

Original Article

Egr1 deficiency disrupts dynamic equilibrium of chondrocyte extracellular matrix through PPAR γ /RUNX2 signaling pathways

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Abstract: Background: This study is to investigate the effect of Egr1 on the mineralization and accumulation of chondrocyte extracellular matrix. Methods: The femoral heads of patients of various heights were collected. Egr1 knockout mice were used. Their limb length and body weight were assessed. The bone characteristics were detected by micro-CT scan and histological staining. Immature murine articular chondrocytes (iMACs) were isolated. Gross morphology was observed by histological staining. Relevant mRNA and protein expression were detected by qRT-PCR and Western blot, respectively. The related proteins were observed by immunohistochemical staining and immunofluorescence assay. Chromatin immunoprecipitation and reporter gene assay were also used. TUNEL was used to detect apoptosis. Results: It was found that shorter patients had reduced Egr1 expression levels in the hypertrophic cartilage zone of the femoral head. In addition, Egr1 knockout mice exhibited reduced body size. Micro-CT analysis showed that these mice also had reduced bone volume. Safranin-O staining showed that the extracellular matrix of these mice exhibited a relatively limited degree of mineralization, and TUNEL staining showed reduced cell apoptosis levels. After transfecting the iMACs with dominant-negative Egr1 adenoviruses to inhibit Egr1, the enzymes of Adamst4, Adamst5, Mmp3 and Mmp13 were significantly upregulated. ChIP and luciferase assays revealed that Egr1 might regulate the chondrocyte extracellular matrix by the PPAR γ /RUNX2 signaling pathways. Conclusion: Egr1 has an important regulatory effect on the dynamic equilibrium of the chondrocyte extracellular matrix, which may be achieved through the PPAR γ /RUNX2 signaling pathways.

Keywords: Egr1, PPAR γ , RUNX2, immature murine articular chondrocytes, MMPs, extracellular matrix

Introduction

The skeleton of vertebrates is primarily composed of bone and cartilage. Most bones, including the long bones of the limbs, are formed through endochondral ossification [1-3]. In this process, chondrocytes replicate and secrete extracellular matrix, which enlarges the

cartilage. Chondrocytes located in the core region of the cartilage will stop replicating, undergo hypertrophy, and secrete collagen X [1, 4, 5]. The enlargement of these hypertrophic chondrocytes is the primary factor responsible for bone growth [3, 6]. Meanwhile, these hypertrophic cells also play an important regulatory role in balance of the bone metabolism. Hy-

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Table 1. Patient demographics

	Normal group (n=5)	Short group (n=5)	<i>p</i> value
Age (year)	62.00 ± 6.6	57.67 ± 3.7	0.6
Gender	Male	Male	
Height (cm)	172.0 ± 1.42	162.0 ± 1.48	0.002
Weight (kg)	77.50 ± 6.8	56.50 ± 4.6	0.04
Body mass index	26.11 ± 1.8	21.46 ± 1.4	0.1

peritrophic chondrocytes induce mineralization of the surrounding matrix and secrete factors such as VEGF, which induce vascularization [4, 7]. They also induce adjoining peri-chondrocytes to differentiate into osteoblasts, which secrete bone matrix and form a bone collar [1, 3, 4]. Next, hypertrophic chondrocytes will undergo apoptosis and the cartilage matrix will provide support for osteoblasts and blood vessels. Thus, the primary ossification center is formed and from this center the resulting bone (cancellous bone) is generated [2, 4]. Chondrocyte cells have unique morphologies, and the matrix they secrete is rich of collagen II and the proteoglycan aggrecan [8]. Matrix metalloproteinases (MMPs), including MMP3 and MMP13, and aggrecanases (ADAMTS-4 and ADAMTS-5), participate in the degradation and remodeling of the chondrocyte extracellular matrix [8, 9]. The dynamic equilibrium of the chondrocyte extracellular matrix plays a key role in endochondral ossification and the development of bones.

Early growth response protein 1 (Egr1), also known as NGFI-A, Zif268, Krox-24 and TIS8, is one of the classic zinc finger transcription factors. It is the product of the immediate early genes [10, 11] and can mediate cellular responses to environmental stimuli, such as mitogenic stimuli and stress signals [12, 13]. It plays an important role in cell differentiation, proliferation, apoptosis, and inflammation [14-17]. Our previous study showed that Egr1 activation was able to decrease the insulin sensitivity of adipocytes by increasing hepatic gluconeogenesis, thus aggravating the progression of type 2 diabetes [18]. Wu *et al.* found that miR-192 could prevent tumor angiogenesis by suppressing the expression of Egr1 and HOXB9, demonstrating that Egr1 may play a role in the formation of blood vessels in tumor tissues [19]. Egr1 also plays a significant role in the homeostasis of skeletal system. For exam-

ple, Gaut *et al.* reported that Egr1 overexpression prevented downregulation of tendon genes such as *Scx* and *Tnmdin* in an environment free from mechanical signals, and promoted tendon and ligament healing [20]. Nuttall *et al.* found that Egr1 was down-regulated more than six-fold in human osteoarthritis cartilage samples compared to normal tissues [21]. It has been suggested that Egr1 may participate in the synthesis and degradation of chondrocyte extracellular matrix [22]. However, the role of Egr1 in the mineralization and accumulation of the chondrocyte extracellular matrix, and in skeletal development is still unclear.

Herein, we first analyzed the expression levels of Egr-1 in cartilage from clinical patients with different heights. Then, the role and mechanism of Egr-1 in the mineralization and accumulation of the chondrocyte extracellular matrix were investigated and discussed.

Materials and methods

Human tissue samples

A total of 10 male patients who was suffering from necrosis of the femoral head subsequent to injury were enrolled (**Table 1**). According to the average male height of China (167.1 cm published by National Health and Family Planning Commission of the People's Republic of China) (<http://www.nhfpc.gov.cn/zwgk/jdjd/201506/4505528e65f3460fb88685081ff158a2.shtml>), the patients were divided into normal group (172.0 ± 1.42 cm) and short group (162.0 ± 1.48 cm). Further inclusion criteria were male, age between 40-70 years old and prepared for total femoral head arthroplasty. Exclusion criteria were: (1) Diagnosed with hypertension and diabetes mellitus; (2) Received glucocorticoid hormone therapy within 3 months; (3) Infections; (4) Diagnosed with autoimmune disease. After total hip arthroplasty, the non-wearing area cartilage of femoral head were obtained and stained with Safranin-O/fast green, hematoxylin-eosin (H&E) and Toluidine blue, respectively. Prior written and informed consent was obtained from every patient and the study was approved by the ethics review board of Nanjing University.

Animals and sampling

C57/Bl6J (B6) male mice were purchased from the Model Animal Research Center of Nanjing

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University (Nanjing, China). Egr1 knockout (KO) male mice were produced as described previously [23]. All of the mice were housed in a 12-h light/dark cycle in a temperature- and humidity-controlled environment and were fed with a standard diet (65% carbohydrate, 4% fat, 24% protein). All mice used in this study were 20 weeks old male. Totally, six wild type (WT) mice and six Egr1 KO mice were used in this study. At the end of the feeding period, mice were anesthetized with halothane and tissues were harvested for analysis as described below. Body weight and the length of limbs were measured immediately. All animal experiments were conducted according to the ethical guidelines of Nanjing University.

Microcomputed tomography (micro-CT) analysis

The left femora dissected from Egr1 KO or WT mice were fixed with 4% paraformaldehyde (PFA) overnight, and washed by PBS, then scanned by a micro-CT scanner (SkyScan, Aarselaar, Belgium). X-ray voltage and current were set to 80 kV and 80 μ A, respectively, with a resolution of 18 μ m per pixel. Cross-sectional images of the mid-diaphysis of femur were used to perform the three-dimensional histomorphometric analysis of trabecular bones. For the distal femur, the region of interest (ROI) selected for analysis was 5% of the femoral length from 0.05 mm below the growth plate and this ROI was used to determine bone mineral density, trabecular bone volume per tissue volume, trabecular number, trabecular separation and trabecular thickness. For cortical bones, the ROI selected for analysis was of 10% of the femoral length in the mid-diaphysis of the femur and this ROI was used to determine the cortical thickness.

Safranin-O/fast greenstaining, HE staining and TUNEL labeling

The dissected right femora of patients and the femoral head cartilage of mice were fixed in 4% PFA overnight, decalcified in 5% EDTA in PBS for 2 weeks, and then embedded in paraffin wax after dehydration. The samples were coronally cut into 5 μ m serial sections and then stained with Safranin-O/fast green or H&E as previously described [24, 25]. For TUNEL labeling, the PFA-fixed paraffin-embedded sections were prepared and labeled according to the In Situ Cell Death Detection Kit (Roche) protocol.

Immunohistochemical staining and immunofluorescence assay

Bone sections were deparaffinized in xylene and rehydrated through graded ethanol, and then incubated in 3% H₂O₂ for 10 min. Antigen-retrieval was performed by heating the sections for 9 min in EDTA buffer (pH=6.0). The slides were incubated with the specific primary antibodies for Caspase 3 (Cell Signaling Technology, USA), Egr1 (Santa Cruz, CA, USA), PPAR γ (Santa Cruz, CA, USA) and RUNX2 (Santa Cruz, CA, USA) overnight at 4°C. The tissue slides were then incubated with secondary antibody of goat anti-rabbit IgG (HRP-labeled) (Santa Cruz, CA, USA), developed by 3,3'-diaminobenzidine and counterstained with haematoxylin. For the immunofluorescence assay, the sections were incubated with specific primary antibodies for Egr-1 (Santa Cruz, CA, USA) and then incubated with a peroxidase-conjugated anti-fluorescein antibody (Cell Signaling Technology, Massachusetts, USA). The above stained sections were then viewed using an Olympus BX51 phase contrast light microscope (Olympus Corporation, Japan) and photographed using an Olympus CC12 camera and Image-Pro Plus software (version 6.1; Media Cybernetics, Inc., Bethesda, MD).

Western blot

The femoral heads were homogenized and lysed in RIPA buffer. Total proteins were collected by centrifugation at 4°C for 15 min at 13,000 \times g. Then, total proteins were subjected to 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Richmond, CA). The membranes were blocked in 4% non-fat milk. The primary antibodies of Egr1, PPAR γ and RUNX2 were added and incubated at 4°C for overnight (Santa Cruz Biotech). Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies.

Isolation and culture of immature murine articular chondrocytes (iMACs)

The iMACs were isolated as previously described [26]. Briefly, femoral heads and tibial plateaus from 12 male mice (C57Bl/6 mice, ages 6 days) were dissected and incubated for 45 minutes with collagenase D (3 mg/ml in Dulbecco's modified Eagle's medium, DMEM) at 37°C in 5% CO₂. And the cartilage pieces were incubated overnight with collagenase D (0.5 mg/ml) at

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Table 2. Primer sequences

Gene symbol	5'-Forward primer-3'	5'-Reverse primer-3'
Caspase 3	ATGGAGAACAACAAACCTCAGT	TTGCTCCCATGTATGGTCTTTAC
Caspase 6	GGAAGTGTTCGATCCAGCCG	GGAGGGTCAGGTGCCAAAAG
Caspase 8	TGCTTGACTACATCCCACAC	TGCAGTCTAGGAAGTTGACCA
Egr1	TCGGCTCCTTTCCTCACTCA	CTCATAGGGTTGTTGCTCGTCGG
ADAMTS4	ATGGCCTCAATCCATCCCAG	GCAAGCAGGGTTGGAATCTTTG
ADAMTS5	CCCAGGATAAAACCAGGCAG	CGGCCAAGGGTTGTAATGG
MMP3	ACATGGAGACTTTGTCCCTTTTG	TTGGCTGAGTGGTAGAGTCCC
MMP13	CTTCTTCTTGTGAGCTGGACTC	CTGTGGAGGTCACTGTAGACT
PPAR-gamma	GGAAGACCACTCGCATTCTT	GTAATCAGCAACCATTGGGTCA
Runx2	ATGCTTCATTCGCCTCACAAA	GCACTCACTGACTCGGTTGG
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

37°C. The resulting chondrocytes were suspended in culture medium (DMEM-Glutamax containing 1 g/L glucose, Gibco) plus 10% fetal calf serum (FCS) and antibiotics. The chondrocytes were then seeded in 10-cm dishes (designated passage 0, PO).

Adenovirus infection

Dominant-negative Egr1 adenovirus (DnEgr1) was constructed as described previously [27]. The iMACs were infected with dominant-negative Egr1 adenovirus or Ad-eGFP in serum-free medium for 6 h and the medium was then supplemented with 2% fetal bovine serum to maintain cell survival for another 24 h.

Treatment with PPAR γ inhibitor

The primary chondrocyte was treated with 50 mmol/L PPAR γ inhibitor, T0070907 (Cayman, Michigan) or DMSO for 18 h. After that, total RNA was isolated as described below.

Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA from treated cells was isolated using Trizolreagent (TaKaRa) and reversely transcribed into cDNA. The qRT-PCR performed on an ABI Viia 7 machine (Applied Biosystems, Carlsbad, CA) using SYBR-Green master mix (TaKaRa). Primer sequences were shown in **Table 2**. The following PCR procedure was used: 2 min activation of the HotGoldStar Taq enzyme (TaKaRa) at 95°C and 40 cycles of amplification: 5 s denaturation at 95°C, 30 s annealing at 60°C, 15 s elongation at 72°C. The relative expression level of each gene was cal-

culated using the 2^{- Δ CT} method and all of the quantitation was independent, conducted in triplicate, and normalized to an endogenous GAPDH control.

Chromatin immunoprecipitation (ChIP) assay

At 48 h after adenovirus infection, chromatin lysates of iMACs were prepared. The lysate was pre-cleared with Protein-A/G agarose beads, and then immunoprecipitated

with antibodies against the corresponding protein or control mouse IgG (Santa Cruz Biotech) in the presence of BSA and salmon sperm DNA. Beads were extensively washed before reverse cross-linking, and the bound DNA was purified using a PCR purification kit (Qiagen). Subsequently, the purified DNA was analyzed by PCR or qRT-PCR using primers flanking the GC element in the mice RUNX2 promoter.

Dual luciferase reporter assay

The iMACs cells were plated into 24-well plates at a concentration of 4×10⁴ cells per well. After 12 hours, the plasmid (240 ng pcDNA3.0 + 250 ng Runx2-pGL3 + 10 ng renilla or 240 ng pcDNA3.0-Egr1 + 250 ng Runx2-pGL3 + 10 ng renilla) was transfected into the cells. After 72 hours of transfection, luciferase activity was measured using the Dual-Glo[®] Luciferase Assay System (E1910, Promega, Madison, USA).

Statistical analysis

All the experiments were performed in triplicate. The data are presented as mean ± standard deviation (SD). Comparison among groups was performed with one-way ANOVA. A *P*<0.05 was considered statistically significant.

Results

Reduced Egr1 expression in cartilage of short patients

The femoral heads were collected from patients with hip replacement operation. The demographic data showed no significant difference between normal group and short group except

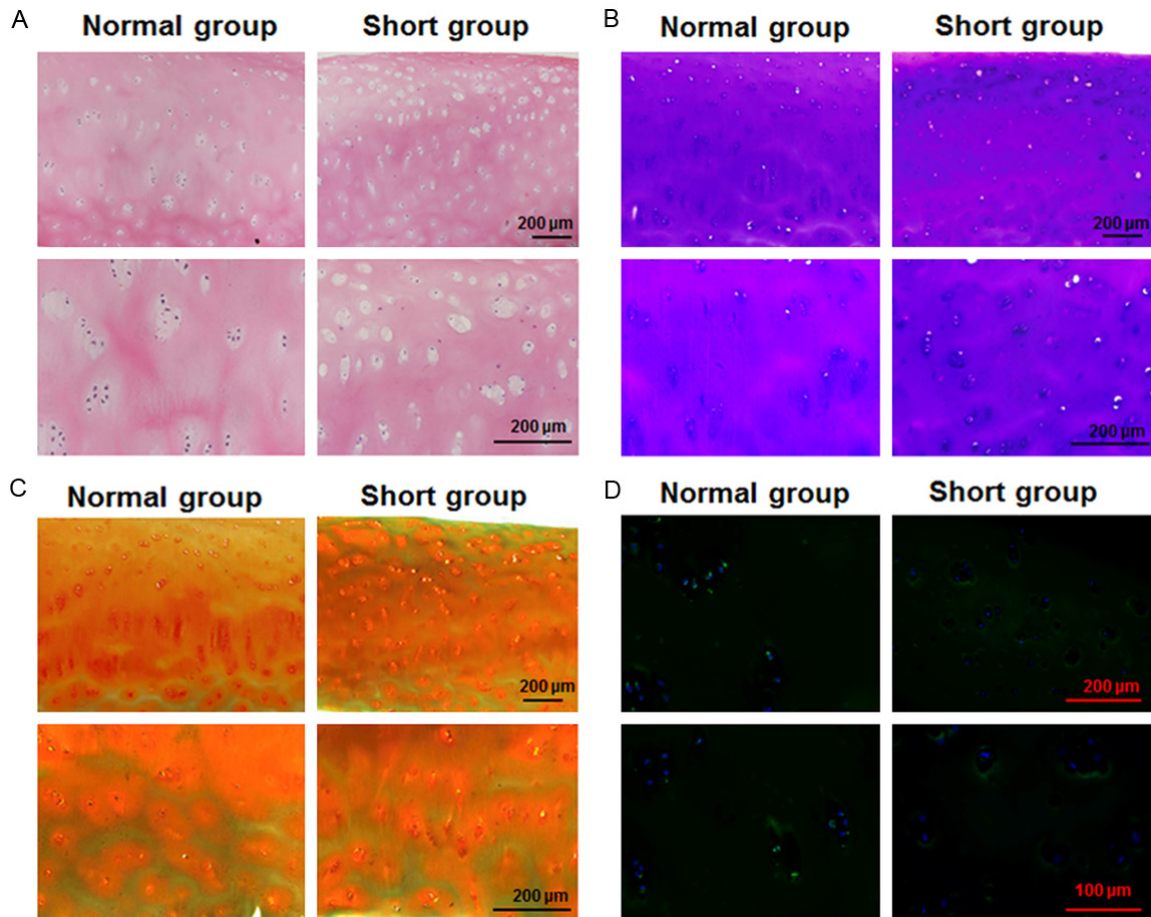


Figure 1. Analysis of femoral cartilage morphology and Egr1 expression in the cartilage of patients. Microscopic appearance of the femoral head cartilage of patients suffering from necrosis of the femoral head was observed. Paraffin-embedded sections were stained with (A) H&E, (B) Toluidine blue or (C) Safranin-O. Magnification was 100 \times and 200 \times . (D) Representative sections from patient cartilage were probed for Egr1 by immunofluorescence. Magnification was 200 \times and 400 \times (Normal height patient N=5, shorter height patient N=5).

the height ($P=0.002$) and weight ($P=0.04$, **Table 1**). The accumulation of the chondrocyte extracellular matrix in the non-wearing areas was observed. The superficial zone chondrocytes of short group exhibited a gross morphology that was not noticeably different from those of the normal group (**Figure 1A** and **1B**). However, the cells in transition zone were larger and disordered in short group (**Figure 1C**). Immunofluorescent staining showed that short patients had reduced Egr1 expression in the hypertrophic cartilage zone of the femoral heads (**Figure 1D**). This suggests that Egr1 may play a role in regulating the accumulation of the chondrocyte extracellular matrix.

Egr1 KO mice shows lower body weight and shorter limb length

The gross morphological indicators of Egr1 KO and WT mice were assessed. It was found that

the body size and weight of KO mice were relatively small (**Figure 2A**) and had a relatively short limb length at 20 weeks (**Figure 2B**). And, the difference in weight and femur length was significant (**Figure 2C** and **2D**). These results indicate that Egr1 may play a role in regulating skeletal development in mice.

Egr1 KO mice shows reduced bone volume

To further analyze the differences in the femurs between Egr1 KO and WT mice, a micro-CT scan was performed. The 3D image reconstruction showed obviously lower density in some trabecular bone regions of the Egr1 KO mice (**Figure 3A**). The bone mineral density and the bone volume fraction of the femur in Egr1 KO mice were significantly lower compared to the Egr1 WT group ($P<0.05$) (**Figure 3B** and **3C**). After assessing trabecular bone microstructure, it was found that the trabecular thickness

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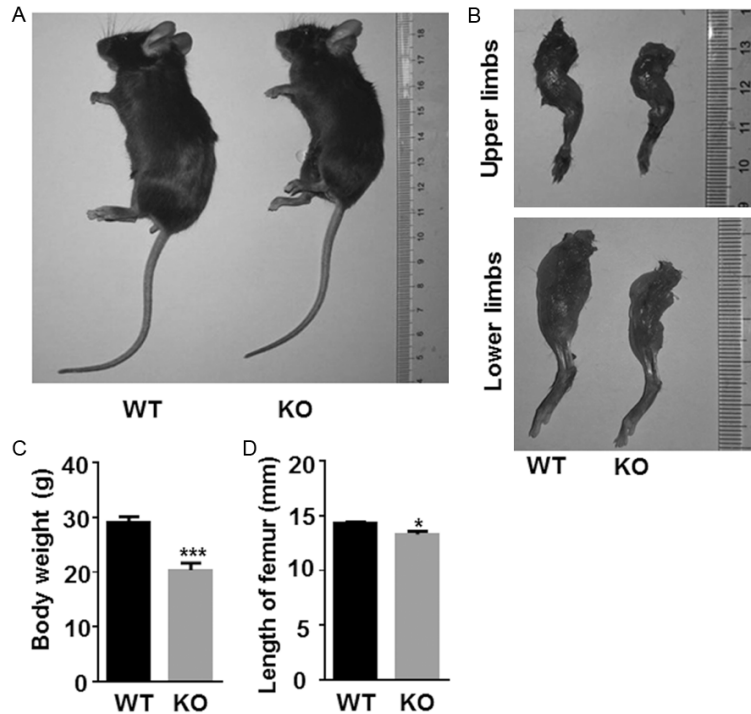


Figure 2. Egr1 KO mice showed lower body weight and shorter limb length. Representative (A) body size and (B) the length of upper and lower limbs of WT and Egr1 KO mice were assessed. The (C) Body weight and (D) the length of femur of 20-week-old Egr1 WT and KO mice were shown. (WT, n=6; Egr1 KO, n=6) (* $P<0.05$; ** $P<0.01$; *** $P<0.001$).

of Egr1 KO mice was significantly decreased than that of the Egr1 WT mice (Figure 3D). However, there was no statistically significant difference in femoral trabecular number, trabecular separation or the cortical thickness between the two groups (Figure 3E-G). These results show that the Egr1 KO mice have significantly lower bone volume compared to Egr1 WT mice.

Egr1 KO mice shows relatively limited chondrocyte extracellular matrix mineralization

Then, a systematic morphological assessment of the articular cartilage of Egr1 KO and WT mice was performed. H&E and Safranin-O staining revealed morphological abnormalities in the subchondral bone cells of the Egr1 KO mice (Figure 4A and 4B). In Egr1 KO mice, the number of hypertrophic chondrocyte was greater, and the hypertrophic chondrocytes were arranged in an irregular, scattered manner (Figure 4A). The extracellular matrix was hard to be stained with Safranin-O and showed a limited degree of mineralization (Figure 4B). TUNEL staining revealed reduced levels of chondrocyte apoptosis in Egr1 KO mice (Figure

4C) and there was significant difference in apoptotic cells between Egr1 WT and KO mice ($P<0.05$) (Figure 4E). The low apoptosis rate was also confirmed by the lower expression of caspase-3 in Egr1 KO mice (Figure 4D). In addition, by infecting iMACs with DnEgr1 virus to suppress Egr1 expression, the mRNA expression of caspase-3, caspase-6, and caspase-8 in the cells was also significantly downregulated (Figure 4F-H). These results indicate that Egr1 KO in mice results in less chondrocyte extracellular matrix mineralization by inhibiting chondrocyte apoptosis.

Suppressing Egr1 in primary chondrocytes upregulates enzyme genes related to chondrocyte extracellular matrix degradation

To detect the expression of enzyme genes related to the degradation of the extracellular

matrix secreted by chondrocytes after Egr1 suppression, qRT-PCR was performed. As expected, Egr1 expression in iMACs was significantly suppressed by DnEgr1 virus transfection (Figure 5A). *Adamts4* and *Adamts5* are two members of the Zn²⁺-dependent, secreted matrix metalloproteinases (MMPs) family, and they are important enzymes found in the cartilage matrix that can degrade proteoglycans [28]. After Egr1 expression suppression, the iMACs showed significantly higher levels of *Adamts4* mRNA (Figure 5B) and *Adamts5* mRNA (Figure 5C) ($P<0.05$). At the same time, the levels of *Mmp13* (Figure 5D) and *Mmp3* mRNA (Figure 5E) were also significantly upregulated ($P<0.05$). These results suggest that suppressing Egr1 expression in iMACs can strongly promote the secretion of related metalloproteinases, thus promoting the degradation of the chondrocyte extracellular matrix.

Egr1/PPAR γ /RUNX2 signaling pathways participate in the regulation of the chondrocyte extracellular matrix

In order to investigate the mechanisms underlying the effects of Egr1 on extracellular

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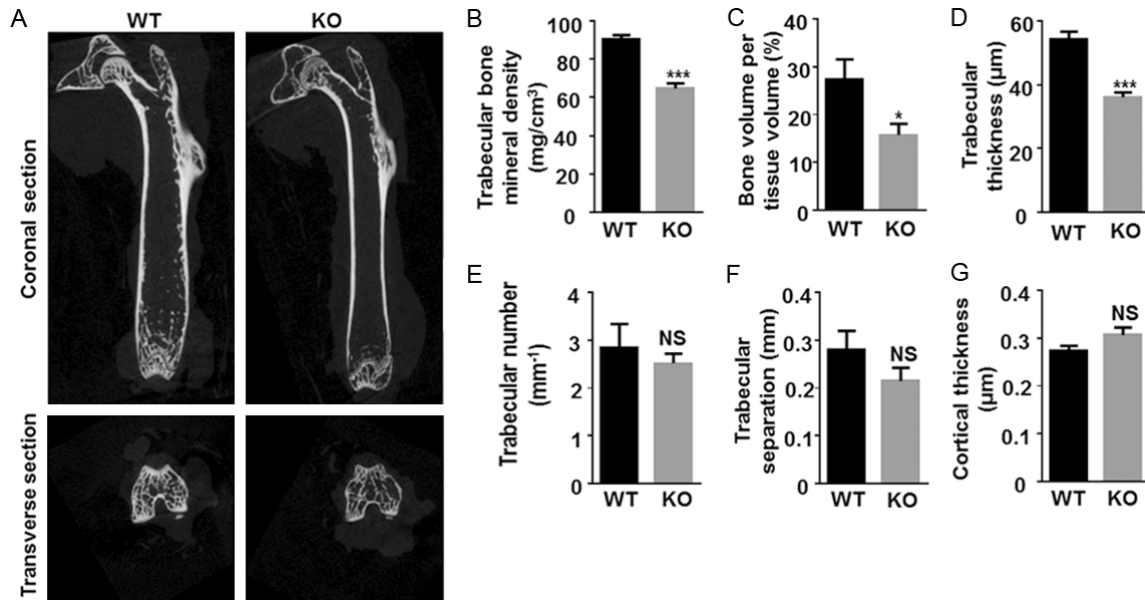


Figure 3. Analysis of bone characteristics of Egr1 WT and KO mice. (A) Representative images of micro-CT analysis of femur showed reduced bone volume of Egr1 KO mice. The (B) bone mineral density, (C) bone volume per tissue volume, (D) trabecular thickness, (E) trabecular number, (F) trabecular separation and (G) cortical thickness of the femur of 20-week-old Egr1 WT and KO mice were shown. (WT, n=6; KO, n=6) (* $P<0.05$; ** $P<0.01$; *** $P<0.001$).

matrix mineralization, the levels of key proteins in the Egr1/PPAR γ /RUNX2 signaling pathways were analyzed. High expression of Egr1 was detected in WT chondrocyte (Figure 6A), which resulted in lower PPAR γ and higher RUNX2 expression (Figure 6B and 6C). However, PPAR γ was increased in Egr1 KO mice (Figure 6B) whereas RUNX2 expression was decreased in Egr1 KO mice (Figure 6C). These results were also confirmed by Western blot (Figure 6D). The luciferase reporter assay revealed that RUNX2 promoter activity was markedly decreased after transfection of pcDNA3.0-Egr1 (Figure 6E). However, the ChIP assay showed that Egr1 did not bind to the promoter region of RUNX2 (Figure 6F). These results also suggest that Egr1 may not achieve its effect by direct binding to RUNX2. Further analysis showed that higher PPAR γ expression was detected in dominant-negative Egr1 adenovirus transfected iMACs cell (Figure 6G). These results above suggest Egr1 KO may inhibit RUNX2 expression indirectly through upregulated PPAR γ . Thus, we treated the primary chondrocytes with T0070907, a PPAR γ inhibitor. The significantly higher *Runx2* mRNA was detected (Figure 6H). Meanwhile, the expression of *Mmp3* and *Adamts 4* mRNA significantly decreased in the primary chondrocytes treated with T0070907

(Figure 6I and 6K), while that of *Mmp13* mRNA had no significant changes (Figure 6J).

Taken together, these results illustrate that the Egr1/PPAR γ /RUNX2 signaling pathway may participate in the regulation of the chondrocyte extracellular matrix.

Discussion

The body height depends mainly on the length of the spine and long bone, whose growth is mainly through endochondral ossification. In this study, the expression of Egr1 in the short group was lower. And the Egr1-KO mice showed relatively lower body weight, shorter limb length and reduced bone volume. Moreover, Egr1-KO mice also showed lower chondrocyte apoptosis rate, which induced the limited chondrocyte extracellular matrix mineralization. Thus, this study suggests that Egr1 has an important regulatory effect on the dynamic equilibrium of the chondrocyte extracellular matrix.

Egr1 is an important transcription factor that primarily participates in cell differentiation, proliferation, apoptosis, inflammation, and other processes [19, 22, 29-31]. In this study, reduced Egr1 expression was found in the chondrocyte extracellular matrix in the non-

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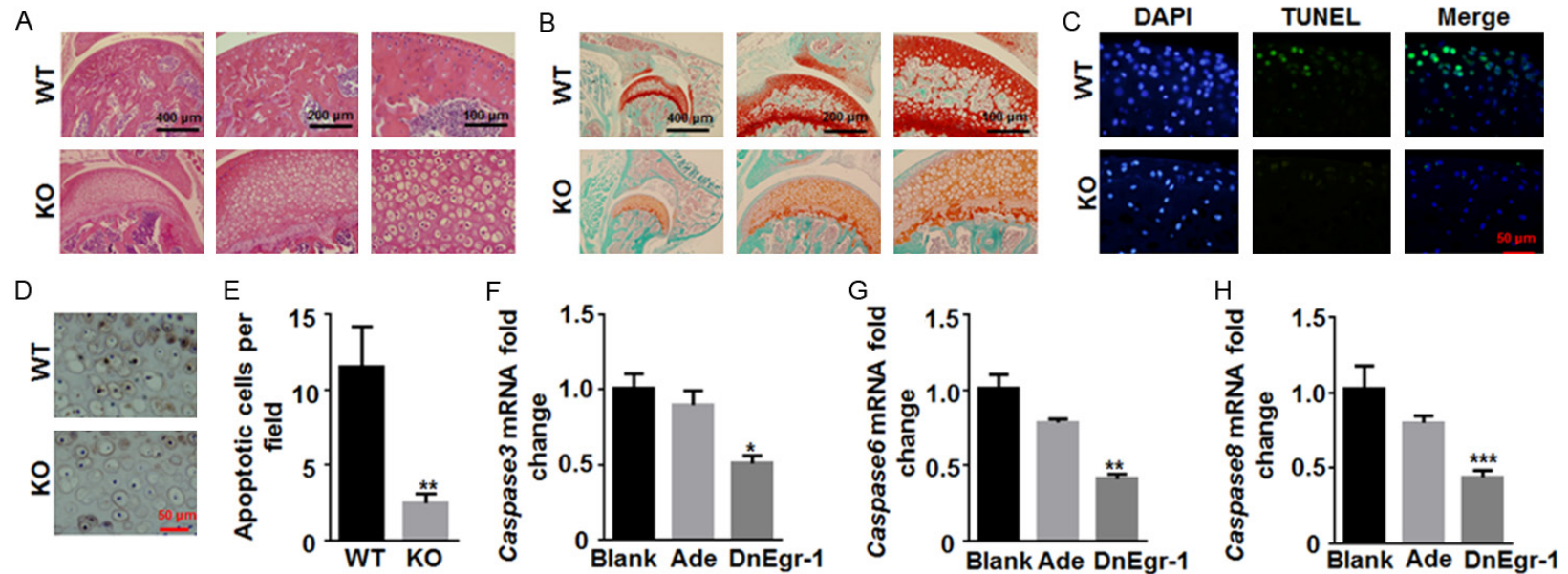
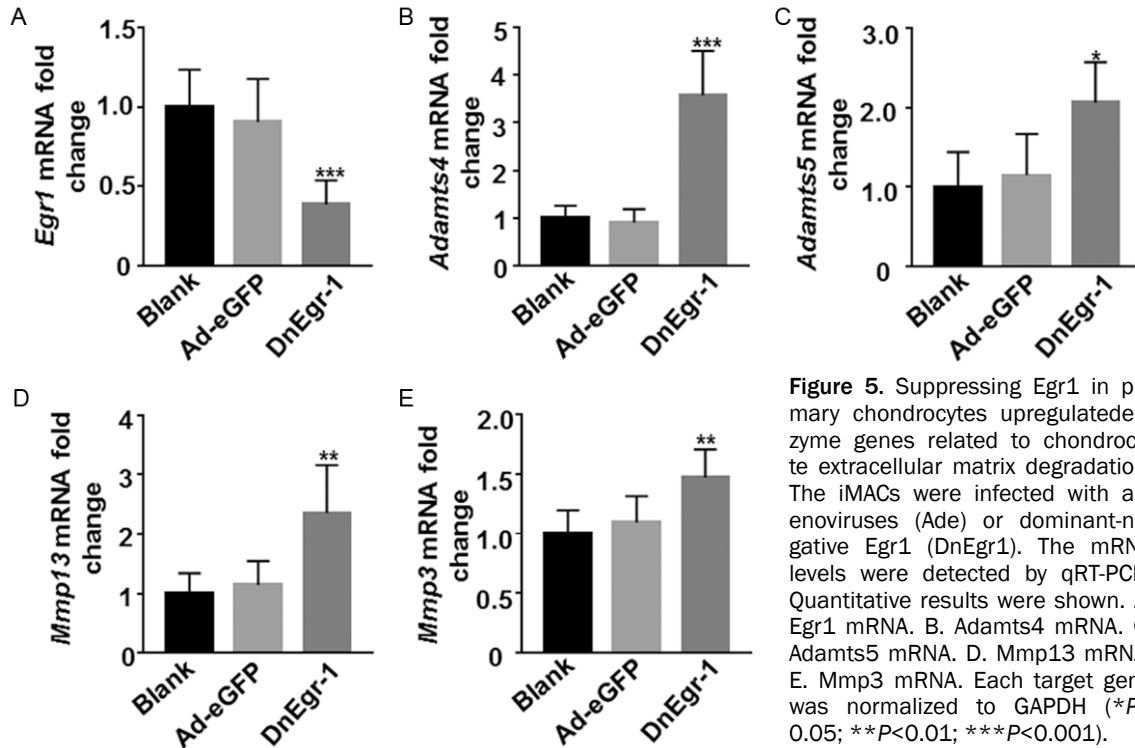


Figure 4. Egr1 KO mice showed relatively limited chondrocyte extracellular matrix mineralization. Microscopic appearance of the femoral head cartilage of Egr1 KO and WT mice was observed. Paraffin-embedded sections were stained with (A) H&E and (B) Safranin-O. Magnification was 100 \times , 200 \times and 400 \times , respectively. (C) Representative sections from Egr1 KO and WT mice cartilage were stained with TUNEL (WT, n=6; KO, n=6). (D) Immunohistochemical staining for the cleaved Caspase 3 in the cartilage of Egr1 KO and WT mice. (E) Apoptotic cells per field were counted from 5 slides (10 fields in each slide). The mRNA expression of (F) Caspase 3, (G) Caspase 6 and (H) Caspase 8 in iMACs transfected with dominant-negative Egr1 (DnEgr1) virus was detected by qRT-PCR. (* P <0.05; ** P <0.01; *** P <0.001).

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wearing areas of the femoral head of relatively shorter patients. We also observed that Egr1 KO mice exhibited reduced body size and femur length at adulthood. Thus, we speculate that Egr1 might play a role in the regulation of limb length.

A research by Chen *et al.* found that ERK1 and ERK2 were able to disrupt endochondral bone formation by influencing chondrocyte terminal differentiation and reducing the secretion of factors such as MMP13 and osteopontin, resulting in the reduced limb length in mice [32]. In this research, suppressing the Egr1 gene in iMACs caused an increased expression of secreted enzymes that related to extracellular matrix degradation, such as *Adamts4*, *Adamts5*, *Mmp3* and *Mmp13*. Morphological abnormalities in the subchondral bone cells of Egr1 KO mice were observed. A research by Dalcq *et al.* [10] also demonstrated the importance of Egr1 in cartilage development. They found that Follistatin A expression was increased in Egr1 KO zebrafish, which inhibited BMP signaling and ultimately affected cartilage development in the pharyngeal region [10]. These findings suggest that Egr1 may affect the chondrocyte proliferation in the period of

endochondral ossification, which may regulate the limb length.

RUNX2 has a major effect on chondrocyte differentiation and chondrocyte extracellular matrix deposition [33, 34]. Yoshida *et al.* found that RUNX2 and RUNX3 might be involved in the development of limb (29). When RUNX2/3 genes were knocked out, reduced chondrocyte proliferation and hypertrophy were observed, leading to decreased Indian hedgehog (*IHH*) expression and reduced limb length [35]. Komori *et al.* revealed that RUNX2, CBFA1 and PEBP2 α A might play a major role during chondrocyte terminal differentiation [36]. Additionally, RUNX2 and AP-1 could regulate MMP13 expression together, thus influencing the process of cartilage matrix degradation during endochondral bone formation [37, 38]. In this study, we found that RUNX2 expression was noticeably reduced in Egr1 KO mice. However, ChIP and luciferase reporter assays revealed that Egr1 did not directly regulate RUNX2 expression. Thus, indirect regulation might be achieved through the use of additional factors. Some researchers have demonstrated that PPAR γ has a similar effect on the regulation of cartilage metabolism. For example, Vasheghani

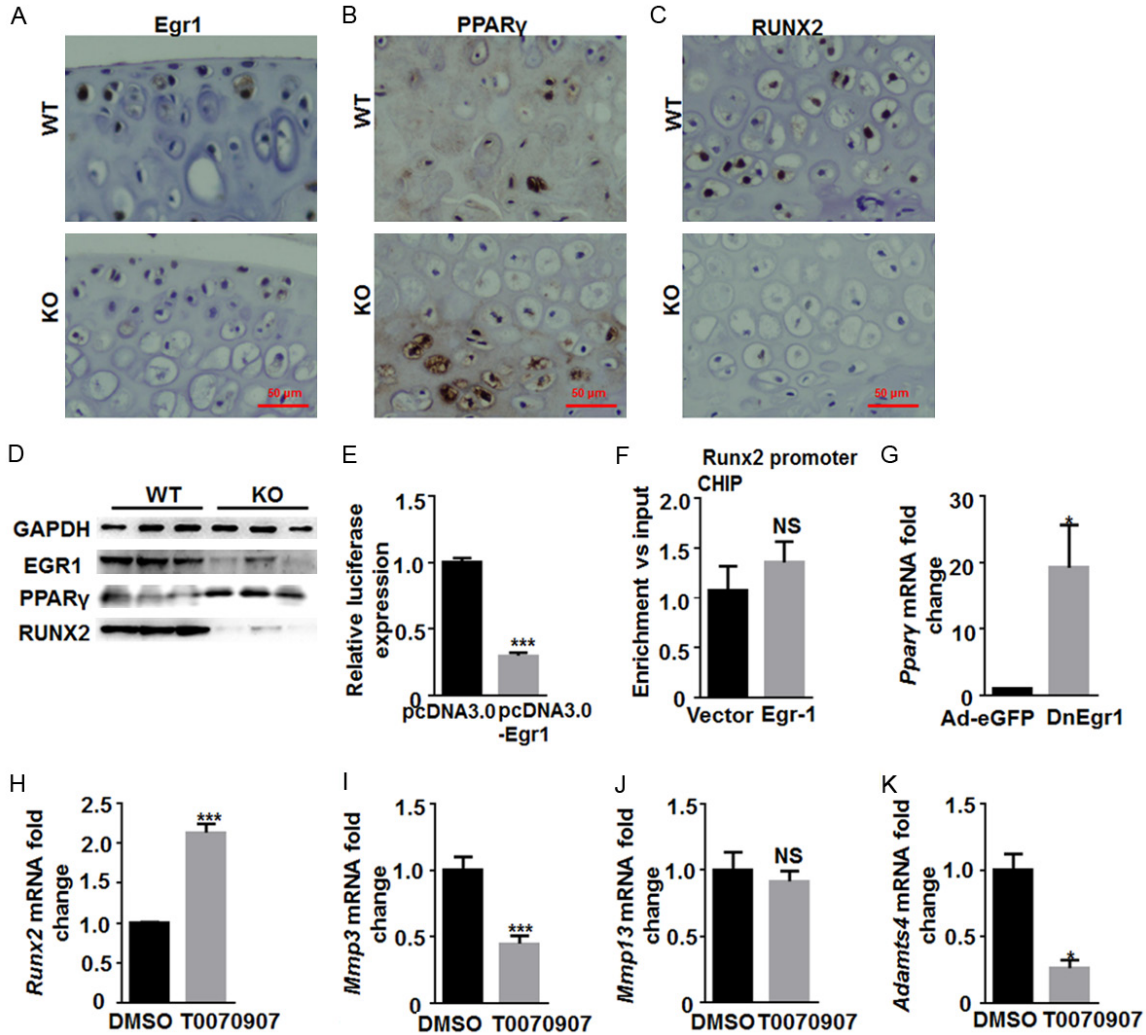


Figure 6. Egr1/PPAR γ /RUNX2 signaling pathways participate in the regulation of the chondrocyte extracellular matrix. Representative images of the immunohistochemical staining for (A) Egr1, (B) PPAR γ and (C) Runx2 in the Egr1 KO and WT mice cartilage. (D) Western blot was used to analyze the Egr1, PPAR γ and Runx2 protein expression in the Egr1 KO and WT mice. GAPDH was used as control. (E) A luciferase reporter gene assay for the detection of Egr1 effect on Runx2. (F) Chromatin immunoprecipitation was performed in iMACs using antibodies of Egr1 to examine the direct interaction between Egr1 and Runx2. (G) qRT-PCR analysis of PPAR γ mRNA was performed after transfected DnEgr1 virus into iMACs. The mRNA expression of (H) Runx2, (I) Mmp3, (J) Mmp13 and (K) ADAMTS like 4 in iMACs were analyzed by qRT-PCR after treated with PPAR γ inhibitors, T0070907 or DMSO. (* P <0.05; ** P <0.01; *** P <0.001).

et al. found that cartilage-specific PPAR γ KO mice exhibited aggravated progression of spontaneous osteoarthritis [39]. In addition, Monemdjou *et al.* observed reduced limb length, reduced body weight and slow skeletal development in cartilage-specific PPAR γ KO mice [22]. In this study, we observed a noticeable increase in PPAR γ expression when suppressed Egr1 expression in primary chondrocytes, suggesting that Egr1 may have an inhibitory effect on PPAR γ . These findings are consistent with

the ones by Zhou *et al.*, which reported that PPAR γ expression in hepatic stellate cells was inhibited when using Egr1 expression was upregulated [40]. In addition, when we inhibited PPAR γ expression in iMACs using the inhibitor T0070907, RUNX2 expression was markedly increased, while Mmp3 and Adamts4 mRNA expression was noticeably reduced. These results indicated that PPAR γ might participate in the regulation of chondrocyte extracellular matrix by inhibiting RUNX2 expression.

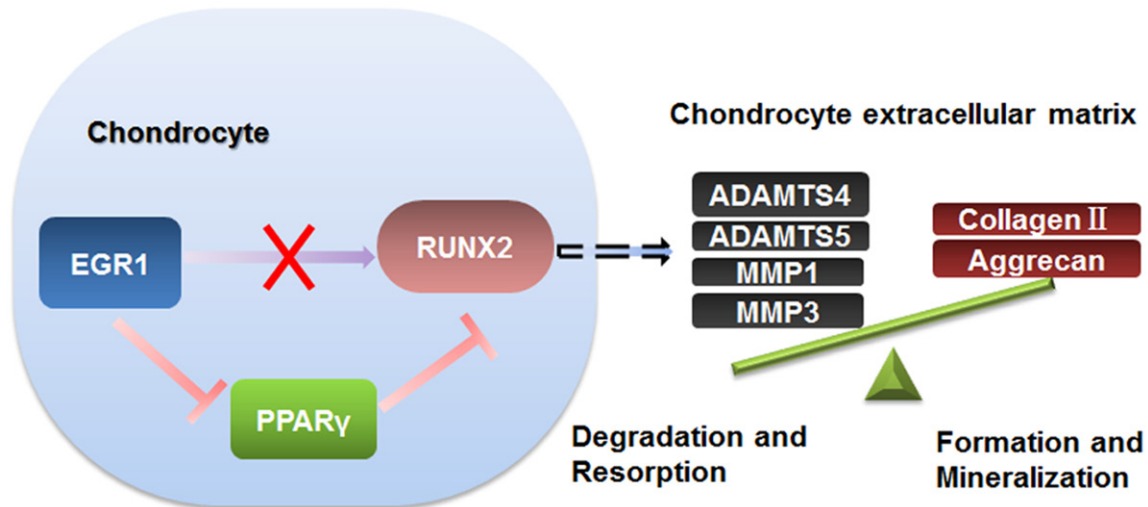


Figure 7. A model for the dynamic equilibrium regulation of chondrocyte extracellular matrix by Egr1. In the process of cartilage enlargement, knockout of Egr1 in chondrocytes disrupts the dynamic equilibrium of chondrocyte extracellular matrix. The inhibition of Egr1 on PPAR γ and the inhibition of PPAR γ on RUNX2 were relieved, which lead to upregulation of the enzyme genes related to chondrocyte extracellular matrix degradation and affect skeletal development.

Conclusion

Egr1 KO mice showed relatively shorter limb length and reduced bone volume. PPAR γ upregulation inhibited RUNX2 expression, leading to the promotion of MMPs family members, disturbing the normal apoptosis of chondrocyte and finally decreasing the equilibrium of chondrocyte extracellular matrix (**Figure 7**). These findings improve our understanding about endochondral bone development abnormalities and the role of Egr1 in regulating this physiological process.

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Disclosure of conflict of interest

None.

Abbreviations

Egr1, Early growth response protein 1; PPAR γ , peroxisome proliferator-activated receptor γ ; RUNX2, Runt-related transcription factor 2; iMACs, Immature murine articular chondrocytes; MMPs, Matrix metalloproteinases; ADAM, Aggrecanases; BMD, Bone mineral density; BV/TV, Bone volume per tissue volume; Tb. N, Trabecular number; Tb. Sp, Trabecular separation; Tb. Th, Trabecular thickness; Ct. Th, Cortical thickness; PFA, Paraformaldehyde; EDTA, Ethylene Diamine Tetraacetic Acid; Qpcr, Quantitative PCR; ECM, Extracellular matrix.

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