated based on quadrants determined from single-stained and unstained control samples.

Statistical Analysis

The statistical significance of the results was determined by ANOVA, using SPSS 23.0 software. The data were presented as means ± standard deviation (SD). Statistical analysis was performed using 1-way ANOVA, and the mean of different groups was compared, followed by LSD-*t* or SNK-*q* for *post hoc* comparisons. *P* values less than 0.05 were considered statistically significant.

Ethics and Patient Consent

This study was approved by the Human and Ethical Committee for Medical Research of Health and Science Center, Xi'an Jiaotong University, and performed according to the principles of the Declaration of Helsinki as revised in 1983. The use of animals in this study was abided by National Institutes of Health publication 85-23 "Guide for Care and Use of Laboratory Animals" (National Research Council, 1996). All donor samples were obtained with written informed consent.

Results

Histomorphological Location of MMP-10 in Growth Plates from Patients with KBD

The H&E staining images of growth plates from children with KBD and the normal control group are shown in **Fig. 1A**. Compared with the controls, the growth plate of KBD manifested cell necrosis characterizing chondrocyte disappearance and red shadow of cell outlines without cytoplasm in the deep zone, and then presenting red-stained unstructured areas. And the focal occurrence of chondral necrosis was mainly in the deep zone close to the bone edge of the articular cartilage from KBD patients, and the chondrocyte clusters occurred in and around the necrotic foci.

The histomorphometry of MMP-10 distribution showed that there were significant differences in MMP-10 distribution between KBD and control samples (**Fig. 1B**). A large number of MMP-10-positive chondrocytes with cytoplasmic red staining were found in the whole layer of growth plate cartilage in the normal group. However, the number of MMP-10-positive chondrocytes was remarkably decreased in the growth plate cartilage of children with KBD (**Table 1**). The percentages of MMP-10-positive chondrocytes in the resting zone, proliferative zone, and upper hypertrophic zone (0.00% ± 0.00%, 13.42% ± 3.39%, 4.28% ± 2.94%) in the KBD group were significantly lower than those in the control group (11.47% ± 0.00%, 27.69% ± 4.72%, 96.34% ± 5.18%). Also, MMP-10-positive staining chondrocytes were barely observed in the lower hypertrophic zone (0.00% ± 0.00%) of the growth plate of samples from KBD children than that in the control group (48.26% ± 6.31%).

The Expression of MMP-10 in the Growth Plate from Se-Deficient and T-2 Toxin–Induced KBD Rat Model

To reveal further insight into the expression pattern of MMP-10 in KBD, we examined the expression of MMP-10 in the growth plate from the Se-deficient and T-2 toxin–induced rat model using the immunohistochemical method. In the control group, the chondrocytes in the growth plate were well organized, and MMP-10 positively stained chondrocytes distributed in all the zones as in human tissue, especially in the proliferation zone and the hypertrophic zone. In the Se-deficient diet group, T-2 toxin group, and Se-deficient diet + T-2 toxin group, the chondrocytes of the growth plate were arranged loosely and disorderly and showed obvious atrophy phenomenon. The hypertrophic layer of the growth plate in the Se-deficient diet + T-2 toxin group showed vacuolar changes. The expression of MMP-10 in the growth plate was significantly decreased in the Se-deficient diet group, T-2 toxin group, and Se-deficient diet + T-2 toxin group compared with the control group (**Fig. 2**). The percentages of positive staining for MMP-10 in the growth plate in the 3 groups were 8.67% ± 5.38%, 6.79% ± 2.37%, and 3.79% ± 3.21%, respectively, which are significantly lower than those in the control group (19.35% ± 4.71%; Table 2).

Expression of Chondrocyte Marker Genes and MMP-10 during Chondrogenic Differentiation of ATDC5 Cells

ATDC5 cell is an ideal model *in vitro* analogous to endochondral ossification *in vivo*. Images taken through optical microscopes showed that ATDC5 cells undergo the multistep processes of chondrocyte differentiation during a 21-day culture period. The ATDC5 cells began to proliferate rapidly and fused into dense monolayer cells after being induced by ITS on day 7. On day 14, the chondrocyte proliferation was significantly increased and started to form chondrocyte clusters. Furthermore, chondrocytes showed apparent clustered proliferation on day 21 that gradually formed cluster-like growth areas and multi-layer edgy growth areas (**Fig. 3A**, arrow).

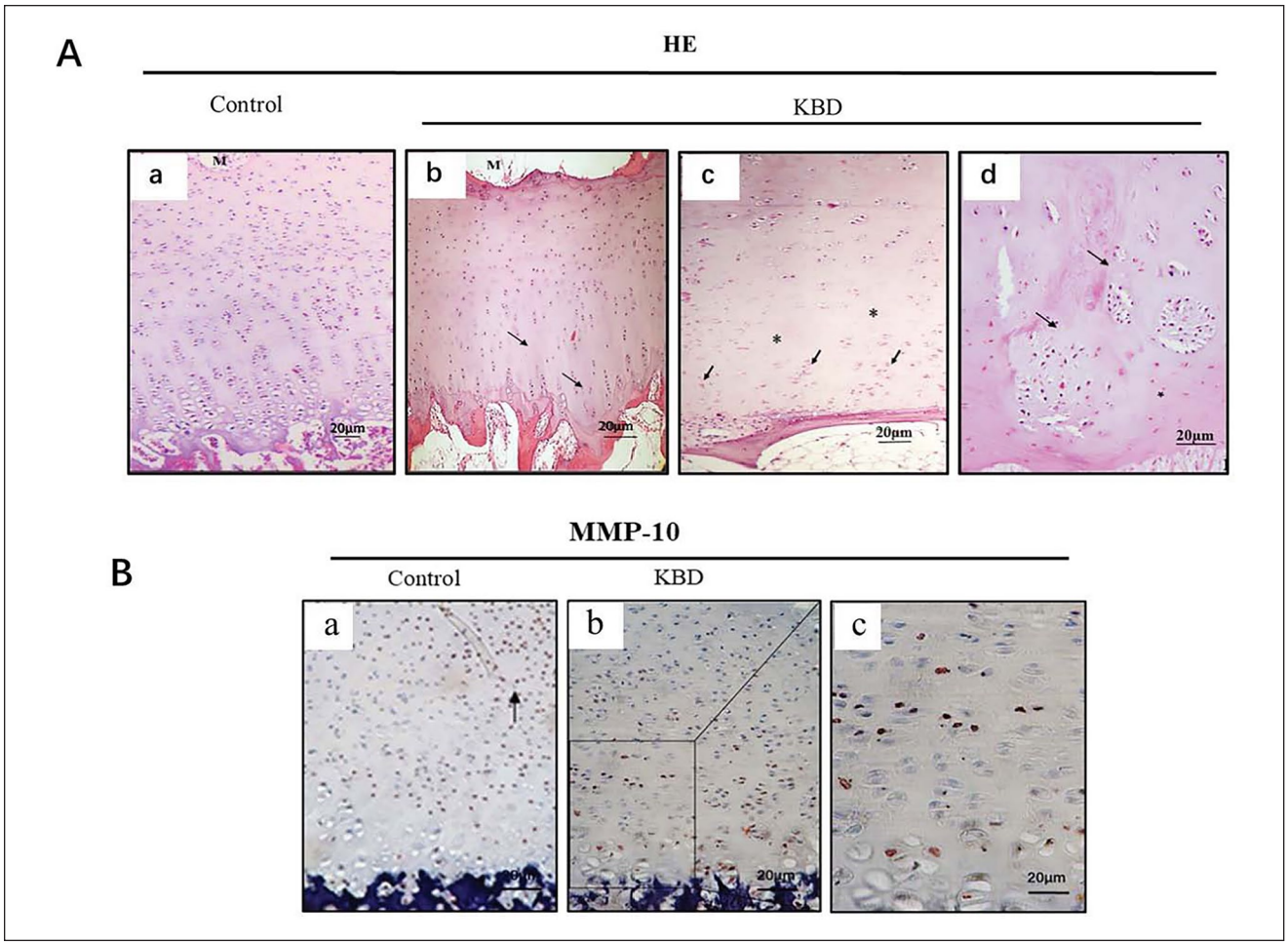

Figure 1. H&E staining and MMP-10 immunohistochemical staining of growth plates of KBD and control children. (**A**) H&E staining of growth plate cartilage. (a) The cartilage is from control child, from a non-KBD area. The cartilage shows no change in deep zone. (b, c, and d) The cartilage is from KBD donor with clinical manifestations of KBD. In the KBD group, the deep area of cartilage showed chondrocyte necrosis and residual red shadow of cell outlines, even unstructured areas (black arrow). M, secondary ossification center. (**B**) Immunohistochemical localization of MMP-10 in the growth plate cartilages from control and KBD child. Positive staining of chondrocytes is in brown, indicated with arrows ($n = 5$). MMP-10 = matrix metalloproteinase-10; KBD = Kashin-Beck disease; H&E = hematoxylin and eosin.

Table 1. The Number of MMP-10-Positive Chondrocytes in KBD and Control Groups $(\bar{x} \pm s)\%$.

Groups	Resting Zone	Proliferative Zone	Upper Hypertrophic Zone	Lower Hypertrophic Zone
Control	11.47 ± 0.00	27.69 ± 4.72	96.34 ± 5.18	48.26 ± 6.31
KBD	0.00 ± 0.00[a]	13.42 ± 3.39[a]	4.28 ± 2.94[a]	0.00 ± 0.00[a]

MMP-10 = matrix metalloproteinase-10; KBD = Kashin-Beck disease.
[a]KBD group compared with the control group in each zone, $P < 0.05$, $n = 5$.

Furthermore, the expression patterns of chondrocyte marker genes show that type II collagen (Col II) and type X collagen (Col X) are indicative of the chondrogenic differentiation of ATDC5 cells after ITS induction (**Fig. 3B**). The expression of Col II increased first and then decreased—it increased to 1.72-fold on day 7 and 10.55-fold on day 14 while decreasing to 1.39-fold on day 21. The Col X expression level increased continuously, and it reached 1.75-fold, 3.08-fold, and 8.21-fold on day 7, day 14, and day 21 after induced differentiation, respectively. Based on this, we concluded that the ATDC5 cell lines can undergo hypertrophy over 21 days and can be used as a hypertrophic differentiation

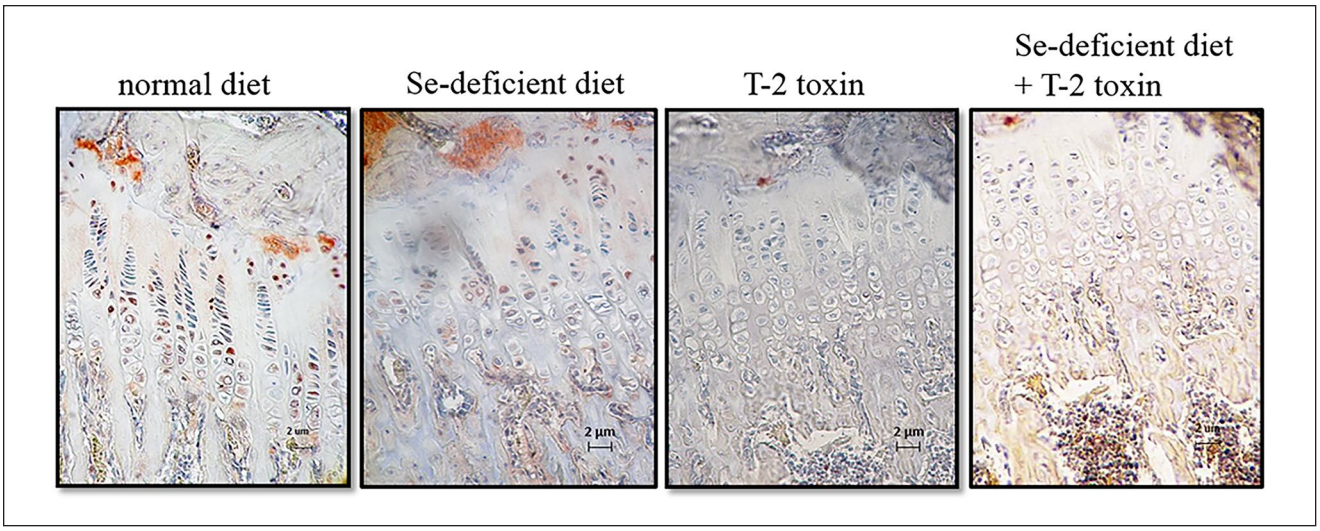

Figure 2. Immunostaining of matrix metalloproteinase-10 expression in the growth plate cartilage from rat model. Positive staining of matrix metalloproteinase-10 appears in orange color in the cytoplasm and the pericellular matrix ($n = 6$). Scale bar: 2 μm.

Table 2. The Number of MMP-10-Positive Chondrocytes in Growth Plate from Rats ($\bar{x} \pm s$).

Groups	Control Group	Se-Deficient Diet Group	T-2 Toxin Group	Se-Deficient Diet +T-2 Toxin Group
Percentages	19.35 ± 4.71	8.67 ± 5.38[a]	6.79 ± 2.37[a]	3.79 ± 3.21[a]

MMP-10 = matrix metalloproteinase-10.
[a]Each group compared with the control group, $n = 6$, $P < 0.05$.

model *in vitro*. The expression of MMP-10 protein during chondrogenic differentiation of ATDC5 cells was measured to characterize the expression patterns of MMP-10 during chondrogenesis. As shown in **Fig. 3C**, the MMP-10 mRNA level increased 1.74-fold at 7 days, 214.2-fold at 14 days, and 1,086.4-fold at 21 days after ITS-induced differentiation compared with that of the cells cultured in the absence of ITS. MMP-10 protein levels increased to 1.32-fold, 1.94-fold, and 16.63-fold at 7 days, 14 days, and 21 days after induction, respectively. That both the MMP-10 mRNA and protein levels were preferentially upregulated during chondrocyte differentiation suggests a potential role for MMP-10 in chondrogenic ATDC5 cells.

Chondrocyte Marker Gene Expression during Chondrogenic Differentiation of ATDC5 Cells after MMP-10 Knockdown

The expression of MMP-10 was divergent during the differentiation process of ATDC5 cells into chondrocytes. Furthermore, we assess the effect of MMP-10 on the chondrogenic differentiation of ATDC5 cells by detecting the chondrocyte marker gene expression under the MMP-10 knockdown condition. After MMP-10 knockdown, the expression of MMP-10 mRNA and protein in ATDC5 cells differentiated for 14 days decreased by more than 50% compared with the control (**Fig. 4A**). The mRNA and protein expression levels of Col II and Col X were decreased in shMMP-10 cells (**Fig. 4B** and **C**). Similarly, the expression of MMP-10 mRNA and protein in shMMP-10 cells differentiated for 21 days decreased to less than 0.5-fold compared with the control (**Fig. 4D**). The mRNA and protein expression levels of Col II and Col X also decreased accordingly in ATDC5-shMMP-10 cells (**Fig. 4E** and **F**). These results imply that MMP-10 deficiency leads to reduced expression of the chondrogenic marker Col II and the hypertrophic marker Col X during hypertrophic cell differentiation to hypertrophic chondrocytes.

Hypertrophy Regulating Gene Expression during Chondrogenic Differentiation of ATDC5 Cells after MMP-10 Knockdown

Runx2 and MMP-13 are 2 important genes regulating hypertrophic differentiation of chondrocytes. The mRNA and protein expression levels of Runx2 and MMP-13 in the

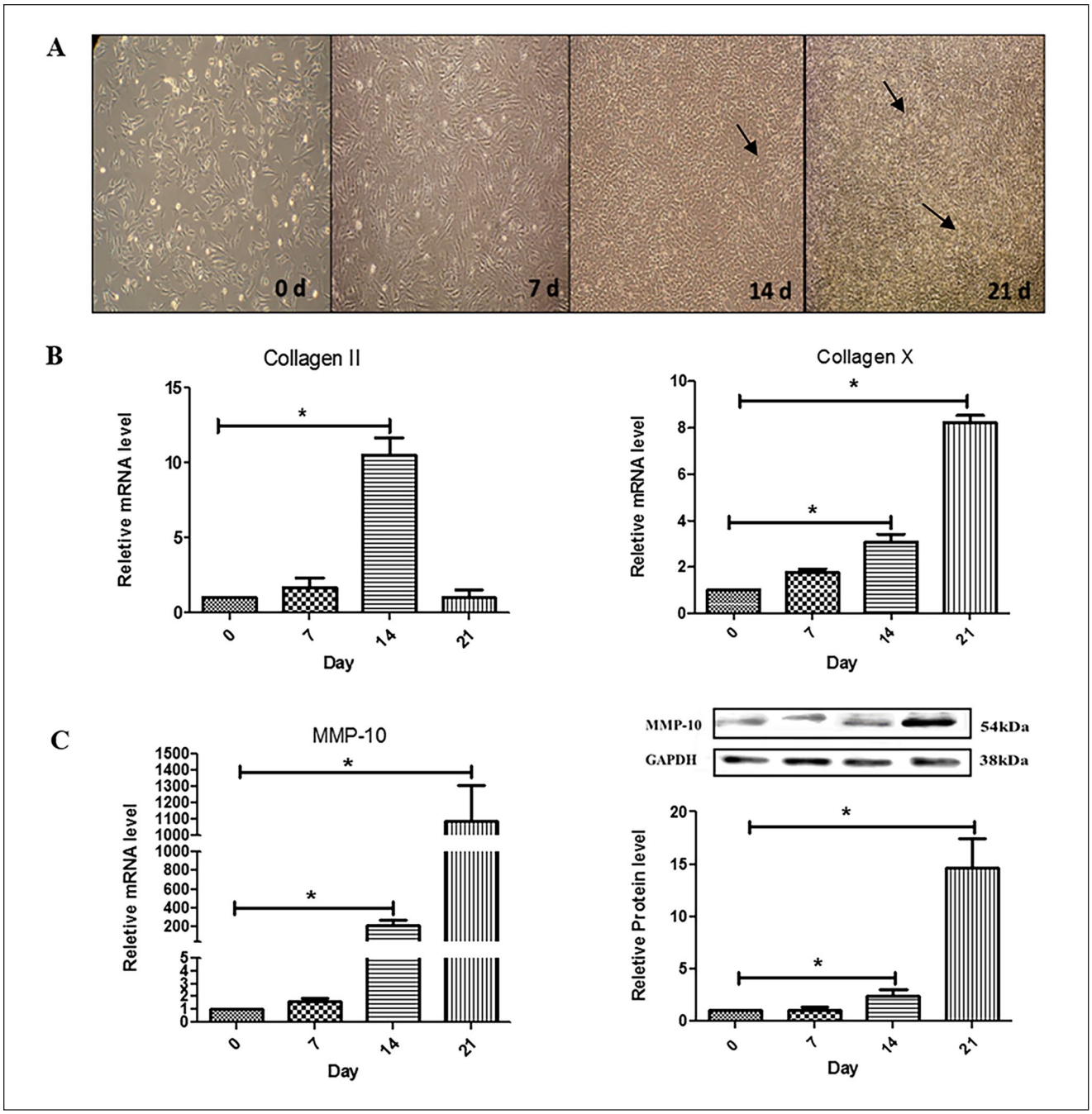

Figure 3. The expression of chondrocyte marker genes and MMP-10 during chondrogenic differentiation of ATDC5 cells. (**A**) The differentiation presentation of ATDC5 cells: day 0, undifferentiated cells; day 7, dense monolayer cells; day 14, nodule formation; day 21, cluster-like growth areas. (**B**) The mRNA expression of Col II and Col X. *$P < 0.05$. (**C**) The mRNA and protein expression level of MMP-10 of ATDC5 cells in different culture times after ITS induction. Results are normalized according to GAPDH levels ($n = 3$). Representative data from 3 independent experiments are shown. Results are considered statistically significant at *$P < 0.05$. MMP-10 = matrix metalloproteinase-10; ITS = insulin-transferrin-selenium; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; mRNA = messenger RNA.

knock down cells were determined by RT-PCR and Western blot to study the mechanism of MMP-10 on chondrogenic hypertrophy differentiation (**Fig. 5**). The expression of Runx2 and MMP-13 in ATDC5-shMMP-10 cells reduced whether it was day 14 or day 21 after ITS induction. Moreover, mRNA and protein levels of Runx2 and

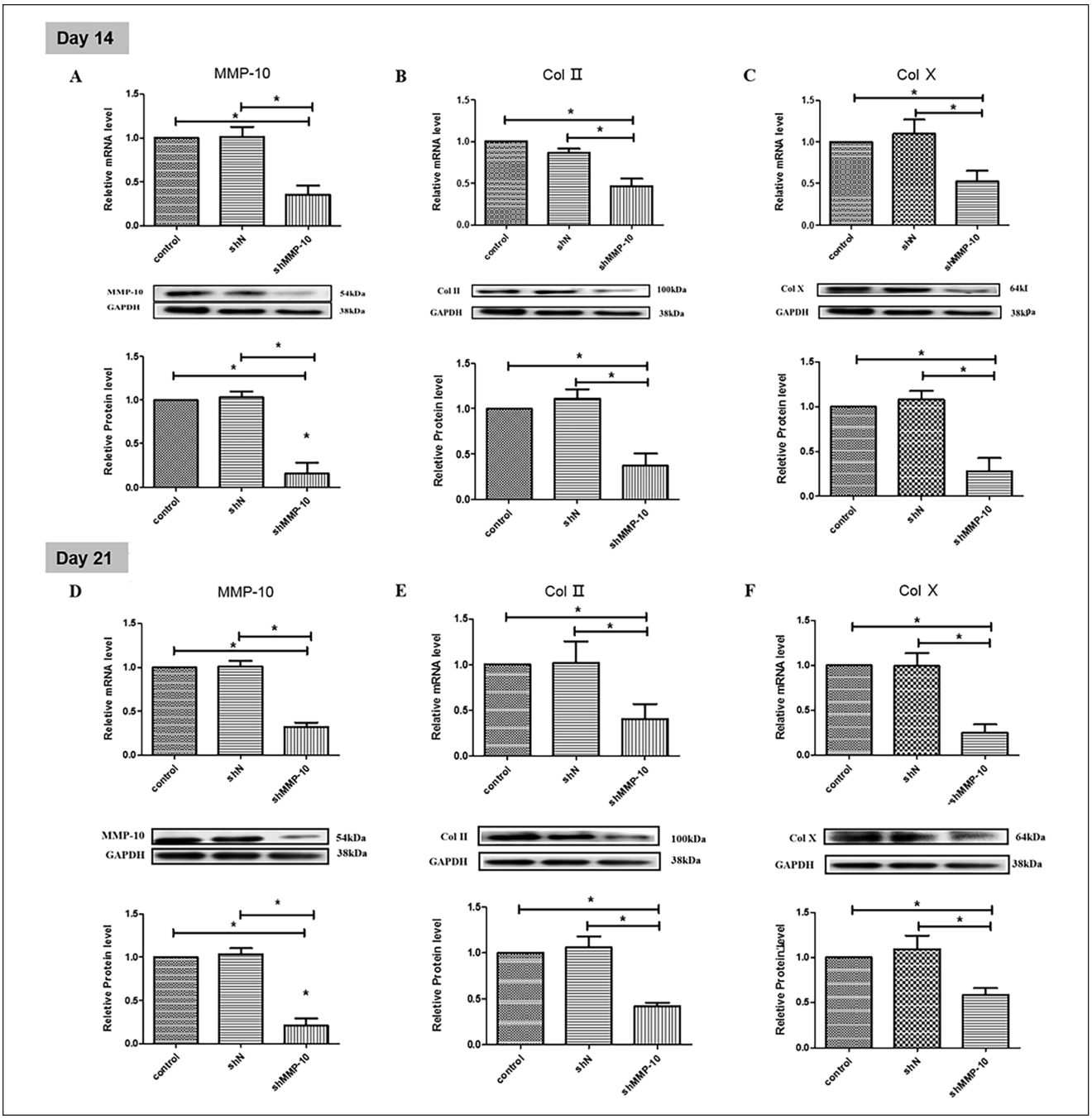

Figure 4. The expression of MMP-10 and chondrocyte marker genes in the cells after MMP-10 knockdown. (**A-C**) mRNA and protein expression of MMP-10, Col II, and Col X in shMMP-10 cells on day 14 after induction, respectively. (**D-F**) mRNA and protein expression of MMP-10, Col II, and Col X in shMMP-10 cells on day 21 after induction, respectively. Results are normalized according to GAPDH levels ($n = 3$). Triplicates of 3 independent protein and mRNA samples were evaluated in each sample. Error bars, SE. *$P < 0.05$ vs. control group. MMP-10 = matrix metalloproteinase-10; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; mRNA = messenger RNA.

MMP-13 decreased more at 21 days after induction than at 14 days after induction (**Fig. 5**).

Chondrocyte Apoptosis after MMP-10 Knockdown

We further examined whether MMP-10 deficiency was related to cell death during chondrocyte differentiation. Cell death was detected by annexin V-PI staining. The results indicated that early apoptosis and late apoptosis were significantly increased in the MMP-10-deficient group both on day 14 and on day 21 after ITS induction (**Fig. 6**). The total apoptosis rates were (23.597 ± 1.569)% and (26.732 ± 2.423)% on day 14 and day 21, respectively, higher than in the control groups ($P < 0.05$). There were no significant changes in necrosis in the MMP-10 deficiency group.

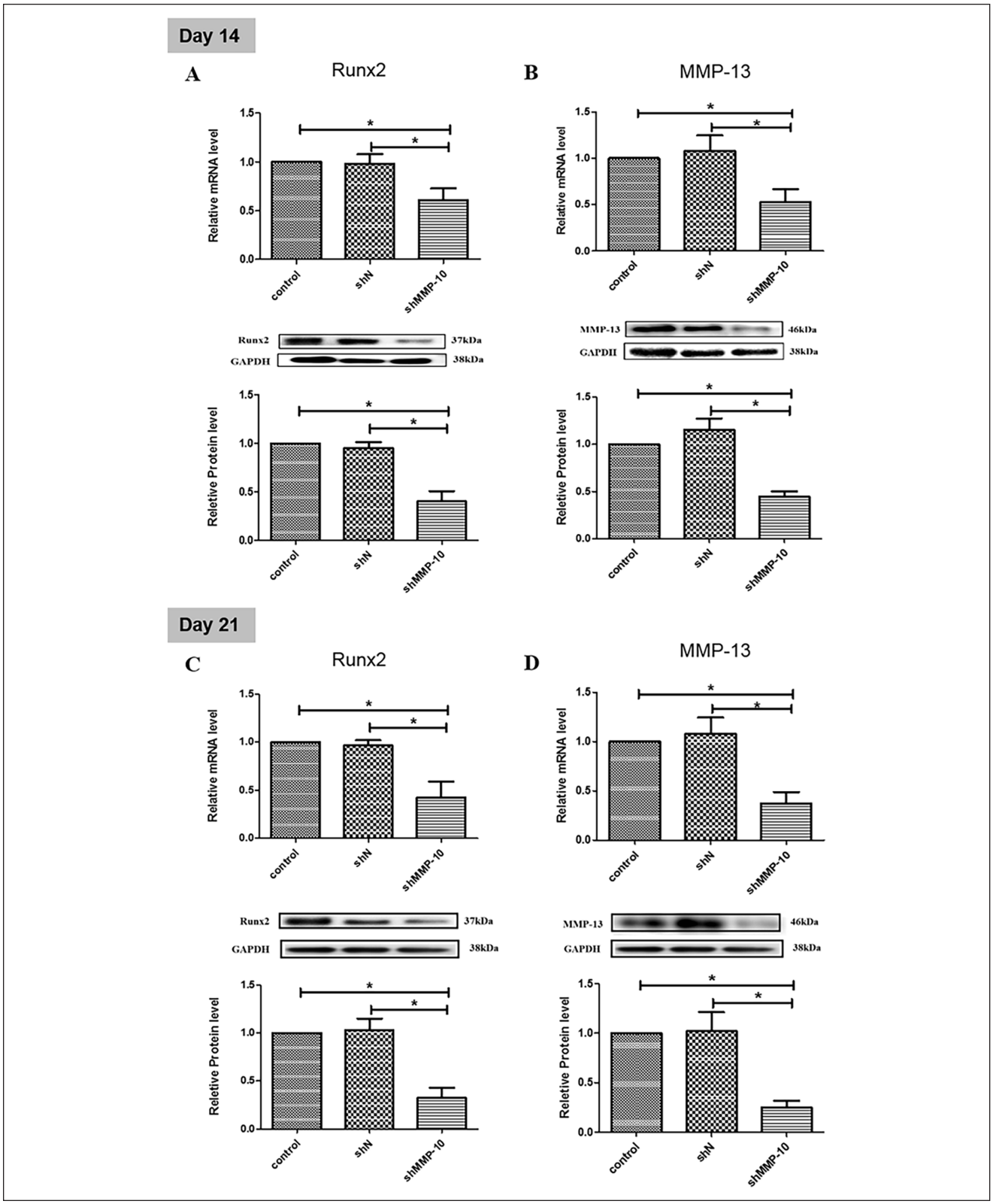

Figure 5. The expression of genes regulated chondrogenic differentiation in the cells after MMP-10 knockdown. (**A**) and (**B**) mRNA and protein expression of Runx2 and MMP-13 in shMMP-10 cells on day 14 after induction, respectively. (**C**) and (**D**) mRNA and protein expression of Runx2 and MMP-13 in shMMP-10 cells on day 21 after induction, respectively. Results are normalized according to GAPDH levels ($n = 3$). Data shown are representative of 3 independent experiments. Error bars, SE. *$P < 0.05$ vs. control group. MMP-10 = matrix metalloproteinase-10; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; mRNA = messenger RNA.

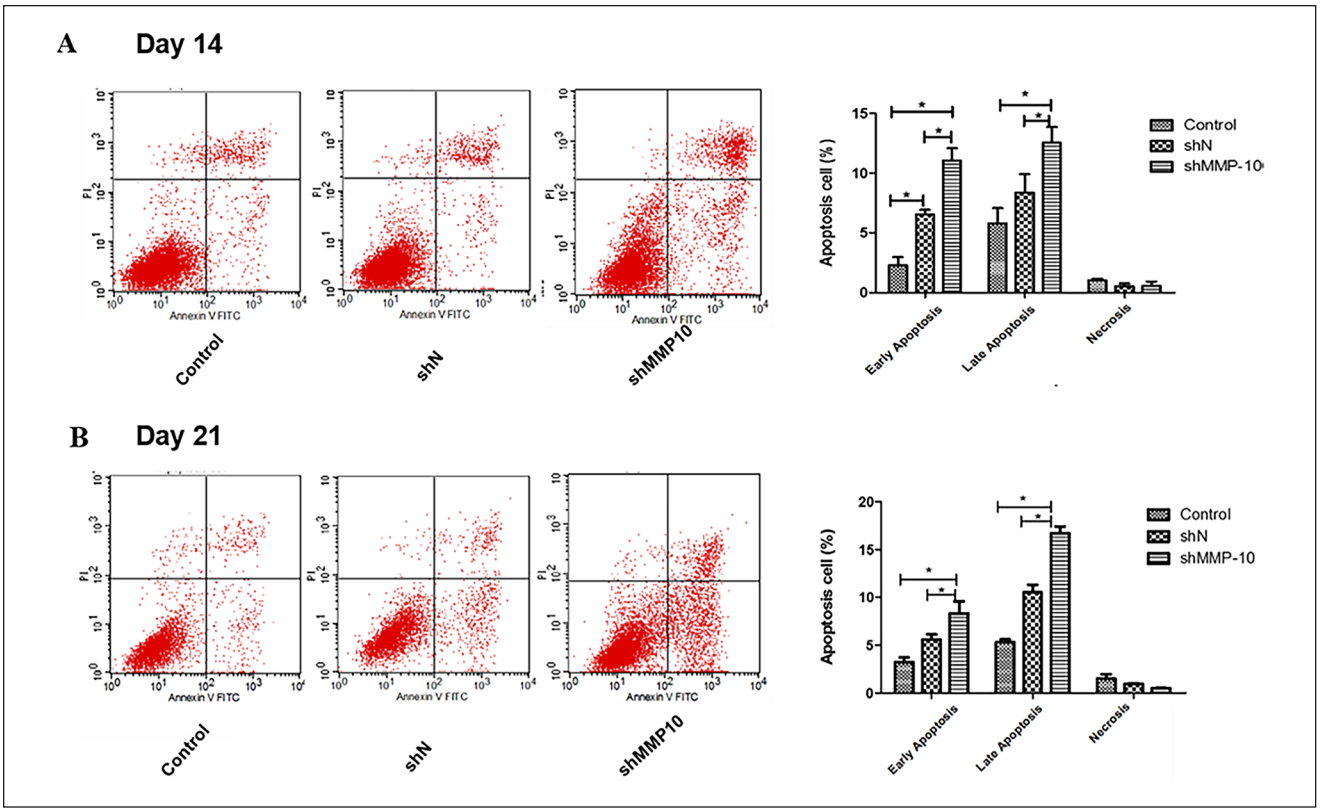

Figure 6. MMP-10 deficiency induces cell death during ATDC5 chondrogenic differentiation. (**A**) MMP-10 was silenced with shRNA in ATDC5 chondrogenic cells on the 14th day post-induction by ITS and cell apoptosis was assayed using an Annexin-V/PI FCM 2 days later after MMP-10 was silenced. (**B**) MMP-10 was silenced with shRNA in ATDC5 chondrogenic cells on the 21st day post-induction by ITS and cell apoptosis was assayed using an Annexin-V/PI FCM 2 days later after MMP-10 was silenced. Representative data from 3 independent experiments are shown ($n = 3$). Error bars, SE. *$P < 0.05$ vs. control group. MMP-10 = matrix metalloproteinase-10; shRNA = short hairpin RNA; ITS = insulin-transferrin-selenium; PI = propidium iodine; FCM = flow cytometry; FITC = fluorescein isothiocyanate.

Discussion

KBD mainly makes inroads on the deep chondrocytes of hyaline cartilage from the endochondral bone in children, including epiphyseal cartilage, epiphyseal plate cartilage, and articular cartilage, which leads to endochondral ossification disorder. And then the enlarged and shortened fingers and deformed enlarged joints occur. The degenerative change in KBD cartilage is characterized by chondronecrosis in multiple foci of the deep zone of cartilage.[2] Another pathologic change in the abnormal endochondral ossification in KBD is shown as the abnormal terminal differentiation of chondrocytes.[24] During the process of endochondral bone formation, the cellular features and expression profiles of the chondrocytes progressively change. Chondrocytes appear in dedifferentiation and premature hypertrophy. In this study, the downregulated MMP-10 expression was found in the growth plate cartilage of KBD children and disease rats fed with a Se-deficient and T-2 toxin diet. Based on the finding of MMP-10 decreased in hypertrophic chondrocytes in human growth plates,[12] herein we propose that MMP-10 deficiency may contribute to the abnormal differentiation in the pathological mechanism of KBD.

The ATDC5 cell line is derived from mouse teratocarcinoma cells and is characterized as a chondrogenic cell line that goes through a sequential process analogy to chondrocyte differentiation.[25] Thus, in this study, the ATDC5 was induced into hypertrophic chondrocytes to clarify the role of the MMP-10 in regulating chondrocyte differentiation. Chondrogenic differentiation is a continuous and delicate process in which different phenotypes of collagen are synthesized in this process and expressed in specific zones of cartilage. Hypertrophic chondrocytes specifically synthesize Col X and Col II, but Col X and Col I were mainly synthesized in the process of post-hypertrophic differentiation of chondrocytes to the formation of osteoblast-like cells. Present data showed that the expression of Col II and Col X significantly increased on day 14 during induction, while the Col II mark decreased and Col X increased on day 21, which indicates that ATDC5 cells differentiated into

hypertrophic chondrocytes on day 14, and after continued induction and differentiation, differentiated into post-hypertrophic chondrocytes on day 21. As the characteristic lesions of KBD mainly occur with hypertrophic chondrocytes in the deep zone of cartilage, our data demonstrated that ATDC5 chondrocytes could be used as a target cell after 14 and 21 days of induction, respectively, to study the chondrocyte necrosis in the deep zone of cartilage in KBD. The expression of MMP-10 mRNA and protein was observed to be divergent during late-stage chondrogenesis of ATDC5 cells (14 days and 21 days). The above results implied that MMP-10 might be related to hypertrophy during chondrogenic differentiation of KBD.

To address the role of MMP-10 in chondrocyte differentiation, we further examined the mRNA and protein expression levels of Col II, Col X, Runx 2, and MMP-13 under MMP-10 knockdown by shRNA in ATDC5 chondrogenic cells. We observed that MMP-10 deficiency in chondrocytes at different differentiation stages suppressed the expression of Col II, the hypertrophic marker Col X, and the regulators of chondrocyte hypertrophy differentiation and later stage, which are Runx2 and MMP-13. Runx2 regulates chondrocyte proliferation and differentiation that drives endochondral ossification toward a hypertrophic-like state of chondrocytes.[26] Col X is a direct transcriptional target of Runx2 during chondrogenesis. COL10A1 (Col X-coding gene) promoter activity was enhanced by both Runx2 alone and Runx2 in combination with the coactivator core-binding factor-beta.[27,28] The regulation of COL10A1 by Runx2 contributes directly to the chondrocyte-specific hypertrophic profile.[29,30] On the other hand, Col X and MMP-13 were closely associated with the phenotypic transition of the cells from the growing stage to hypertrophic and matrix mineralizing stages.[14] Hypertrophic chondrocytes lose the expression of Col10a1and Col X and then express MMP-13, vascular endothelial growth factor A, and other factors. It heralds the invasion of the growth plate by endothelial cells, osteoclasts, and osteoblast precursors.[31] MMP-13 is expressed by chondrocytes in human osteoarthritis and plays a critical role in cartilage destruction through the degradation of aggrecan and collagens.[32] On the other side, MMP-13 loss associated with impaired ECM remodeling disrupts chondrocyte differentiation by concerted effects on multiple regulatory factors.[33]

Chondrocyte hypertrophy is an essential contributor to longitudinal bone growth. Hypertrophic chondrocytes play fundamental roles in signaling to other skeletal cells, as regulators of endochondral ossification.

MMP-10 showed intense cell-associated staining at sites of resorption in areas of endochondral ossification and in resorptive cells at the chondro-osseous junction, which correlated with enzyme activity detected by zymography, which indicates its important role in cartilage and bone development.[16] Previous evidence revealed that IκB kinase (IKK) α activates NF-κB dimers that regulate the expression of specific target genes involved in extracellular matrix remodeling and terminal differentiation of chondrocytes. Importantly, MMP-10 acts as a target gene that makes IKKα maintain the maximum activity of MMP-13 which regulates the chondrocyte differentiation state.[34] Together with reduced Col X, MMP-13, and Runx2 levels in MMP-10 knockdown chondrocytes in the present study, we proposed that MMP-10-dependent Runx2/MMP-13 decrease would affect terminal chondrocyte differentiation and growth plate chondrocyte maturation, making further efforts on endochondral ossification.

Chondrocyte hypertrophy and cell death are both crucial steps during the natural process of endochondral bone formation. Unexpectedly, in our study, MMP-10 deficiency not only affected chondrocyte hypertrophy but also induced chondrocyte death via apoptosis. The classic pathological changes of KBD include focal necrosis in the deep cartilage and apoptosis of chondrocytes.[2,35] In recent years, more and more studies have shown that apoptosis is the main way of chondrocyte death in KBD. KBD chondrocyte presented an increased apoptosis rate compared with the normal control and expressed Bcl-2, Bax, and Fas.[36,37] Hypermethylation and low gene expression of Gpx3 were found to be related to the apoptosis of KBD chondrocyte.[38] Apoptosis in KBD chondrocytes also can be dependent on FAS/DR4-FADD-caspase signaling.[39] Our results proposed a new clue on the mechanism of KBD chondrocyte apoptosis. However, this study did not reveal the detailed signaling pathways through which MMP-10 affects cell death. This will become one focus of our further research.

Together, our study shows that MMP-10 regulated chondrogenic differentiation and chondrocyte death via apoptosis. It indicates that MMP-10 deficiency may play a pivotal role in chondrocyte hypertrophy and death, with potential consequences for abnormal endochondral ossification of KBD pathobiology. These findings have implications both for understanding the effects of MMP-10 on the basic biology of cartilage and bone and for understanding how disruption of this finely tuned process of chondrocyte maturation results in various skeletal pathologies such as KBD.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Approval for this study was obtained from the Human and Ethical Committee for Medical Research at Xi'an Jiaotong University (#0075). The rats' corpses were collected and processed by the Experimental Animal Center of Xi'an Jiaotong University in accordance with standard principles of animal experimental ethics.

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