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ORIGINAL RESEARCH

RETRACTED ARTICLE: TMF inhibits miR-29a/Wnt/β-catenin signaling through upregulating Foxo3a activity in osteoarthritis chondrocytes

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Background: miR-29a, a downstream factor of Wnt/β-catenin signaling, promotes the activity of the Wnt/β-catenin signaling in a positive feedback loop. Our previous work showed that 5,7,3′,4′-tetramethoxyflavone (TMF), a major constituent from *Murraya exotica* L., exhibited chondroprotective activity by inhibiting the activity of Wnt/β-catenin signaling.
Purpose: To investigate whether TMF showed the inhibitory effects on miR-29a/β-catenin signaling by up regulation of Foxo3a expression.
Methods: Rat knee OA models were duplicated by using Hulth's method. TMF (5 μg/mL and 20 μg/mL) was used for administration to cultured cells, which were isolated from the rat cartilages. Analysis of chondrocytes apoptosis, gene expression, and protein expression were conducted. In addition, miR-29a mimics and pcDNA3.1(+)-Foxo3a vector were used for transfection, luciferase reporter assay for detecting the activity of Wnt/β-catenin signaling, and co-immunoprecipitation for determining proteins interaction.
Results: TMF down regulated miR-29a/β-catenin signaling activity and cleaved caspase-3 expression and up regulated Foxo3a expression in OA rat cartilages. In vitro, miR-29a mimics down regulated the expression of Foxo3a and up regulated the activity of Wnt/β-catenin signaling and cleaved caspase-3 expression. TMF ameliorated miR-29a/β-catenin-induced chondrocytes apoptosis by up regulation of Foxo3a expression.
Conclusion: TMF exhibited chondroprotective activity by up regulating Foxo3a expression and subsequently inhibiting miR-29a/Wnt/β-catenin signaling activity.
Keywords: osteoarthritis, chondrocytes apoptosis, miR-29a, Wnt/β-catenin, Foxo3a, TMF

Introduction

MicroRNAs (miRNAs), a set of endogenous non-protein-coding RNA molecules, are approximately 22 nucleotides in length. The long primary transcripts (pri-miRNAs) transcribed from the genome are processed into small hairpin precursor miRNAs (pre-miRNAs), which are cleaved into mature and functional miRNAs by Dicer after being transported into the cytoplasm.[1] miRNAs typically mediate the post-transcriptional expression of certain genes by binding to the 3′ un-translated region (3′-UTR) of target mRNAs, therefore regulating various physiological and pathological processes.[2] miRNAs can be a master in regulating the expression of genes and influencing cell activities and events virtually. However, the regulatory activities of miRNAs depend on the extent of sequence complementarity between miRNAs and 3′-UTR of target mRNAs.[3] Thus, the regulatory profiles of miRNAs are greatly enriched, and they become as the signatures to identify and predict the

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outcome of some diseases, such as cancers.[4] The molecular mechanisms of miRNAs in affecting the processes of diseases are still poorly understood. miRNAs have been demonstrated to biologically function in the development and homeostasis in cartilage.[5]

Osteoarthritis (OA) is one of the most common age-related degenerative diseases with characteristic signs, such as pain, transient morning stiffness, and crepitus.[6,7] More than 10% of the population above 60 years old in the world are estimated to have OA, indicating a large socio-economic burden.[8] The epidemiology of OA is complex and multi-factorial, with genetic, biological, and biomechanical components.[7,9] Recently, it has been reported that OA is not the absolute consequence of joint mechanical use. The implication of inflammatory cytokines contributes to the development and progression of OA.[10] Cartilage, subchondral bone, and synovium probably have critical roles in OA pathogenesis. Cartilage homeostasis is essential for joint functionality and might be maintained by balanced molecular network of signaling pathways.[11] Biological pathways in cartilage could be modifiable and offer a potential strategy for intervention. Enhanced expression of Wnt/β-catenin signaling has been shown in OA cartilage chondrocytes, and inhibition of Wnt/β-catenin signaling is implicated in maintaining chondrocytes phenotypic stability. In absence of Wnt signaling, β-catenin in cytosol is degraded. Binding of Wnt to the receptors Frizzled and LRP5/6, β-catenin stabilized and accumulates in cytoplasma and translocated into the nucleus to induce genes transcription.[12] Our previous work demonstrated that Wnt/β-catenin signaling played a critical role in the development of OA.

Epigenetic modification has been involved in OA pathogenesis at all of the levels including DNA methylation, histone modification, miRNAs, and long non-coding RNA.[10] Accumulating evidence has demonstrated that miRNAs play a crucial role as regulators of cartilage biology and OA pathogenesis.[14] Microarray analysis by Miyaki (2009) found that miR-140 expression was downregulated in OA chondrocytes. Deletion of miR-140 predisposed mice to develop age-related OA-like changes.[15] Recently, it has been demonstrated that miR-29b regulates chondrogenesis homeostasis and enhances hyperptrophic phenotype.[16] It has been shown that the promoter of miR-29a contains TCF/LEF binding sites and that the expression of miR-29a is induced by activation of Wnt/β-catenin signaling. In addition, miR-29a targets to degrade the negative regulators of Wnt signaling, such as DKK1, Kremen2, and sFRP2, leading to formation of a positive feedback loop in human osteoblasts.[17]

Recently, it is shown that the transcription factor Forkhead box class O 3a (Foxo3a) can directly bind to β-catenin and inhibit the formation of β-catenin/TCF4 complex, resulting in attenuation of Wnt/β-catenin signaling activity.[18] Interestingly, Foxo3a is a direct target of miR-29a.[19] However, whether miR-29a promotes the activity of Wnt/β-catenin signaling through targeting Foxo3a is still unknown. Our previous work showed that 5,7,3′,4′-tetramethoxyflavone (TMF), a major constituent from Murraya exotica L., exhibited chondroprotective effects by inhibiting Wnt/β-catenin signaling activity.[13] In this paper, we further investigated whether TMF inhibited miR-29a/Wnt/β-catenin signaling through upregulating Foxo3a activity in OA chondrocytes.

Materials and methods

General

The study was approved by the Institutional Animal Care and Use Committee of Gannan Medical University and performed in accordance with the guideline of Animal Care and Use issued by the Institutional Animal Care and Use Committee of Gannan Medical University. Male rats were kept under standard environmental conditions. Rats in the treated groups were intragastrically administered with TMF (25 mg/kg and 100 mg/kg, respectively), which was prepared as our previous work.[13,20] The doses of TMF for in vivo study have been investigated in OA rat models, according to the level changes of inflammatory cytokines, such as IL-1β and TNFα, in the synovial fluid.[13] Rats in the controlled group received the same doses of vehicle as those in the treated groups.

Rat knee OA models

Rat knee OA models were duplicated by using Hulth's method.[21] Simply, rats were anesthetized with 3% pentobarbitone (30 mg/kg) by intravenous injection. After routine disinfection, longitudinal incision (1 cm) was conducted at the medial parapatella for separating and cutting off the tibial collateral ligament. Then, the articular cavity was opened. The cruciate ligament was cut off, and the medial meniscus was removed. After rinsing, the cavity was sutured. 8 weeks later, rats were sacrificed, and joint cartilages were collected for gross observation, histomorphological examination, and primary cells harvest.

Cell cultures

Under sterile conditions, joint cartilages were cut into small pieces and digested with 0.25% pancreatic enzyme and 0.2% collagenase II at 37°C for 4 hrs. Cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) (low glucose) (Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (Life Technologies) at 37°C with 5% CO_2. The second and third passages of cells were employed for the following assays. TMF (5 μg/mL and 20 μg/mL, respectively) was ready for administration to cultured cells. The doses have been investigated by MTT in our previous study.[20]

Quantitative analysis of apoptosis

Annexin V-FITC apoptosis assay was used for determining the apoptotic changes by flow cytometry according to the procedures instructed by the apoptosis detection kit (Nanjing KeyGEN Biological Technology Development Co., Ltd, Nanjing, China). Simply, TMF-treated chondrocytes were harvested and incubated in the buffer containing Annexin V-FITC and PI. The apoptotic ratio of chondrocytes was determined by a flow cytometer (FACSCalibur BD, San Jose, CA, USA).

miRNA, plasmid construction and transfection

Chondrocytes were grown to 60% confluence (1×10^5 cells/well) and transfected with miR-29a mimics (RiboBio, Guangzhou, China), or miRNA mimic negative control (miR-NC) (RiboBio) by using lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the instructions of kit. miR-29a mimics and miR-NC were used at a final concentration of 100 nM. The sequence of miR-NC was 5′-guaauguugcuca, which did not produce any effects on chondrocytes. pcDNA3.1(+)-Foxo3a vector was prepared by clone of the full length of Foxo3a open reading frame into pcDNA3.1(+) vector. Chondrocytes were transfected with pcDNA3.1(+)-Foxo3a vector by using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection for 8 hrs, the standard medium containing 10% FBS was employed to culture the transfected chondrocytes for another 48 hrs.

Luciferase reporter assays

Chondrocytes (5×10^4 cells/well) were suspended in the 48-well plate with serum-free culture medium. Then, they were transfected with Wnt/β-catenin reporter plasmid (Upstate, Lake Placid, NY, USA) (Topflash, encoding 7 copies of TCF/LEF binding sites linked to firefly luciferase and indicating the activity of Wnt/β-catenin signaling). Meanwhile, chondrocytes were co-transfected with renilla luciferase plasmid (pRL-CMV, Thermo Fisher Scientific) to normalize the results to the transfection efficiency. After 4 hrs of transfection, cells were cultured in the standard medium overnight. Then, TMF (5 μg/mL and 20 μg/mL, respectively) was added to the medium for culturing 24 hrs. Passive Lysis Buffer (Promega, Madison, WI, USA) was used to lyse the cultures. A dual luciferase assay kit (Promega) was used for determining the luciferase activities of both Topflash and Fopflash reporters by using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

RNA extraction and real-time quantitative PCR

TRIzol reagent (Invitrogen) was employed to extract the total RNA according to the manufacturer's instructions. For each sample, 2 μg of total RNA was reverse-transcribed using M-MLV (Promega) to synthesize the first-strand of cDNA following the standard protocols. For miRNA detection, EzOmics SYBR qPCR kits and miR-29a primer were obtained from Biomics in Mastercycler (Eppendorf). The procedures for amplification were conducted as follows: 95°C for 10 mins, followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 10 mins. For mRNA detection, qRT-PCR was used to determine the expression levels of Foxo3a, β-catenin, and caspase-3 using the ABI PRISM7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The sense and anti-sense primers were synthesized by Biomics (Eppendorf) and showed as follows: Foxo3a forward: 5′-cgactatgcagtgacaggttgtg-3′, reverse: 5′-cgactatgcagtgacaggttgtg-3′; β-catenin forward: 5′-acagcaccttcagcactct-3′, reverse: 5′-aagttcttggctattacgaca-3′; caspase-3 forward: 5′-agcaataaatgaatgggctgag-3′, reverse: 5′-gtatggagaaatgggctgtagg-3′; U6 forward: 5′-ctcgcttcggcagcaca-3′, reverse: 5′-aacgcttcacgaatttgcgt-3′; 18S rRNA forward: 5′-cctggataccgcagctagga-3′, reverse: 5′-gcggcgcaatacgaatgcccc-3′. U6 and 18S rRNA were used as the internal control genes to normalize miRNAs and mRNA levels, respectively. All reactions were performed with optimized conditions in triplicate. Fold changes of miRNAs and mRNA were calculated to normalize to U6 and 18S rRNA, respectively, by using $2^{-\triangle\triangle CT}$ method.

Western blot

Chondrocytes were lysed by the lysis buffer (Life Technologies) on ice. The lysates were then centrifuged at 12,000×g at 4°C for 15 mins. BCA assay (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the protein concentrations. The denatured equal protein (30 μg) of each sample was subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and subjected to the standard Western blot procedures. The primary antibodies against Foxo3a, β-catenin, Cleaved-caspase-3, and GAPDH, respectively, and the secondary antibody HRP-conjugated goat anti-rat IgG were purchased from Cell Signaling Technology. The enhanced chemiluminescence detection system (Applied Biosystems) was used to detect proteins, and Quantity One software (Bio-Rad) was used to quantify and analyze the bands.

Co-immunoprecipitation assay

Transfected chondrocytes were harvested by the immunoprecipitation lysis buffer. Then, they were centrifugated at 12,000 rpm for 30 mins. 10% of chondrocytes lysates were kept and used as the input. The retained proteins were immunoprecipitated by incubating with normal goat IgG or β-catenin antibodies (Cell Signaling Technology) at 4°C overnight. Goat IgG control immune serum was used for background evaluation. Next, they were incubated with the pre-cleared protein A-sepharose beads at 4°C for After five rinses in immunoprecipitation (IP) washing buffer, 30 μg proteins of the eluted samples were then subjected to immunoblotting (IB, Western blot) as mentioned above by using the following antibodies: anti-Foxo3a, anti-TCF4, anti-β-catenin (Cell Signaling Technology).

Statistical analysis

Data were shown as mean ± SD. The results were analyzed by a two-tailed unpaired *t*-test. $P<0.05$ was considered to be statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

Results

TMF downregulated the activity of miR-29a/β-catenin signaling and upregulated the expression of Foxo3a in OA rat cartilages

No rats died during the experiment. 8 weeks after establishment of OA models, rats were sacrificed. Histomorphological examination by hematoxylin-eosin (HE) staining showed that the thickness of cartilages in the model groups was significantly decreased (Figure 1). Hypertrophic chondrocytes with disorder array were also found in the model groups. TMF effectively reversed these pathological changes in a dose-dependent manner. The in situ immunofluorescence analysis showed that the expression of β-catenin was upregulated and the expression of Foxo3a was downregulated in rats' OA model groups (Figure 1). The expression of miR-29a, a downstream factor of Wnt/β-catenin signaling, in chondrocytes in vivo was detected and found to be increased in the model groups. Similarly, TMF exhibited protective effects on OA chondrocytes as indicated by downregulation of miR-29a and β-catenin and upregulation of Foxo3a.

TMF protected chondrocytes against apoptosis induced by miR-29a

To investigate whether miR-29a promoted the activity of Wnt/β-catenin signaling, transfection of miR-29a mimics into chondrocytes was employed. qRT-PCR results showed that the expression of miR-29a was dramatically increased in chondrocytes transfected with miR-29a mimics, compared with that in the groups transfected with negative control (Figure 2A). This indicated the successful transfection of miR-29a mimics. The effects of miR-29a on chondrocytes apoptosis were determined. As shown in Figure 3, miR-29a mimics significantly promoted chondrocytes apoptosis (37.16±2.52%, $P<0.01$). TMF at doses of 5 μg/mL and 20 μg/mL showed counteractive effects against miR-29a and decreased chondrocytes apoptosis (20.38±1.89%, $P<0.01$ and 7.99±1.47%, $P<0.01$, respectively), compared to that in the miR-29a mimics group.

Then, we detected the mRNA and protein levels of β-catenin, Foxo3a, and caspase-3 in miR-29a mimics-transfected chondrocytes (Figure 2B–D). Results showed that the mRNA expressions of β-catenin were not statistically changed, compared with that in the negative control group. In contrast, TMF could significantly suppress the expression of β-catenin. Foxo3a mRNA expression was significantly downregulated in miR-29a mimics-transfected group, and caspase-3 mRNA expression was greatly upregulated. At protein expression levels, similar trends were found with those at mRNA levels. TMF dose-dependency enhanced the expression of Foxo3a at gene

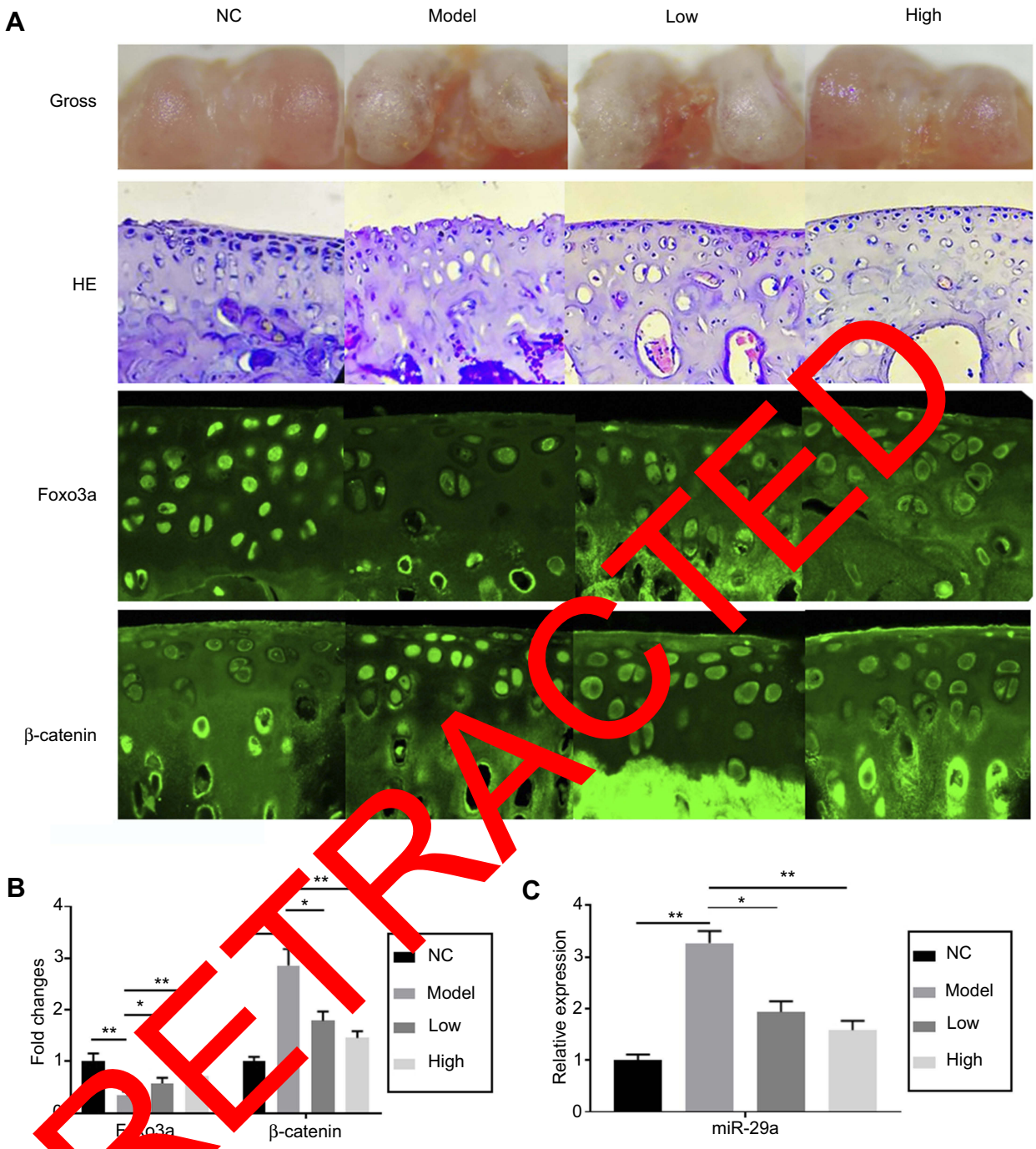

Figure 1 The gross observation, hitomorphological examination, and immunofluorescence assays in rat joint cartilage. The groups were divided in NC (negative control group), Model (OA model group), Low (OA model +25 mg/kg TMF treatment), and High (OA model +100 mg/kg TMF treatment). After 8-week OA model establishment, rats were sacrificed. (**A**) Joint cartilage was collected for gross observation (the first array), hitomorphological examination by HE staining (the second array), and immunofluorescence assays of Foxo3a (the third array) and **β**-catenin (the fourth array). The fluorescence intensities of Foxo3a and **β**-catenin were indicated in the column figure (**B**), of which the data in the model and TMF-treated groups were compared with those in the NC group. Cartilage tissues were collected for extracting miR-29a, which was determined by qRT-PCR (**C**). Data were presented by mean ± SD of 6 replicates. $P<0.05$ was considered to be statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$).
Abbreviations: OA, Osteoarthritis; NC, negative control.

and protein levels, counteracting the effects of miR-29a on Foxo3a. Cleaved caspase-3 was significantly upregulated in miR-29a mimics-transfected group. The luciferase reporter activity of Wnt/β-catenin signaling was also dramatically increased in miR-29a mimics-transfected group (Figure 2E) and decreased significantly by TMF dose-dependency. Collectively, TMF could effectively reverse the effects of miR-29a, as indicated by

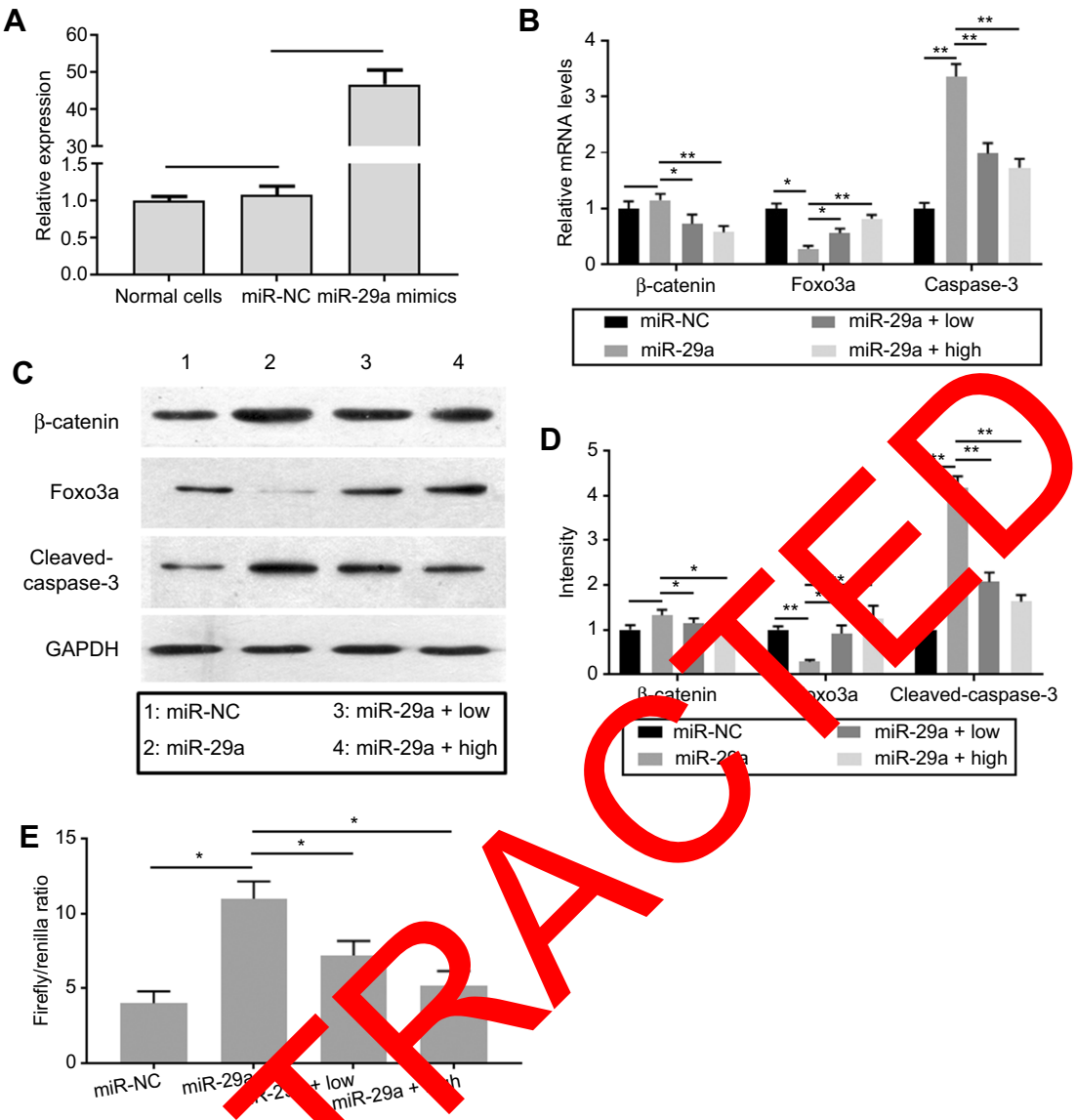

Figure 2 Changes of β-catenin, Foxo[illegible], and cleaved-caspase[illegible] expression in miR-29a mimics-transfected chondrocytes.

Notes: (**A**) miR-29a mimics and [illegible]NA mimics negative control (miR-NC) were transfected with chondrocytes. The expression of miR-29a was quantified by qRT-PCR. (**B**) After transfection of miR-29a [illegible]ics, the [illegible]A expressions of β-catenin, Foxo3a, and cleaved-caspase-3 were determined by qRT-PCR. (**C** and **D**) After transfection of miR-29a mimics, the protein expr[illegible]ons [illegible]β-catenin, F[illegible]xo3a, and cleaved-caspase-3 were determined by Western blot. The intensity of protein bands was indicated in the column. 1 (miR-NC grou[illegible]/miR-NC[illegible]/miR-29a [illegible]ics-transfected group) (miR-29a), 3 (miR-29a mimics-transfected group +5 μg/mL TMF treatment) (miR-29a + low), and 4 (miR-29a mim[illegible] trans[illegible]ted gro[illegible]+20 [illegible]L TMF treatment) (miR-29a + high). (**E**) Chondrocytes were co-transfected with miR-29a mimics and Topflash or Fopflash luciferase [illegible]porters. [illegible]nsfected c[illegible]s were treated with 5 μg/mL (low) or 20 μg/mL TMF (high) for 24 hrs. The values were indicated as Firefly/Renilla ratio in the column. Da[illegible]were pres[illegible]ed by mean ± SD of 3 replicates. $P<0.05$ was considered to be statistically significant (*$P<0.05$, **$P<0.01$).

downregulation [illegible] miR-29a and β-catenin expression. These might be ass[illegible]ciated with upregulation of Foxo3a expression by TMF.

TMF attenuated miR-29a/β-catenin signaling activity by upregulation of Foxo3a expression

To investigate the effects of Foxo3a on Wnt/β-catenin signaling and whether TMF attenuated the activity of miR-29a/β-catenin signaling by upregulation of Foxo3a expression, co-transfection of miR-29a mimics and pcDNA3.1(+)-empty vector or pcDNA3.1(+)-Foxo3a vector was employed. The expressions of miR-29a and Foxo3a were determined for verification of successful transfection (Figure 4). As indicated in Figure 5, overexpression of Foxo3a greatly downregulated the expression of caspase-3. However, it did not affect the mRNA and protein expressions of miR-29a (Figure 4) and β-catenin statistically. The co-immuno-precipitation assay showed that TMF could promote binding of β-catenin to Foxo3a dose-dependency. In contrast, TMF decreased the

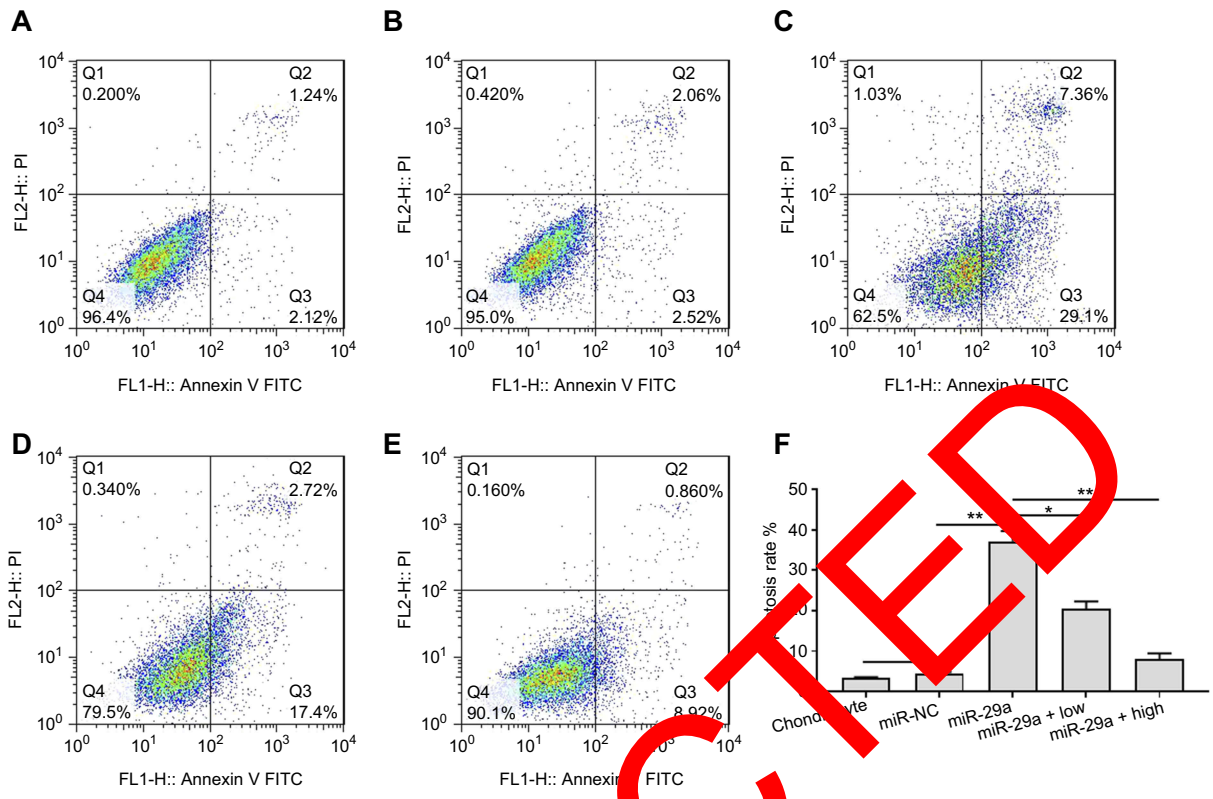

Figure 3 Inhibitory effects of TMF on apoptosis in miR-29a mimics-transfected chondrocytes. Group (**A**) was normal untreated chondrocytes. Group (**B**) was miRNA mimic negative control. Group (**C**) was miR-29a mimics-transfected chondrocytes. Group (**D**) was miR-29a mimics-transfected chondrocytes +5 μg/mL TMF (low) treatment. Group (**E**) was miR-29a mimics-transfected chondrocytes +10 μg/mL TMF (high) treatment. Figure (**F**) was the summarized data indicating the rate of chondrocytes apoptosis detected by flow cytometry. Data were presented by mean ± SD of 3 replicates. $P<0.05$ was considered to be statistically significant (*$P<0.05$, **$P<0.01$).

interaction between β-catenin and TCF4 which was increased by miR-29a by targeting to Foxo3a (Figure 5C). These indicated that TMF promoted Foxo3a to compete with TCF4 for binding to β-catenin, leading to negatively regulating the activity of Wnt/β-catenin signaling. In addition, the luciferase reporter assay also showed that Foxo3a overexpression attenuated the activity of Wnt/β-catenin signaling (Figure 5D). TMF could further promote the effects of Foxo3a, as shown by downregulating the expression of caspase-3 and the activity of Wnt/β-catenin signaling (Figure 5C and D). Thus, TMF might protect chondrocytes against miR-29a/β-catenin signaling activity by upregulation of Foxo3a expression.

Discussion

Chondrocyte is the unique cell type presented in cartilage and responsible for maintaining the homeostasis of articular cartilage. A substantial body of evidence indicates that the overwhelming apoptosis of chondrocytes is involved in OA development.[22] Wnt/β-catenin signaling has been associated with multiple steps during the formation and maturation of chondrocytes. Gain- or loss-of-function of β-catenin may impair the homeostasis of articular cartilage and induce pathological changes, such as increased apoptosis.[23] Activation of Wnt/β-catenin signaling induces hypertrophic differentiation of articular chondrocytes which, in turn, promotes cartilage degradation and subsequent OA aggravation.[24] Wnt/β-catenin signaling is also associated with mechanical force-induced damage in OA cartilage, and hydrostatic pressure may attenuate the expression of β-catenin.[25] However, Wnt inhibition may potentially be powerful for re-directing MSC chondrogenesis towards chondrocytes by silence of BMP and IHH signaling pathways.[26] Interestingly, at the late stage of OA, methylation alternations have been found to be enriched in Wnt/β-catenin signaling pathway genes, indicating the self-repairing capability of chondrocytes.[27] Our previous work shows that TMF ameliorates chondrocytes apoptosis by attenuation of Wnt/β-catenin signaling.[13]

Recently, interaction between miRNAs and Wnt/β-catenin signaling has been comprehensively discussed.[28] miR-138 may downregulate the expression

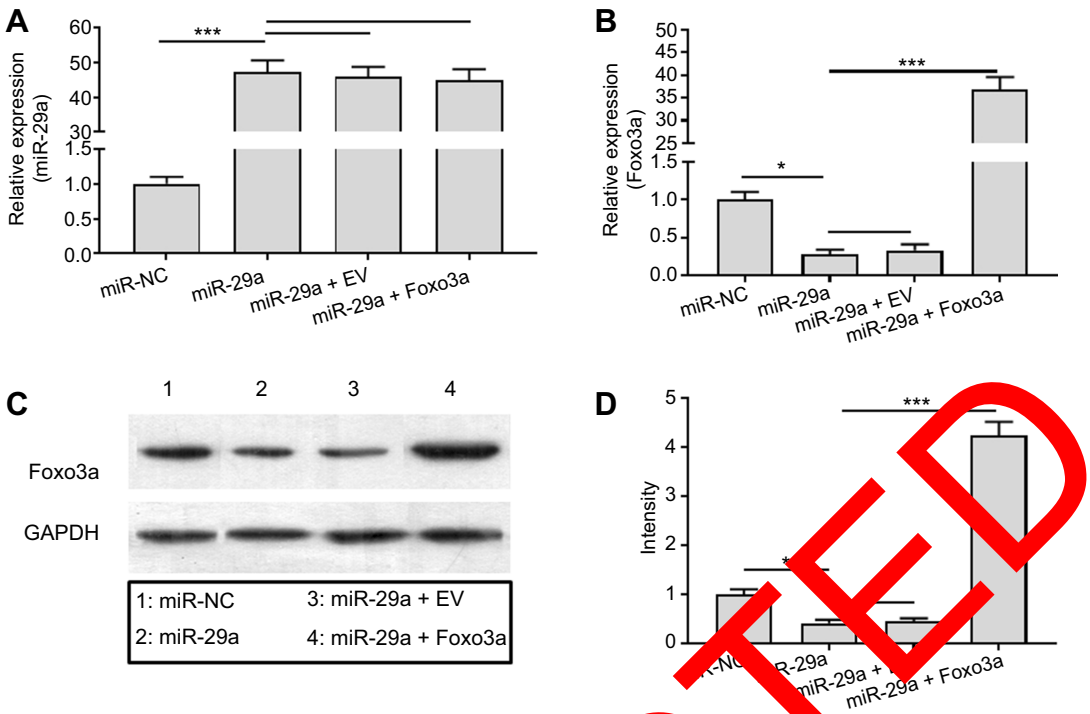

Figure 4 miR-29a targeted to degrade the expression of Foxo3a in chondrocytes. Chondrocytes were co-transfected with miRNA mimic negative control (miR-NC), miR-29a mimics, pcDNA3.1(+)-empty vector (EV), and pcDNA3.1(+)-Foxo3a. The expressions of miR-29a (**A**) and Foxo3a (**B**) were detected by qRT-PCR. The protein level of Foxo3a was determined by Western blot (**C**). Summary of the protein bands intensity (**D**). Data were presented as mean ± SD of 3 replicates. $P<0.05$ was considered to be statistically significant (*$P<0.05$, ***$P<0.001$).

of β-catenin through targeting to degrade NIMA-related kinase 2 (NEK2), which is related to Wnt/β-catenin signaling.[29] MiR-29 family has three members, including miR-29a, miR-29b, and miR-29c. They evolve with a conserved region, AGCACCA, and act to similar biological functions.[30] It has been demonstrated that activation of Wnt/β-catenin signaling upregulates the expression of miR-29a by binding of β-catenin/TCF4/LEF1 complex to its promoter.[17] miR-29a also modulates the activity of Wnt/β-catenin signaling in a positive feedback loop. Recently, it has been found that miR-29a decreases the expression of PI3K, p-AKT, and p-GSK3β, leading to stabilization and translocation of β-catenin into nuclei in malignant glioma.[31] However, the biological functions of miR-29a might be controversial. It has been shown that miR-29a downregulates the activity of Wnt/β-catenin signaling in colon cancer.[32] In addition, the cytokines, including FZD3, FZD5, DVL3, FRAT2, and CK2A2, involved in Wnt/β-catenin signaling have been showed to be the potential targets of miR-29 in miR-29 gain- and loss-of-function microarray experiments in primary human articular chondrocytes.[5] These suggest that the functions of miR-29a might depend on cell types and their situations. In chondrocytes, we found that overexpression of miR-29a dramatically upregulated the activity of Wnt/β-catenin signaling and induced cell apoptosis.

Foxo3a, a transcription factor, has been associated with the upregulation of ROS-scavenge enzymes, such as SOD2, protecting cell against oxidative stress.[33,34] Foxo3a competes with TCF4 for the interaction with β-catenin, resulting in repression of β-catenin/TCF4 transcriptional activity.[35] Thus, β-catenin is a co-activator of Foxo3a for resistance to oxidative stress. Foxo3a may form a complex with PPARγ to bind to β-catenin, abrogating canonical Wnt/β-catenin signaling.[36,37] However, Foxo3a may also bind to the promoter and induce the expression of PUMA, which is a BH3-only pro-apoptoic factor antagonizing Bcl-2 and promoting p53-independent apoptosis in colorectal cancer cells.[38] In addition, induction of PUMA by Foxo3a requires active GSK-3β.[39] Therefore, Foxo3a might regulate a large scale of genes expression. In our study, we found that in TMF-treated groups, overexpression of Foxo3a significantly downregulated the activity of miR-29a/Wnt/β-catenin signaling.

Wnt/β-catenin signaling has been the therapeutic target in clinic. Several drugs and many natural occurring compounds are reported to modulate the activity of Wnt/β-catenin signaling.[40] However, the underlying mechanisms of these

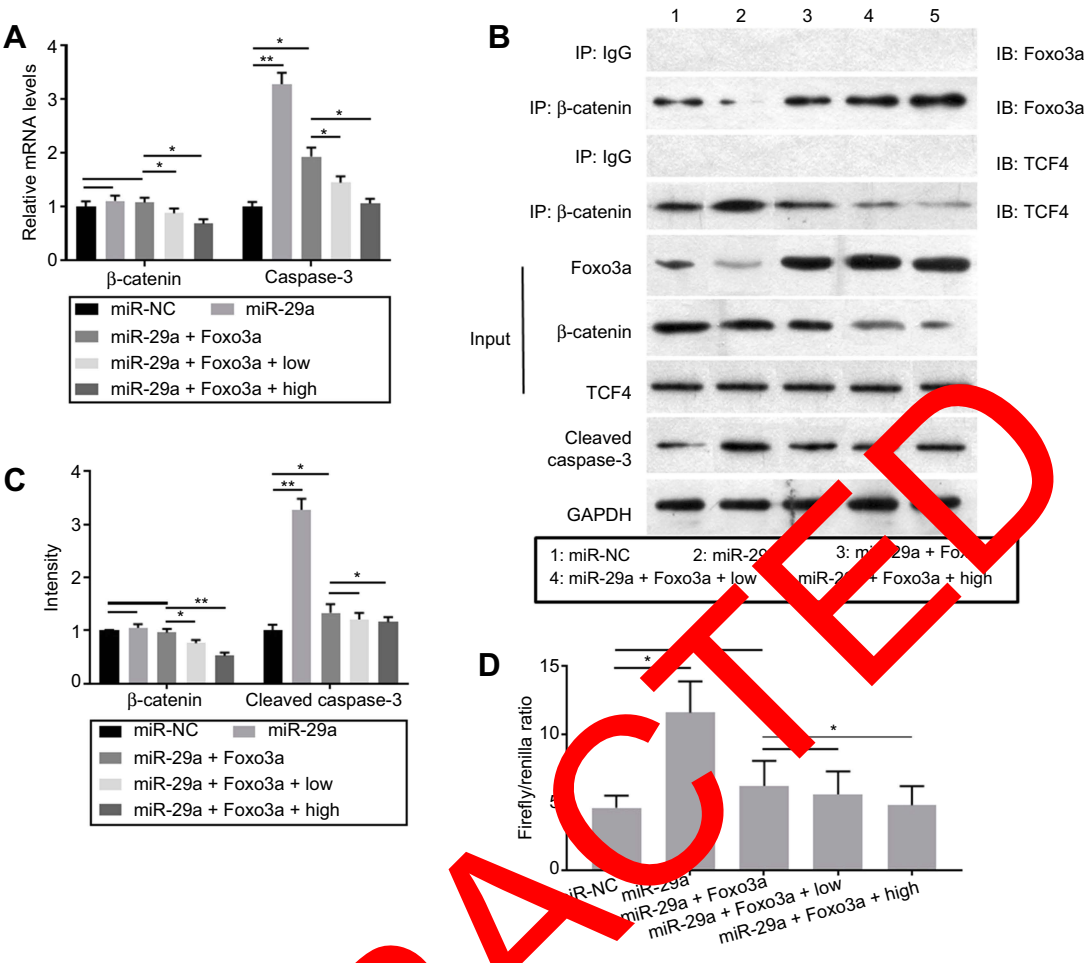

Figure 5 Foxo3a overexpression attenuated the activity of Wnt/β-catenin signaling by competing with TCF4 for binding to β-catenin. Chondrocytes were co-transfected with miR-29a mimics and pcDNA3.1(+)-Foxo3a. After treatment with 5 (low) or 2 μg/mL (high) TMF for 24 hrs, the mRNA (**A**) and protein (**B**) expression were detected by RT-PCR and Western blot, respectively. (C) was the summary of protein bands intensity. (**B**) The co-immuno-precipitation assay was conducted for detecting the interaction between β-catenin and Foxo3a or TCF4. 10% input chondrocytes protein extracts were used as the input, which was subjected to Western Blot. The remaining protein extracts were subjected to IP by using control goat IgG or β-catenin antibody, followed by IB with anti-Foxo3a, anti-TCF4, and anti-β-catenin. (**D**) Chondrocytes were co-transfected with miR-29a mimics or pcDNA3.1(+)-Foxo3a and Topflash or Fopflash luciferase reporters. Transfected cultures were treated with 5 or 20 μg/mL TMF for 24 hrs. The values were indicated as Firefly/renilla ratio in the column. Data were presented by mean ± SD of 3 replicates. $P<0.05$ was considered to be statistically significant (*$P<0.05$, **$P<0.01$).

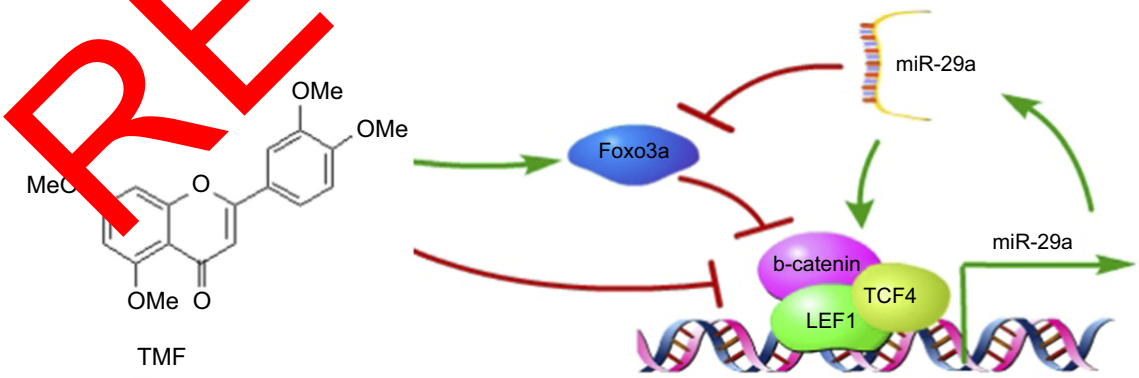

Figure 6 TMF inhibited miR-29a/Wnt/β-catenin signaling activity by up regulating Foxo3a expression. Wnt/β-catenin signaling up regulated the expression of miR-29a, which in turn promoted the activity of Wnt/β-catenin signaling. miR-29a targeted to degrade Foxo3a. However, TMF up regulated the activity of Foxo3a, leading to attenuation of miR-29a/Wnt/β-catenin signaling.

candidates are still poorly understood. Several strategies have been developed, such as prevention of stabilization of Axin2, reduction of β-catenin stability, and blockage of β-catenin/TCF4 interaction.[40,41] Quercetin has been shown to inhibit Wnt/β-catenin signaling activity by disruption of β-catenin /TCF4 interaction.[42] TMF, a tetramethoxyflavone isolated from *Murraya exotica*, has been demonstrated to downregulate the activities of PKA and β-catenin.[13] In this article, we further investigate the inhibitory effects of TMF on miR-29a/β-catenin signaling activity by upregulation of Foxo3a expression (see Figure 6). However, the potential target of TMF in inhibiting Wnt/β-catenin signaling activity still needs to be clearly elucidated.

Data availability statement

The experimental data used to support the findings of this study are included within the article.

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Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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