TMF inhibits extracellular matrix degradation in osteoarthritis cartilage by regulating the Sirt1/STAT3 signaling pathway

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Abstract: **Objective**: Osteoarthritis (OA) is a degenerative joint disease characterized by extracellular matrix (ECM) degradation, chondrocyte apoptosis, and chronic inflammation. Cartilage destruction and ECM degeneration contribute to joint function loss and disability. Signal transducer and activator of transcription 3 (STAT3) up-regulates the expression of MMP-13, which degrades collagen Ⅱ. Our previous study found that 5,7,3',4'-tetramethoxyflavone (TMF) exhibited protective effects on OA chondrocytes. This study aims to investigate the protective role of TMF in inhibiting ECM degradation by mediating the Sirt1/STAT3 signaling pathway. **Methods**: Rat OA models were established by the injection of monosodium iodoacetate (MIA). Hematoxylin & eosin (HE) staining and immunohistochemistry (IHC) analysis were performed. IL-1β stimulated C28/I2 cells were used as OA-like chondrocyte cell model. Western blotting assays were used to determine the protein expression. **Results**: The expression of MMP-13 was upregulated while type Ⅱ collagen expression is downregulated, and the phosphorylation level of STAT3 is increased in rat OA models. TMF reverses the STAT3-mediated expression of MMP-13 and type v collagen. Activation of STAT3 or inhibition of Sirt1 function attenuates the inhibitory effect of TMF on ECM degradation. **Conclusion**: TMF can inhibit ECM degradation mediated by the STAT3 signal pathway by activating Sirt1 expression in OA cell and animal models.

Key words: Osteoarthritis; Extracellular matrix degradation; Chondrocytes; 5, 7, 3', 4'-tetramethoxyflavone; Signal transduction and activator of transcription
CLC Number: R684 **Document code**: A **Article ID**: 2097-7174(2026)01-0007-09
DOI: 10.3969/j.issn.2097-7174.2026.01.002

TMF通过调控Sirt1/STAT3信号通路抑制骨关节炎软骨胞外基质降解

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摘 要:**目的**:骨关节炎(Osteoarthritis, OA)是一种以胞外基质(Extracellular matrix, ECM)降解、软骨细胞凋亡及慢性炎症为特征的退行性关节疾病。软骨破坏及ECM降解是造成关节功能丧失和残疾的重要因素。信号转导和转录激活因子3(Signal transduction and activator of transcription 3, STAT3)可上调MMP-13的表达,而后者可降解Ⅱ型胶原蛋白。前期研究表明,5,7,3′,4′-四甲氧基黄酮(5,7,3′,4′-tetramethoxyflavone, TMF)对软骨细胞具有保护性。本研究旨在探讨TMF通过调控Sirt1/STAT3信号通路而抑制ECM降解。**方法**:通过关节腔注射碘乙酸钠构建大鼠OA模型,HE染色和免疫组织化学分析研究软骨病理变化。采用IL-1β刺激C28/I2细胞建立OA样软骨细胞模型。利用Western blot检测蛋白质表达。**结果**:在大鼠OA模型中,MMP-13表达上调,而Ⅱ型胶原蛋白表达是下

Founding: Project Supported by Jiangxi Provincial Natural Science Foundation(20212ACB206002)

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调。STAT3的磷酸化水平提高。TMF可逆转STAT3调控的MMP-13和Ⅱ型胶原蛋白的表达。活化STAT3或抑制Sirt1功能可减弱TMF对ECM降解的抑制作用。**结论**:OA细胞和动物模型表明,TMF通过激活Sirt1表达而抑制STAT3信号通路介导的ECM降解。

关键词:骨关节炎;胞外基质降解;软骨细胞;5,7,3′,4′-四甲氧基黄酮;信号转导及转录激活因子

Osteoarthritis (OA) is a progressive joint disease characterized by degenerative extracellular matrix (ECM) of cartilage, chondrocyte apoptosis, and chronic inflammation. The risk factors for OA development include aging, obesity, genetics, gender, and trauma. The prevalence of OA increases with the growth of the aged population[1]. OA has become the 11th cause of years lived with disability worldwide, according to the WHO Global Burden of Disease Study[2]. Joint pain, swelling, and morning stiffness are the common clinical symptoms of OA. There are not any disease-modifying drugs available to cure OA. Inhibition of ECM degradation and promotion of ECM synthesis are useful strategies to maintain joint functions.

The joint articular cartilage is composed of chondrocytes and ECM, and they occupy 5% and 95%, respectively, of the space in the cartilage[3]. However, the homeostasis of ECM is maintained by chondrocytes, the main cell type in the cartilage. The cartilage ECM is constructed by a network of biological macromolecules, such as collagens and proteoglycans. Collagen Ⅱ accounts for 90%-95% of collagens and acts as the structural backbone in the ECM. Aggrecan is the main component of proteoglycan in the joint cartilage and forms a macromolecular aggregate by interacting with collagen fibers and hyaluronic acid[4]. Matrix metalloproteases (MMPs) have been known to degrade collagens. In joint cartilage, MMP-13 exhibits a strong affinity to collagen Ⅱ and degrades it[5]. MMP-13 is a catabolic factor for OA pathogenesis, and inhibition of MMP-13 can reduce the severity of OA[6]. MMP-13 has become a drug target for OA treatment.

Signal transducer and activator of transcription 3 (STAT3), a transcription factor in the STAT family, regulates a diverse of signaling pathways in various diseases, such as OA. It has been reported that STAT3 is highly active in OA chondrocytes and promotes OA progression by activating the NF-κB signaling pathway[7]. One study reports that STAT3 can up-regulate the expression of MMP-13 and ADAMTS-5, which exert catabolic activity to degrade ECM in OA cartilage[8]. Another study reports that IL-6 can increase the transcriptional expression of MMP-1, MMP-3, and MMP-13 by promoting the binding of STAT3 dimers to their promoters in synovial fibroblasts[9]. Sirt1, a deacetylase, has been reported to interact with STAT3 and inhibit its activation through deacetylation[10]. The roles of the Sirt1/STAT3 signaling in the ECM degradation in OA cartilage are still unclear. Natural flavonoids have been reported to activate Sirt1[11]. Our previous study found that 5, 7, 3′, 4′-tetramethoxyflavone (TMF) can also activate Sirt1 and ameliorate cholesterol dysregulation in OA chondrocytes[12]. In this study, we further investigated the protective activity of TMF against ECM degradation in OA chondrocytes through modulating the Sirt1/STAT3 signaling pathway. This study will help to understand the pharmacological activity of TMF and the pathogenesis of OA cartilage.

1 Materials and methods

1.1 General information The study was approved by the Institutional Animal Care and Use Committee of Gannan Medical University under the guidelines of the Declaration of Helsinki Principles and the principles for laboratory animal use. The male rats (200±20) g, obtained from the Laboratory Animal Center of Gannan Medical University, were kept under the SPF environment [(22±1) ℃ of the temperature, 45%-55% of the humidity, and 12 h of the light/dark cycle]. Rats were free access to water and food.

1.2 Duplication of rat OA models The rat OA models were established by using the intra-articular injection of monosodium iodoacetate [MIA: 1 mg/(20 μL)][13]. The rats in the negative control group received the same volume of vehicle. The OA rats in the treated group were orally administered 50 mg/kg and 100 mg/kg of TMF, respectively, for

one month. After that, all rats were sacrificed by cervical dislocation. The knee articular cartilage tissues were collected and prepared for further analysis.

1.3 Hematoxylin & eosin (HE) staining and immunohistochemistry (IHC) analysis The collected articular cartilage was fixed using 4% paraformaldehyde and decalcified using 10% EDTA. Next, the articular cartilage was embedded in the paraffin and cut into 5 μm slices. The slices were dewaxed using xylene and dehydrated using 100%, 90%, and 80% ethanol sequentially. Then, HE staining was performed.

IHC analysis: The dewaxed slices were processed to block the endogenous peroxidase activity using 3% H_2O_2. After 30 min incubation with the goat serum (10%), the slices were further co-incubated overnight at 4 °C with the primary antibodies, including MMP-13 (Cat. no. AF5355; 1∶100 dilution; Affinity) and collagen Ⅱ (Cat. no. AF0135; 1∶100 dilution; Affinity). After being washed with PBS three times, the slices were further co-incubated with the goat anti-rabbit HRP-conjugated secondary antibody. DAB was employed for staining.

1.4 Cell culture C28/I2 cells were cultured in DMEM (Gibco, Germany) containing 10% FBS (Gibco), 100 U penicillin (Gibco), and 100 U streptomycin (Gibco) at 37 °C in an incubator with 5% CO_2. IL-1β (10 ng/mL; Sigma, MO, USA) was added to stimulate chondrocytes at 37 ℃ for 24 h. Cells were treated with TMF (5 μg/mL and 20 μg/mL, respectively) at 37 ℃ for 24 h, according to the protocol in our previous studies[12,14]. A STAT3 activator Colivelin and a Sirt1 inhibitor EX527 were, respectively, used for intervention.

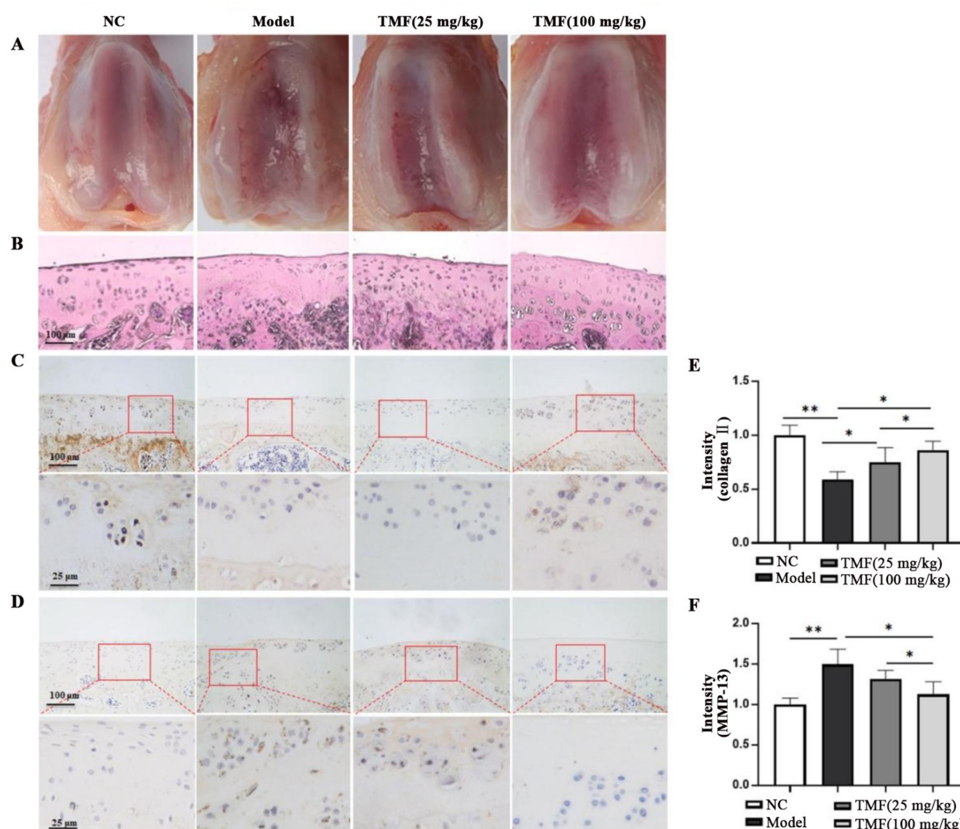
1.5 Western blot Cells were harvested and lysed, and the total protein was collected. 30 μg of the total proteins was separated by 10% SDS-PAGE and then transferred onto PVDF membranes. The primary antibodies against MMP-13 (1∶1 000 dilution; Affinity, cat. no. AF5355), collagen Ⅱ (1∶1 000 dilution; Affinity, cat. no. AF0135), STAT3 (1∶1 000 dilution; Affinity, cat. no. AF6294), p-STAT3 (1∶1 000 dilution; Affinity, cat. no. AF3293), and Sirt1 (1∶1 000 dilution; Affinity, cat. no. DF6033) and β-actin (1∶1 000 dilution; Affinity, cat. no. AF7018) were added for incubation overnight at 4 ℃. Then, the goat anti-rabbit HRP-conjugated secondary antibody (1∶2 000 dilution; Boster Biological Technology, Wuhan, China) was added. The protein bands were analyzed by the enhanced chemiluminescence detection system and analyzed by the Fiji ImageJ (an open-source image processing package).

1.6 Statistical analysis Data are shown as mean ± standard deviation. GraphPad Prism v8 Software was utilized for statistical analysis. One-way analysis of variance (ANOVA) prior to Bonferroni's multiple comparisons test was employed for statistical analysis. A value of $P<0.05$ represented a statistically significant difference.

2 Results

2.1 TMF inhibited ECM degradation in rat OA cartilage The rat OA models were established by the intra-articular injection of MIA. The results (Figure 1A) showed that the surface of the cartilage in the model group was erosive. HE staining indicated that the thickness of cartilage and the number of chondrocytes were significantly reduced in the model group (Figure 1B). The results from the IHC examination showed that the expression of collagen Ⅱ was down-regulated (Figure 1C-1D) and that of MMP-13 was up-regulated (Figure 1E-1F) in the model group. However, TMF exhibited protective activity, as shown by the ameliorated pathological changes, the up-regulated collagen Ⅱ expression, and the down-regulated MMP-13 expression. Thus, TMF exhibited protective activity against MIA-induced ECM degradation in rat OA cartilage.

2.2 TMF inhibited ECM degradation in IL-1β-treated C28/I2 cells Human normal chondrocyte C28/I2 cells were treated with IL-1β to establish OA chondrocytes in vitro. The expression of MMP-13 (Figure 2A) was up-regulated, and collagen Ⅱ (Figure 2B) was down-regulated. However, TMF inhibited ECM degradation by reversing IL-1β-mediated expression of collagen Ⅱ and MMP-13 in



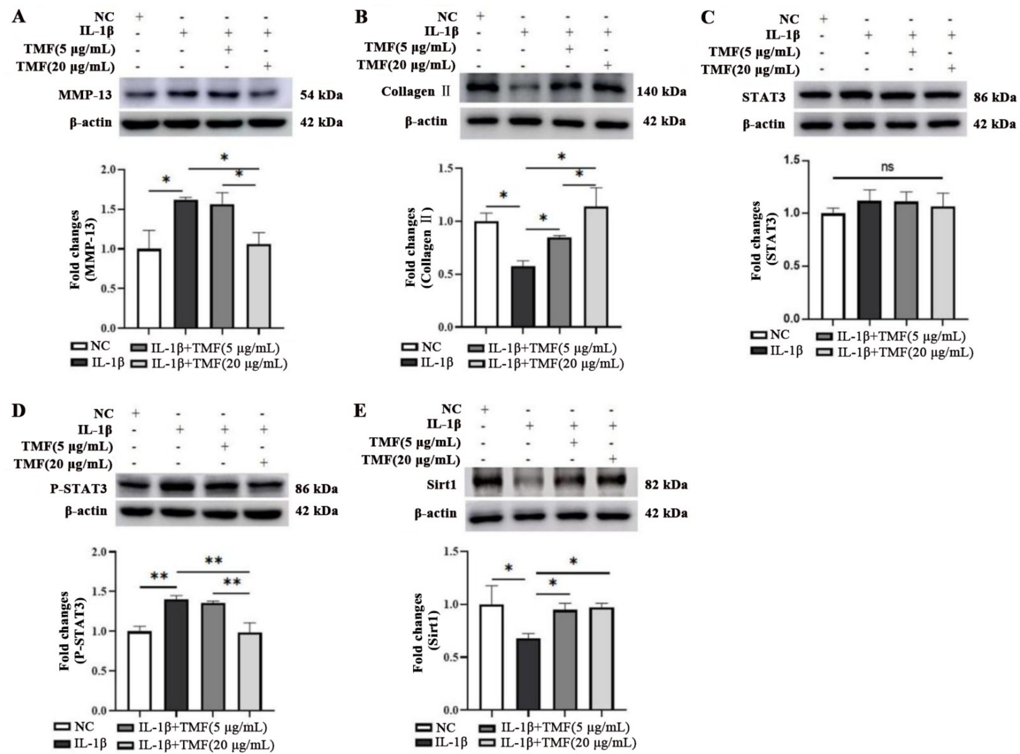
(A) Tissues morphology of knee cartilage under microscope (magnification ×4). (B) General histomorphological architecture of OA cartilage by HE stain. The expression of collagen Ⅱ (C–D) and MMP–13 (E–F) by IHC. *P<0.05 and **P<0.01.

Figure 1 The pathological changes of TMF on MIA-induced ECM degradation in rat OA cartilage

C28/I2 cells. In addition, TMF also reversed IL-1β-mediated expression of STAT3 (Figure 2C), p-STAT3 (Figure 2D), and Sirt1 (Figure 2E). Thus, the protective activity of TMF in inhibiting IL-1β-stimulated ECM degradation might be associated with the regulation of the Sirt1/STAT3 pathway in C28/I2 cells.

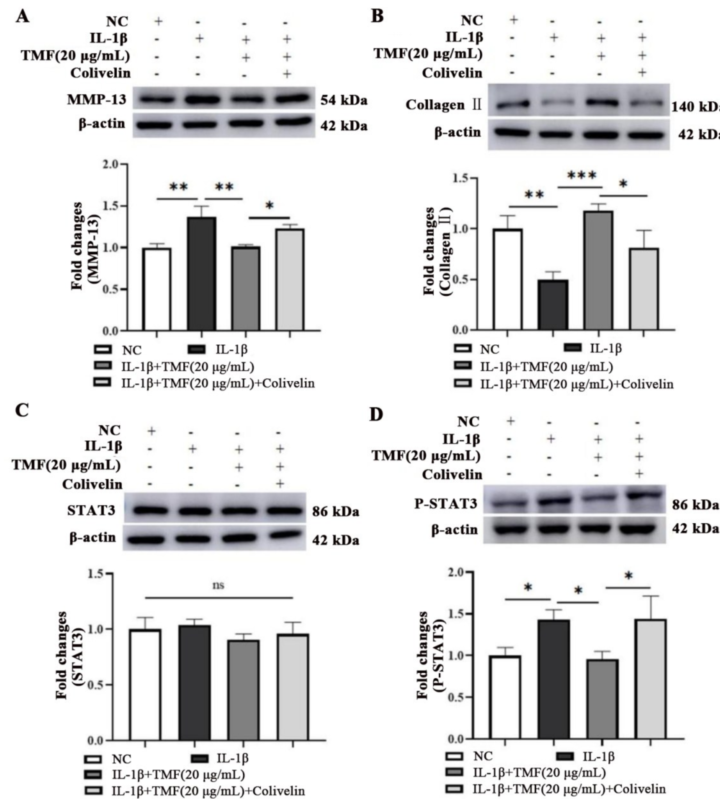
2.3 TMF inhibited ECM degradation by inhibiting STAT3 activity in IL-1β-treated C28/I2 cells To investigate the underlying mechanism of TMF in protecting against ECM degradation in OA chondrocytes, a STAT3 activator Colivelin was used. The results showed that Colivelin compromised the protective activity of TMF, as shown by the up-regulated expression of MMP-13 (Figure 3A) and the down-regulated expression of collagen Ⅱ (Figure 3B). In addition, Colivelin could not markedly affect the protein expression of total STAT3 (Figure 3C) but increased the phosphorylation levels of STAT3 (Figure 3D). Thus, Colivelin blocked the effects of TMF on the expression of collagen Ⅱ and MMP-13 in IL-1β-treated C28/I2 cells.

2.4 TMF ameliorated STAT3-induced ECM degradation by activating Sirt1 expression To investigate how TMF exhibited protective activity against ECM degradation, a Sirt1 inhibitor EX527 was employed. The results showed that EX527 treatment blocked the protective effects of TMF on ECM degradation, as shown by the up-regulated expression of MMP-13 (Figure 4A) and the down-regulated expression of collagen Ⅱ (Figure 4B). In addition, EX527 treatment did not affect the protein expression of STAT3 (Figure 4C) but enhanced the phosphorylation levels of STAT3 (Figure 4D). EX527 decreased the expression of Sirt1 (Figure 4E) in IL-1β-treated C28/I2 cells. Collectively, EX527 blocked the effects of TMF on the expression of collagen Ⅱ and MMP-13 in IL-1β-treated C28/I2 cells.



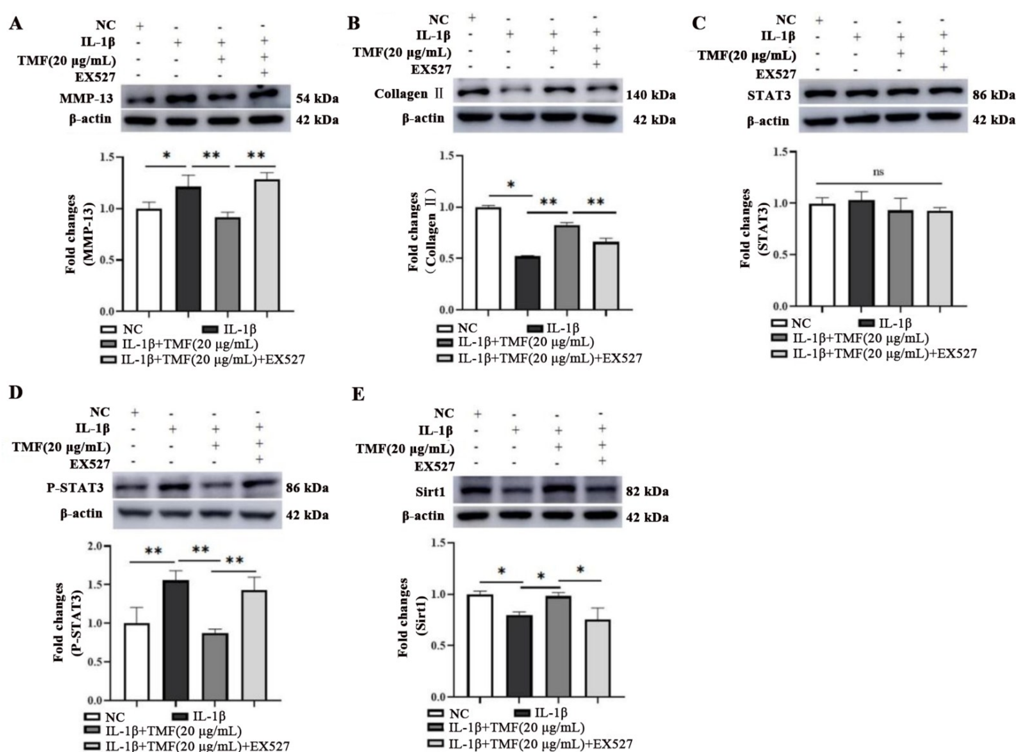
The protein expression level of MMP-13（A）, collagen Ⅱ（B）, STAT3（C）, p-STAT3（D）, and Sirt1（E）. *P<0.05, **P<0.01, and ***P<0.001.

Figure 2　The effects of TMF on ECM degradation in IL-1β-treated C28/I2 cells by Western blot



The protein expression level of MMP-13（A）, collagen Ⅱ（B）, STAT3（C）, and p-STAT3（D）. *P<0.05, **P<0.01, and ***P<0.001.

Figure 3　TMF inhibited ECM degradation by inhibiting STAT3 activity in IL-1β-treated C28/I2 cells by Western blot



The protein expression level of MMP-13 (A), collagen Ⅱ (B), STAT3 (C), p-STAT3 (D), and Sirt1 (E). *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

Figure 4 TMF ameliorated STAT3-induced ECM degradation by activating Sirt1 expression by Western blot

3 Discussion

OA has caused the social, economic, and physical burdens on patients. Currently, non-steroidal anti-inflammatory drugs (NSAIDs) are the first-line treatments for relieving clinical symptoms. However, NSAIDs cannot modify the progression of OA, and some side effects, such as gastrointestinal risk, have significantly compromised the effects of NSAIDs[15]. It is urgent to explore the effective and safe agents for OA treatment. In the current study, we found that TMF could inhibit ECM degradation in vivo and in vitro. The expression of collagen Ⅱ was down-regulated, and that of MMP-13 was up-regulated in OA chondrocytes. TMF improved the pathological changes of OA chondrocytes. Activation of STAT3 could significantly reverse the effects of TMF on ECM degradation, and inhibition of Sirt1 blocked the protective effects of TMF. These highlighted that TMF could be a potential agent to inhibit ECM degradation in OA chondrocytes and verified the pharmacological effects of TMF in OA cartilage by mediating the Sirt1/STAT3 pathway.

In normal cartilage tissues, the metabolism of ECM is kept in a dynamic equilibrium. Collagen has a complex structure with a tightly wound triple helix, and it can be degraded by MMPs. MMPs can interact with the substrate collagen and access the cleavage site via its C-terminal hemopexin domain[16]. Numerous subtypes of collagen, including types Ⅰ, Ⅱ, Ⅲ, Ⅳ, Ⅴ, Ⅵ, Ⅸ, Ⅺ, Ⅻ, ⅩⅥ, and ⅩⅩⅧ, have been reported in the articular cartilage[17]. Collagen Ⅱ accounts for 90%-95% of hyaline cartilage collagen, and it is the main component of the cartilage matrix. Collagen Ⅱ can form polymers with collagen Ⅸ, Ⅺ, Ⅻ, and ⅩⅣ to be a collagen network, which increases cartilage tensile strength[18]. ECM plays an important role in maintaining the integrity and functionality of articular cartilage. Thus, collagen becomes a target for cartilage degeneration. The important role of MMP-13 in OA cartilage degeneration has been reported. MMP-13 expression is significantly up-regulated in OA cartilage tissues[19]. Consistently, our study also showed that the expression of MMP-13 was up-regulated, and the expression of collagen Ⅱ was down-regulated in rat

OA models.

STAT3 is a highly conserved protein associated with inflammatory responses and cell growth mediation. STAT3 has six conserved domains, including the N-terminal domain, coiled-coil domain, DNA-binding domain, linker domain, SRC homology 2 (SH2) domain, and C-terminal transactivation domain (TAD)[20]. The SH2 domain recognizes and interacts with the phosphorylated tyrosine motif, resulting in STAT3 dimerization. Tyr705 and Ser727 are the two phosphorylation sites in the TAD. After phosphorylation, STAT3 dimerizes and translocates into the nucleus for transcriptional regulation[21]. STAT3 can be activated by various factors, such as hormones, cytokines, and growth factors. For example, IL-6, IL-11, and TLRs can activate STAT3, which mediates the gene expression of VEGF, MMP-2, and MMP-9[22]. It has been reported that STAT3 can be stimulated by various cytokines in the OA chondrocytes. STAT3 activation facilitates OA development. Inhibition or knockdown of STAT3 expression can alleviate angiogenesis-mediated chondrocyte lesions and osteogenic differentiation. STAT3 blockade might be a strategy for OA treatment[23]. In this study, we found that the phosphorylation levels of STAT3 were enhanced, indicating the activation of STAT3 in OA chondrocytes.

It has been reported that STAT3 signaling can up-regulate the expression of downstream genes, such as MMP-2 and MMP-9, inhibiting cell metastasis[24]. In nucleus pulposus cells, IL-1β up-regulates MMP-13 and ADAMTS-5 expression and down-regulates collagen Ⅱ and aggrecan expression. Cyaniding can mitigate disc degeneration and suppress IL-1β-mediated ECM degradation by inhibiting the JAK2/STAT3 signaling pathway[25]. Down-regulation of STAT3 expression has been associated with the decreased inflammatory responses, the attenuated ECM degradation, and the improved chondroprotection[26]. Inhibition of STAT3 can suppress the apoptosis of OA chondrocytes and prevent or slow OA development[27]. In addition, inhibition of STAT3 also blocks p21-induced MMP-13 expression, protecting against OA development[28]. Another study reports that STAT3 dimer can interact with the promoter of MMP-13 and promote its expression[9]. In this study, we found that activation of STAT3 could down-regulate collagen Ⅱ expression and up-regulate MMP-13 expression.

It has been reported that deletion of Sirt1 expression may induce the enhancement of p-STAT3 and MMP-13 in vivo and in vitro. Sirt1 functions as a suppressor of STAT3, opposing the damaging effects of STAT3[29]. Sirt1-mediated deacetylation may reduce the ability of STAT3 to translocate into the nucleus and transcriptionally regulate the target gene expression[30]. In addition, p300-mediated STAT3 acetylation may promote its transcriptional activity, and p300 silencing abolishes it[31]. In this study, we found that inhibition of Sirt1 increased the phosphorylation level of STAT3, up-regulated the expression of MMP-13, and down-regulated the expression of collagen Ⅱ in OA chondrocytes. Many natural flavonoids, such as genistein[32] and nobiletin[33], have been reported to activate Sirt1. Our previous study also showed that TMF could activate Sirt1 expression and protect against OA development[12]. In this study, we found that TMF up-regulated Sirt1 expression, suppressed STAT3 phosphorylation, and attenuated ECM degradation. However, inhibition of Sirt1 might abolish the protective effects of TMF on ECM.

There are some limitations in this study. The transcriptional regulation of STAT3 on the expression of MMP-13 still needs more investigation. The mechanism of IL-1β in promoting the phosphorylation of STAT3 should be studied. The mechanism of TMF in activating Sirt1 still needs further investigation. Many signaling pathways, including the STAT3 pathway, can be the downstream factors of Sirt1. Inhibition of Sirt1 may induce complex changes in molecular processes. Our current study merely focused on the Sirt1/STAT3 signaling in OA cartilage. The effects of TMF on the distribution and function of STAT3 should be elucidated. Sirt1 can deacetylate STAT3. However, we did not determine the expression of deacetylated or acetylated STAT3. How TMF activates Sirt1 to inhibit STAT3 phosphorylation is still unclear.

4 Conclusion

In this study, the down-regulated expression of collagen Ⅱ and the up-regulated expression of MMP-13 and STAT3 were observed in OA chondrocytes. STAT3 activation could up-regulate MMP-13 expression, promoting OA development. Sirt1 inhibition promoted STAT3 phosphorylation and ECM degradation. However, TMF inhibited STAT3-mediated ECM degradation by activating Sirt1 expression, protecting against OA development.

All authors have declared that no conflicts of interest exist.

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(收稿:2025-04-30)(修回:2025-11-30)

(责任编辑:眭荣燕)