

Chondrogenic differentiation of rat bone marrow mesenchymal stem cells induced by puerarin and tetrandrine

Xin-Ran Dong¹, Meng-Jiao Hu¹, Hui-Xin Pan¹, Ke-Feng Li^{2,*}, Yuan-Lu Cui^{1,*}

Abstract

Objective: This study aims to clarify the effect of the active components puerarin and tetrandrine on the chondrogenic differentiation of bone marrow mesenchymal stem cells (BMSCs).

Methods: Using network pharmacology, protein targets of puerarin and tetrandrine were predicted, and a database of cartilage formation targets was established. The protein target information related to disease was then collected, and the drug-targeting network was constructed by analyzing the protein-protein interactions. Genes related to chondrogenesis induced by puerarin and tetrandrine and chondroblast differentiation signaling pathways were searched. Finally, potential drug- and disease-related genes, as well as proteins, were screened and verified using real-time RT-PCR and western blotting.

Results: Network pharmacological studies have shown that puerarin and tetrandrine are involved in BMSCs cartilage differentiation. The experimental results showed that puerarin and tetrandrine could regulate the expression of cartilage differentiation-related genes and proteins. Puerarin increased the protein expression of COL2A1, COL10A1, MMP13, and SOX-9, as well as the gene expression of *Col2a1*, *Mmp13*, *Tgfb1*, and *Sox-9*. Tetrandrine increased the protein expression of COL2A1, COL10A1, MMP13, and SOX-9, as well as the gene expression of *Col10a1*, *Tgfb1*, *Sox-9*, and *Acan*. The combination of puerarin and tetrandrine increased the protein expression of COL2A1, COL10A1, MMP13, and SOX-9 and the gene expression of *Col2a1*, *Col10a1*, *Sox-9*, and *Acan*.

Conclusions: Puerarin, tetrandrine, and their combination can promote the proliferation of BMSCs and induce their differentiation into chondrocytes, and they are thus expected to be inducers of chondrogenic differentiation. These results suggest that puerarin and tetrandrine have potential therapeutic effects on osteoarthritis.

Keywords: Bone marrow mesenchymal stem cells (BMSCs), Chondrogenic differentiation, Network pharmacology, Puerarin, Tetrandrine

Introduction

Osteoarthritis is a common chronic degenerative disease characterized by articular cartilage degeneration and secondary osteoporosis. It mainly occurs in the knee joints, hip joints, spine, and other parts that bear the weight of middle-aged and elderly individuals^[1]. Chondrocyte depletion and extracellular matrix degradation are the main pathological changes. Approximately 33.9% of people over 65 years of age suffer from

osteoarthritis, which has been a main cause of disability among those over 50 years old, second only to coronary heart disease^[2]. This is partially due to the difficulty of repairing and regenerating articular cartilage after injury. Existing treatments, such as cartilage tissue engineering or matrix-induced autologous chondrocyte transplantation, cannot treat large areas of cartilage defects^[3]. As such, bone marrow mesenchymal stem cells (BMSCs) are at present widely used for osteoarthritis treatment.

BMSCs are adult stem cells in the bone marrow, in addition to hematopoietic stem cells and early mesodermal cells, first reported in 1976^[4]. BMSCs have the common characteristics of stem cells, such as self-renewal ability, low immunogenicity, multi-organ homing ability, and multi-directional differentiation, including mesodermal cells such as adipocytes, osteoblasts, chondrocytes, tendon cells, endothelial cells, cardiomyocytes, liver and lung epithelial cells, and ectodermal cells such as neurons^[5–6]. BMSCs can differentiate into chondrocytes under specific conditions and actively migrate to the cartilage defect site for repair and reconstruction under the influence of the *in vivo* microenvironment. They can also activate cell and vascular regeneration pathways^[7], thereby achieving anti-inflammatory effects by inhibiting T lymphocyte activity and natural killer cell differentiation^[8]. BMSCs transplantation therapy has attracted considerable attention for the treatment of osteoarthritis^[9–10]. Bone morphogenetic protein (BMP)-2 can promote the differentiation of BMSCs into osteoblasts,

Xin-Ran Dong and Meng-Jiao Hu contributed equally to this work.

¹ State Key Laboratory of Component-based Chinese Medicine, Research Center of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China; ² School of Medicine, University of California, San Diego, USA.

* Corresponding author. Yuan-Lu Cui, State Key Laboratory of Component-based Chinese Medicine, Research Center of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 301617, China, E-mail: cuiyl@tju.edu.cn; Ke-Feng Li, School of Medicine, University of California, San Diego 92103, USA, E-mail: kli@ucsd.edu.

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significantly increase alkaline phosphatase activity, and promote the expression of collagen type-I and osteocalcin^[11]. Transforming growth factor-beta 1 (TGF- β 1), TGF- β 3, and insulin-like growth factor 1 (IGF-1) proprotein can promote extracellular matrix synthesis, increase cell proliferation, and promote the differentiation of BMSCs into chondrocytes^[12]. IGF-1 can delay matrix degradation and inhibit chondrocyte apoptosis^[13]. However, cytokines are both expensive and relatively unsafe. It is thus necessary to look for substitutes as inducers of BMSCs differentiation, for which natural drugs are good candidates. Further, network pharmacology is a powerful tool for the screening of natural drugs.

Network pharmacology is based on the concept of systems biology theory, which helps to broaden the knowledge of disease manifestation and the role of drugs in its cure^[14]. In recent years, it has often been used in the integrated analysis of disease-related targets and drug action targets and is a favorable tool to realize the transformation of drug action points from a single target to multiple targets^[15]. This helps solve the unclear material basis and mechanism of action of traditional Chinese medicine, favored by researchers of this branch of medicine^[16].

Puerarin, a major flavonoid isolated from *Pueraria lobata* (Willd.) Ohwi, has the functions of improving immunity^[17], enhancing myocardial contractility, protecting myocardial cells, lowering blood pressure, and inhibiting platelet aggregation, among others^[18]. It can also induce embryonic stem cells to differentiate into cardiomyocytes^[19] and umbilical cord mesenchymal stem cells to differentiate into osteoblasts^[20], in addition to enhancing the viability of osteoblasts by stimulating differentiation^[21]. Puerarin can also upregulate the expression of SOX-9 in BMSCs through the TGF- β 1/Smad pathway^[22]. SOX-9, regulated by fibroblast growth factor, TGF- β , BMP, and Wnt family members, is an early marker of cartilage differentiation that increases the expression of key genes, including *collagen type-II* (*Col2a1*)^[23] and *aggrecan* (*Acan*), thus promoting the differentiation of BMSCs into cartilage^[24]. Puerarin in the collagen matrix can locally affect new bone formation^[25] and has also been shown to prevent bone loss in castrated male mice^[26]. Based on these reasons and the results of network pharmacology analysis, we speculate that puerarin has the potential to induce BMSCs to differentiate into cartilage.

Tetrandrine is a bibenzylisoquinoline alkaloid extracted from *Stephania tetrandra* S. Moore, which has anti-inflammatory and immunosuppressive effects by inhibiting the function of inflammatory cells, free radical damage, and the release of inflammatory mediators^[27]. Low concentrations of tetrandrine can promote the proliferation of chondrocytes, increase their activity, and promote the secretion of COL2A1 and glycosaminoglycan by chondrocytes, which is conducive to the repair and reconstruction of cartilage tissue *in vitro*^[28]. It might also affect the treatment of osteoarthritis through the Wnt/ β -catenin signaling pathway^[29–30]. Based on these reasons and the results of network pharmacology analysis, we speculated that tetrandrine could promote the differentiation of BMSCs into cartilage. Therefore, puerarin and tetrandrine were used together to induce the chondrogenic differentiation of BMSCs in this study.

Materials and methods

Database construction

The traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP, <http://lsp.nwu.edu.cn/tcmsp.php>), the encyclopedia of traditional Chinese medicine (<http://www.nrc.ac.cn:9090/ETCM>), an integrative database of traditional Chinese medicine enhanced by symptom mapping (<http://www.symmap.org>), the database and analytical system for network pharmacology analysis for TCM preparations (<http://mesh.tcm.microbioinformatics.org>), and a library of relevant target sites for puerarin and tetrandrine was established by combining literature extraction methods. Targets for chondrogenesis were collected from the Online Mendelian Inheritance in Man (OMIM, <https://www.omim.org/>) database. The targets of puerarin and tetrandrine were compared with the relevant targets of chondrogenesis, and the targets predicted to have beneficial effects were summarized. All target information related to the active ingredients of target proteins was obtained using the universal protein knowledgebase (UniProtKB) search function in the UniProt database (<https://www.uniprot.org/>).

Network construction and analysis

In this study, the targets of puerarin and tetrandrine were predicted using multiple databases and the targets of chondrogenesis were retrieved from the OMIM database. The UniProt database was used to obtain target information related to the active ingredients of the target proteins. A string database was used for protein interaction analysis. Cytoscape software (version 3.2.1) was used to construct the drug–target network to cross-fuse the target protein–protein interaction (PPI) network and disease target PPI network. Puerarin and tetrandrine target genes related to chondrogenesis were screened, and chondrogenic differentiation signaling pathways were identified. Potential drugs, disease-related genes, and proteins were selected for verification.

Experimental verification

Reagents and animals

Puerarin (95.5%) and tetrandrine (99.2%) were purchased from the National Institute for Food and Drug Control (Beijing, China). Trizma[®] base, real-time RT-PCR kits were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The anti-transcription factor SOX-9 antibody, anti-COL2A1 antibody, anti-collagenase 3 (MMP 13) antibody, and anti-glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) antibody, HRP-conjugated goat anti-rabbit IgG antibody, and UNIQU-10 column Trizol total RNA extraction kit were purchased from Sangon Biotech, Co., Ltd. (Shanghai, China). Anti-collagen type-X (anti-COL10A1) antibody was purchased from Abcam (Cambridge, UK). Trypsin solution (0.25%) was purchased from Solarbio Co. Ltd. (Beijing, China). Anti-CD29 (PE Hamster anti-Rat CD29), anti-CD45 (PE-CyTM5 Mouse anti-Rat CD45), anti-CD54 (PE Mouse anti-Rat CD54), anti-CD90 (APC Mouse anti-Rat CD90/Mouse CD90.1), and anti-CD11b (FITC Mouse anti-Rat CD11b) antibodies were purchased from BD Biosciences (Bedford, MA, USA).

Healthy specific pathogen-free (SPF) male Sprague Dawley (SD) rats aged 4 to 6 weeks (180–220 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (SCXK2016–0006, Beijing, China).

Animals were placed in a pathogen-free environment at room temperature (20°C–22°C), with light control (12 h light/12 h dark, lights on at 07:00 AM) and with free access to clean food and water. All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Animal Ethics Committee of the Tianjin University of Traditional Chinese Medicine (TCM–2017–037–E10). Significant efforts have been made to minimize the number of experimental animals and their suffering.

Isolation and culture of primary cells

The rats were sacrificed by cervical dislocation and soaked in 75% alcohol for 10 min. The femur and tibia were separated on a super clean bench and washed three times with phosphate-buffered saline (PBS) buffer (Solarbio, Beijing). The epiphytic ends of the femur and tibia were cut off to expose the marrow cavity. The marrow cavity was rinsed with serum-free α -MEM (Hyclone, La, USA), and the rinsed solution was placed in a Petri dish. The rinsed solution with marrow cells was pipetted repeatedly to obtain a single-cell suspension. The suspension was centrifuged at $250 \times g$ for 10 min, and the supernatant was discarded. The cells were resuspended in a medium containing 10% fetal bovine serum and inoculated in a 25 cm² tissue culture flask. The primary cells were cultured at 37°C, with 5% CO₂ and saturated humidity. Non-adherent cells were removed after 2 days, after which morphological changes were observed under a microscope. Cells were used for experiments at passage levels between 3 and 4.

The cells were digested with 0.25% trypsin, and a single cell suspension was prepared. The supernatant was discarded after centrifugation at $1,000 \times g$ for 5 min, and the pellet was washed twice with PBS buffer. The cell count was measured and the cell density was calculated to be 1×10^6 cells/mL.

Identification of BMSCs

Passage 3 BMSCs (approximately 1×10^5 cells) were divided into 1.5 mL microcentrifuge tubes. CD45 and CD11b were used as negative markers, CD29, CD54 and CD90 were used as positive markers. Diluted antibodies (500 μ L, anti-CD29, anti-CD54, anti-CD90, anti-CD11b, and anti-CD45) were added to BMSCs, and the cells were investigated by flow cytometry. Cells at passage 3 were cultured in cartilage induction medium for 21 days until cell pellets formed. The cell pellets were fixed and embedded in paraffin, cut into 5 μ m slices, and stained with toluidine blue solution. The cells and slices were observed under a microscope, and digital micrographs were obtained.

Effects of puerarin and tetrandrine on cell viability

BMSCs (passage 3) were seeded in 96-well cell culture plates. New phenol red-free media with puerarin at different concentrations (10, 1, 0.1, 0.01, 0.001 μ M), tetrandrine at different concentrations (10, 5, 2.5, 1.25, 0.625 μ M), and a combination of puerarin and tetrandrine at different concentrations (1 μ M + 5 μ M, 0.1 μ M + 2.5 μ M, 0.01 μ M + 1.25 μ M) were added respectively. Dose–response curves were calculated using a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay following the manufacturer's instructions (Sigma-Aldrich, Co., St. Louis, MO, USA). The absorbance at 570 nm was measured using a plate reader.

Verification for gene and protein levels

Real-time RT-PCR analysis

BMSCs at passage 4 were seeded into 6-well plates. The cells were divided into a blank control group, active drug group (10 μ g/L, TGF- β), puerarin group (1 μ M), tetrandrine group (5 μ M), and combined puerarin and tetrandrine group (1 μ M + 5 μ M) for treatment, with four parallel wells in each group. Total RNA was extracted from BMSCs using a Trizol total RNA extraction kit after 3 days and 7 days of continuous culture. Oligonucleotide primer sequences are listed in Supplementary Table 1, <http://links.lww.com/AHM/A18>. GAPDH was used as the internal control. The cycling conditions for amplification were as follows: denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Amplification and analysis were performed using the ABI Prism 7500 real-time PCR system. The samples were analyzed using the relative C_T method. The fold increase or decrease in the targeted genes was calculated based on the formula $2^{-\Delta\Delta C_T}$.

Western blotting

Passage 4 BMSCs were divided into a blank control group, active drug group (10 μ g/L, TGF- β), puerarin group (1 μ M), tetrandrine group (5 μ M), and compatibility of puerarin and tetrandrine group (1 μ M puerarin and 5 μ M tetrandrine) to intervene with medicines. Total proteins were isolated from BMSCs after 3 and 7 days of continuous culture using the mammalian protein prep kit (Sangon Biotech, Co., Ltd., Shanghai, China). Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein bands were blotted onto PVDF membranes (Millipore, MIT, USA). The membranes were blocked with 5% skim milk at 25°C for 1 h. The protein bands were incubated with primary antibodies (anti-SOX-9, anti-COL2A1, anti-COL10A1, anti-MMP13, and anti-GAPDH) overnight at 4°C. The membranes were then incubated with goat anti-rabbit IgG antibody at 25°C for 1 h. After development with horseradish peroxidase substrate and a fluorescent agent, the protein bands were exposed, and the images generated from the exposure were scanned and analyzed. ImageJ software was used to quantify protein concentrations. Each experiment was performed in triplicate. GAPDH was used as the internal control. The primary antibodies and antibody concentrations used are listed in Supplementary Table 2, <http://links.lww.com/AHM/A19>.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance and Origin Pro 8.0 (OriginLab, Northampton, MA, USA). Experimental data are expressed as the mean $\pm$ standard deviation. $P < 0.05$ or $P < 0.01$ was considered to indicate significant differences.

Results

Network construction and analysis

To verify that puerarin and tetrandrine have chondroinductive activity, according to the hypothesis generated, puerarin and tetrandrine targets relevant to chondrogenesis were compared, and their common targets were summarized. Puerarin and tetrandrine target genes

related to chondrogenesis were screened from an analysis of the compound–target and target–pathway networks. Based on the gene screening results, we concluded that puerarin and tetrandrine might induce the chondrogenic differentiation of BMSCs. Next, we investigated signaling pathways related to chondrogenesis, and six targets were selected, including TGF β 1, ACAN, SOX-9, COL2A1, COL10A1, and MMP13, for real-time RT-PCR and western blot validation (Figure 1). The six selected targets interacted with each other and regulated the chondrogenic differentiation of BMSCs (Figure 2).

Experimental verification

Sorting and identification of BMSCs

Through continuous cleaning and fluid exchange, the suspended hybrid cells gradually disappeared, and the adherent cells gradually changed from triangular to polygonal. With an increase in the cell fusion rate, the cells grew in the same direction as a whirlpool. In the third passage, the morphology of all cells turned to a spindle-shape, which could be used in the following experiments (Figure 3A–C).

After the cells were induced to differentiate into chondrocytes, glycosaminoglycan, proteoglycan, and collagen were produced in the extracellular matrix.

Therefore, the induced cells aggregated and grew into cell pellets. Cell pellets were stained with toluidine blue, and the extracellular matrix was blue-purple (Figure 3D). This showed that the cells could be induced to differentiate into chondrocytes.

Flow cytometry results showed that the proportions of cells exhibiting the positive expression of stromal cell and stem cell antigens including CD29, CD54, and CD90 were 99.8%, 92.1%, and 98.3%, respectively. CD11b and CD45 were virtually absent in the cells (Figure 3E–I).

Effects of puerarin and tetrandrine on the viability of BMSCs

The MTT assay was used to determine the effect of puerarin, tetrandrine, and their combination on the viability of BMSCs. Compared to that in the blank control group, puerarin at concentrations of 0.001 μ M, 0.01 μ M, 0.1 μ M, and 1 μ M increased the viability of cells, among which, the concentration of 1 μ M had a better effect. Tetrandrine at a concentration of 5 μ M promoted the viability of BMSCs, which were used in a follow-up study. The combination of puerarin and tetrandrine (1 μ M of puerarin and 5 μ M of tetrandrine) could promote the viability of BMSCs, and the effect was similar to that in the tetrandrine groups (Figure 3J–L).

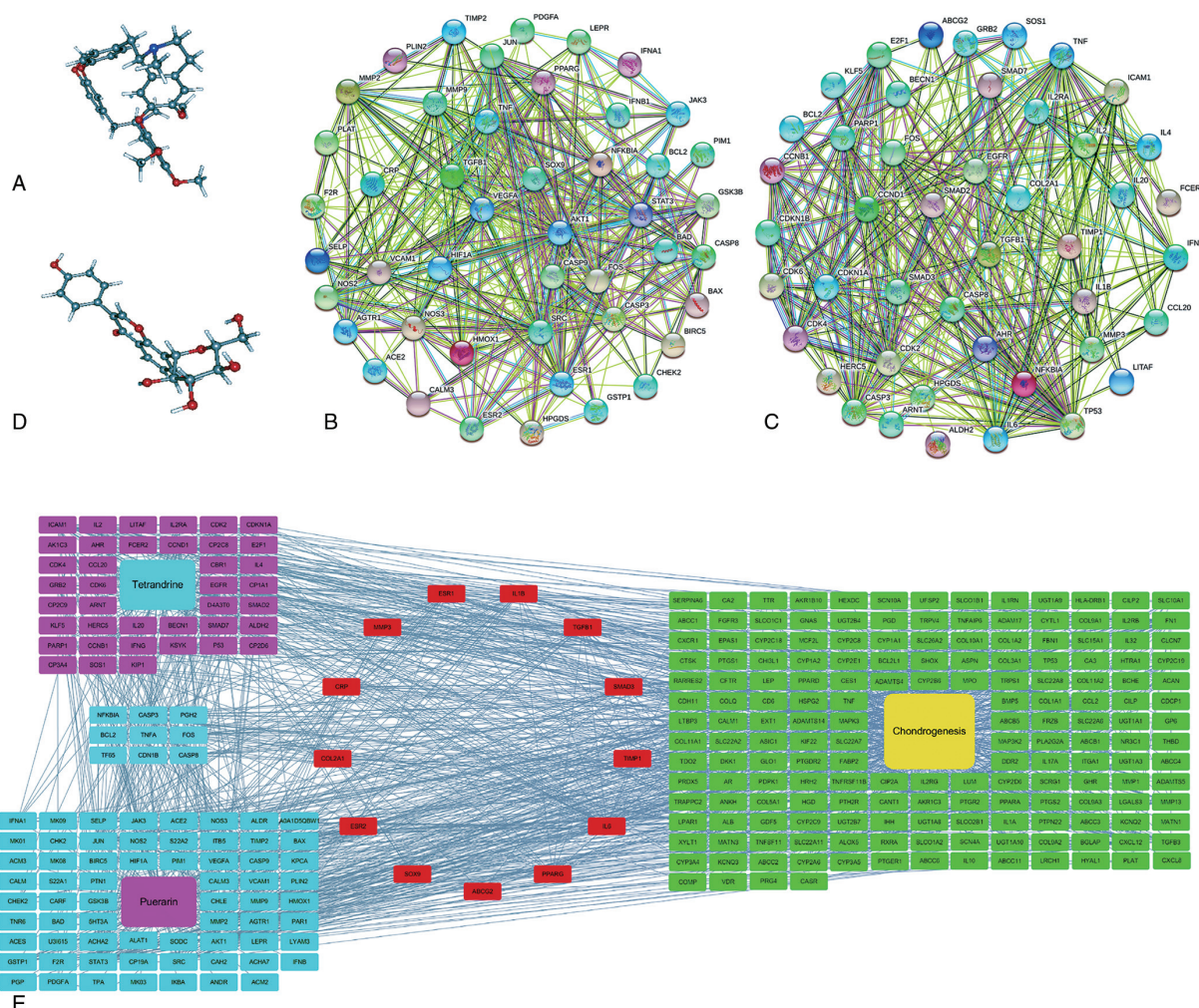


Figure 1. (A) 3D structural formula of tetrandrine. (B) Protein interaction map of tetrandrine targets. (C) Protein interaction map of puerarin targets. (D) 3D structural formula of puerarin. (E) Action target protein interaction (PPI) network, cross-fuse of the drug–target PPI network, and disease target PPI network.

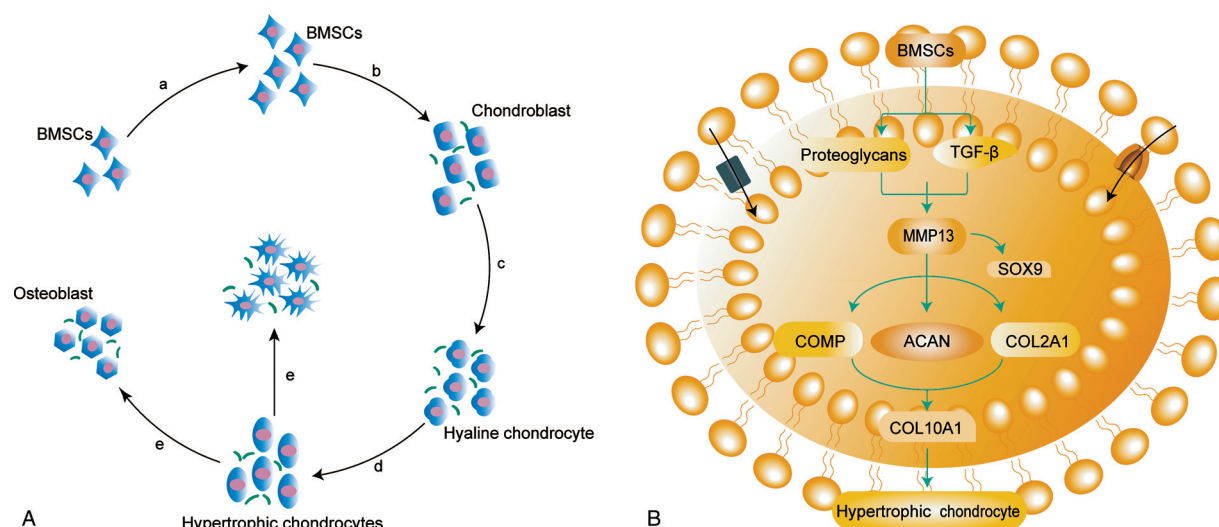


Figure 2. (A) Schematic representation of chondrogenesis and endochondral ossification. (a) Dense cell mass of bone marrow mesenchymal stem cells (BMSCs). (b) BMSCs proliferated and differentiated into chondroblasts. (c) Chondroblasts secreted cartilage extracellular matrix (ECM), and the cells grew into mature chondrocytes. (d) Chondrocytes turned hypertrophic. When endochondral ossification occurred in tissue, (e) Cartilage become vascularized, the ECM was degraded, hypertrophic chondrocytes become apoptotic, and osteoblasts invaded the tissue. (B) Upstream and downstream regulation of SOX-9. Chondrogenesis begins with the upregulation of N-cadherin and SOX-9 by paracrine factors like TGF- β , whereas Wnts downregulate the expression of SOX-9. The expression of N-cadherins and SOX-9 is accompanied by the condensation and proliferation of BMSCs, a process called mesenchymal condensation. SOX-9 induces the BMSCs to differentiate into chondroblasts after condensation. Chondroblasts produce cartilage-specific ECM, including collagen type-II (COL2A1) and proteoglycans such as aggrecan (ACAN).

Effects of puerarin on the expression of chondrogenic differentiation-related genes and proteins

When chondrogenic differentiation of BMSCs was induced by puerarin for 3 and 7 days, the mRNA expression in the puerarin group showed no significant difference, except for *Col2a1* on the seventh day. The protein expression of COL2A1, COL10A1, MMP13, and SOX-9 was promoted after induction for 3 and 7 days, especially the protein expression of SOX-9 and MMP13 (Figure 4).

Effects of tetrandrine on the expression of chondrogenic differentiation-related genes and proteins

When chondrogenic differentiation of BMSCs was induced by tetrandrine for 3 days, the mRNA expression of *Col10a1*, *Tgfb1*, and *Sox-9* increased. The expression of COL2A1, COL10A1, and SOX-9 was also promoted. Seven days after induction, the mRNA expression of *Acan*, *Col10a1*, and *Sox-9* was promoted. Moreover, protein expression of COL2A1, MMP13, and SOX-9 was promoted. The mRNA expression of *Sox-9* increased dramatically at both 3 and 7 days, whereas the protein expression of COL2A1 and SOX-9 was significantly higher (Figure 5).

Effects of combination of puerarin and tetrandrine on the expression of chondrogenic differentiation-related genes and proteins

When the combination of puerarin and tetrandrine was used to induce the chondrogenic differentiation of BMSCs for 3 days, the mRNA expression of *Sox-9* showed a slight increase. The expression of COL2A1, COL10A1, and SOX-9 was also promoted. After being induced for 7 days of induction, the mRNA expression of *Col2a1*, *Acan*, *Col10a1*, and *Sox-9* increased. Further, protein expression of COL2A1, COL10A1, MMP13, and SOX-9 was promoted (Figure 6).

Discussion

Osteoarthritis is the leading cause of chronic disability worldwide. Finding BMSCs chondrogenic differentiation inducers is the ideal strategy to treat osteoarthritis, and the advantages of traditional Chinese medicine in this field have been highlighted. Puerarin can regulate the expression of genes associated with the chondrogenic differentiation of BMSCs^[30]. Low concentrations of tetrandrine can also promote the proliferation of chondrocytes, increase their activity, and promote the secretion of COL2A1 chain and glycosaminoglycan by chondrocytes, which is conducive to the repair and reconstruction of cartilage tissue *in vitro*^[31]. Combined with the results of the network pharmacology analysis, puerarin and tetrandrine could have the potential to induce the differentiation of BMSCs into chondrocytes. Moreover, compatibility of puerarin and tetrandrine was the basis for testing whether it had a better effect on inducing chondrogenic differentiation.

Based on the results of network pharmacological analysis, we generated signaling pathway maps and selected *Tgfb1*, *Acan*, *Sox-9*, *Col2a1*, *Col10a1*, and *Mmp13*, and related proteins SOX-9, COL2A1, COL10A1, and MMP13 for mRNA and protein expression validation. ACAN, also known as the aggrecan core protein, is present in the cartilage matrix and participates in stabilizing the tissue structure. SOX-9 is an early marker of chondrogenic differentiation^[32]. It also regulates *Col2a1* mRNA expression, a cartilage-specific gene^[33–34]. COL2A1 is a cartilage-specific collagen^[35–36] and an important marker of chondrogenic differentiation^[22]. COL10A1 is a marker of hypertrophic chondrocytes. TGF β 1 can promote cell proliferation, chondrogenic differentiation of BMSCs, and the synthesis of extracellular matrix proteoglycans and COL2A1. It can also promote generation of the extracellular matrix of cartilage, downregulate the expression of COL10A1, inhibit the final differentiation of chondrocytes, and

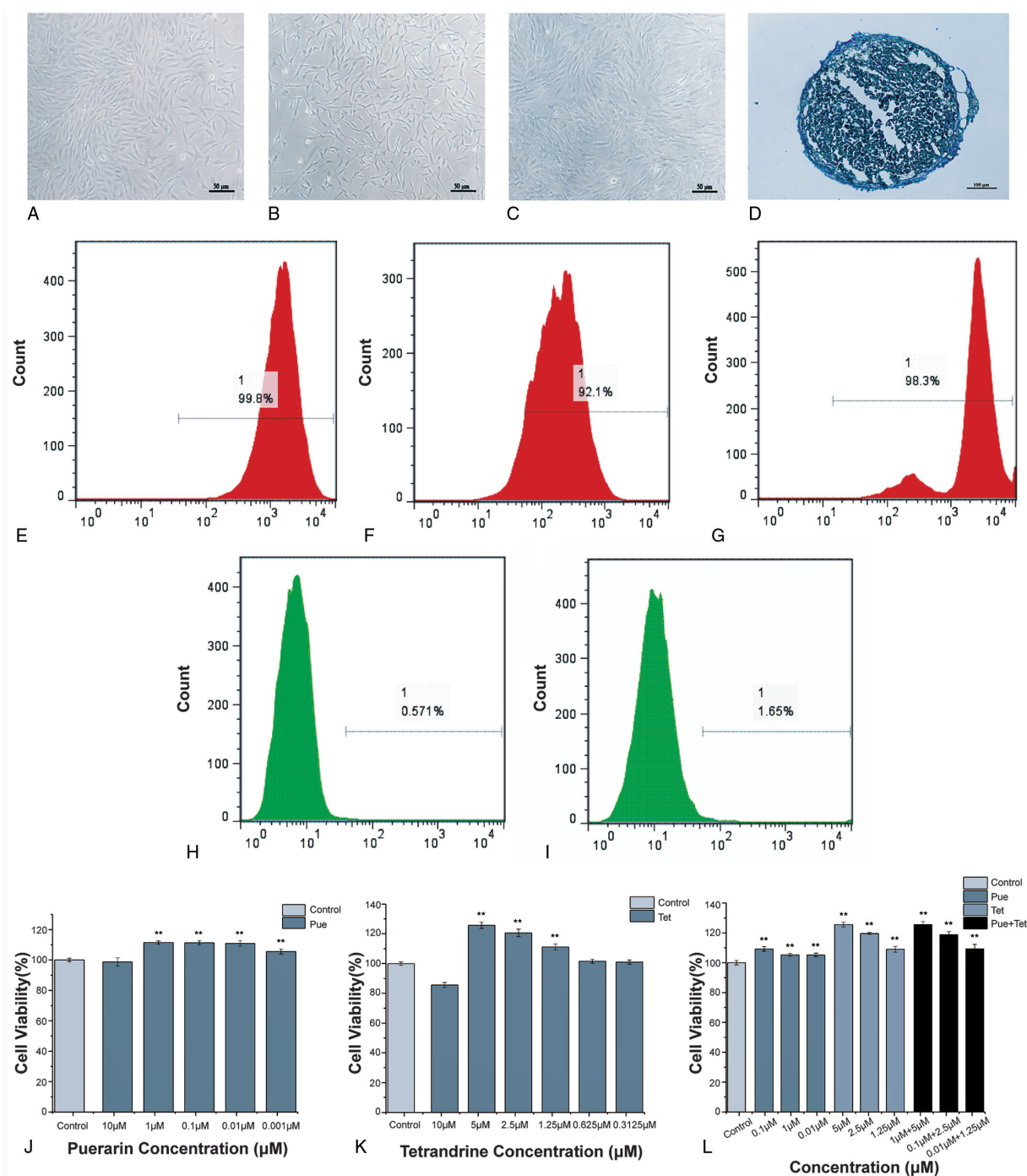


Figure 3. (A–C) Morphological charts of P0, P3, and resuscitation P6 bone marrow mesenchymal stem cells (BMSCs) from SD rats cultured *in vitro* ($\times 100$). (D) Chondrogenic induction of BMSCs from SD rats for 21 d, based on frozen sections and toluidine blue staining ($\times 100$). (E–I) Expression of the surface markers of BMSCs. (J–L) Effects of puerarin and tetrandrine on the proliferation of BMSCs. * $P < 0.05$ or ** $P < 0.01$ was considered to indicate a significant difference.

maintain chondrocytes in the pre-hypertrophic state^[35,37]. MMP13 is a substrate-specific marker of COL2A1 that can promote the absorption and reconstruction of the extracellular matrix and hypertrophy of chondrocytes^[36,38] (Figure 2).

BMSCs are the starting point for chondrogenic differentiation^[39]. With appropriate signaling regulation, along with the interactions between the extracellular matrix and surrounding cells, BMSCs assemble and produce collagen type-I, fibronectin, and ACAN^[40] before differentiating into chondrocytes. At this point,

the assembled BMSCs begin to express SOX-9, which can regulate downstream genes such as *Col2a1* in cartilage formation and promote progenitor cells to secrete cartilage-specific extracellular matrix components^[41–44]. BMSCs then differentiate into hyaline cartilage together with ACAN, COL2A1, collagen type-IX and XI. Hyaline chondrocytes differentiate into hypertrophic chondrocytes through a series of morphological and physical changes. At this point, synthesis of the extracellular matrix is completed, and COL10A1 and MMP13 are secreted. Then, the extracellular matrix begins to degrade,

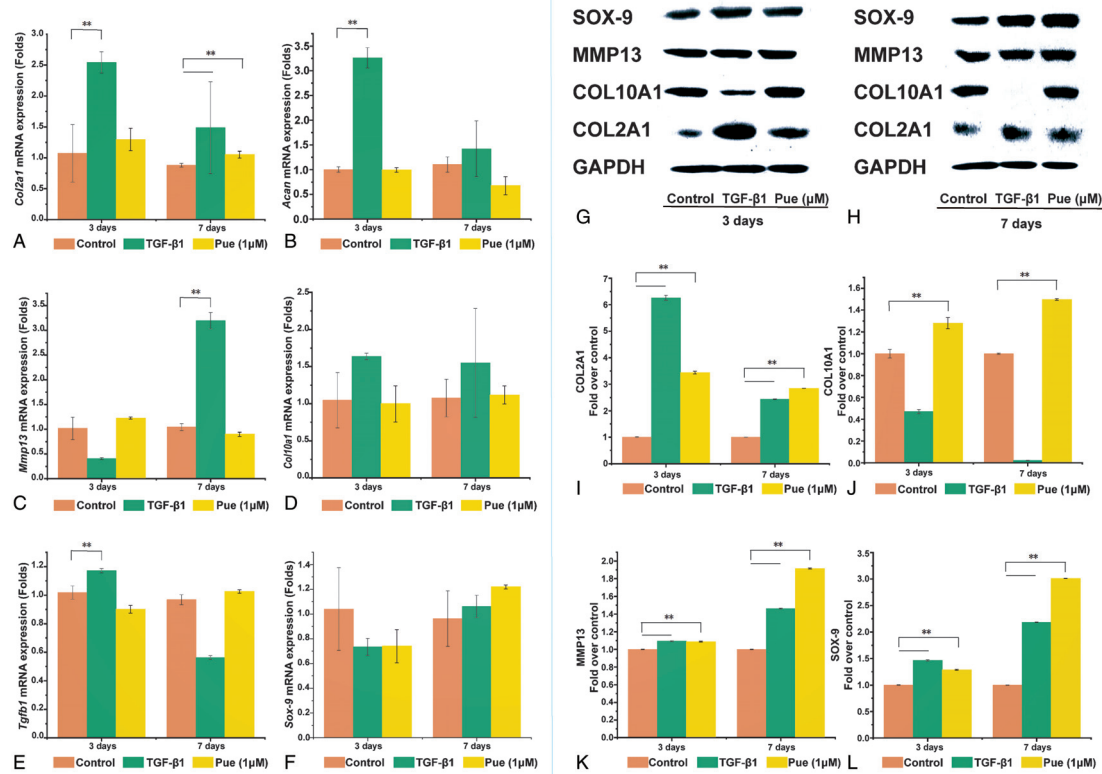


Figure 4. Effects of puerarin (Pue) after 3 days or 7 days of administration. (A–F) After 3 days or 7 days of Pue administration, the mRNA expression of *Col2a1*, *Acan*, *Mmp13*, *Col10a1*, *Tgfb1*, and *Sox-9* was tested. (G–L) After 3 days or 7 days of Pue administration, the protein expression of COL2A1, COL10A1, MMP13, and SOX-9 was tested. * $P < 0.05$ or ** $P < 0.01$ was considered to indicate a significant difference.

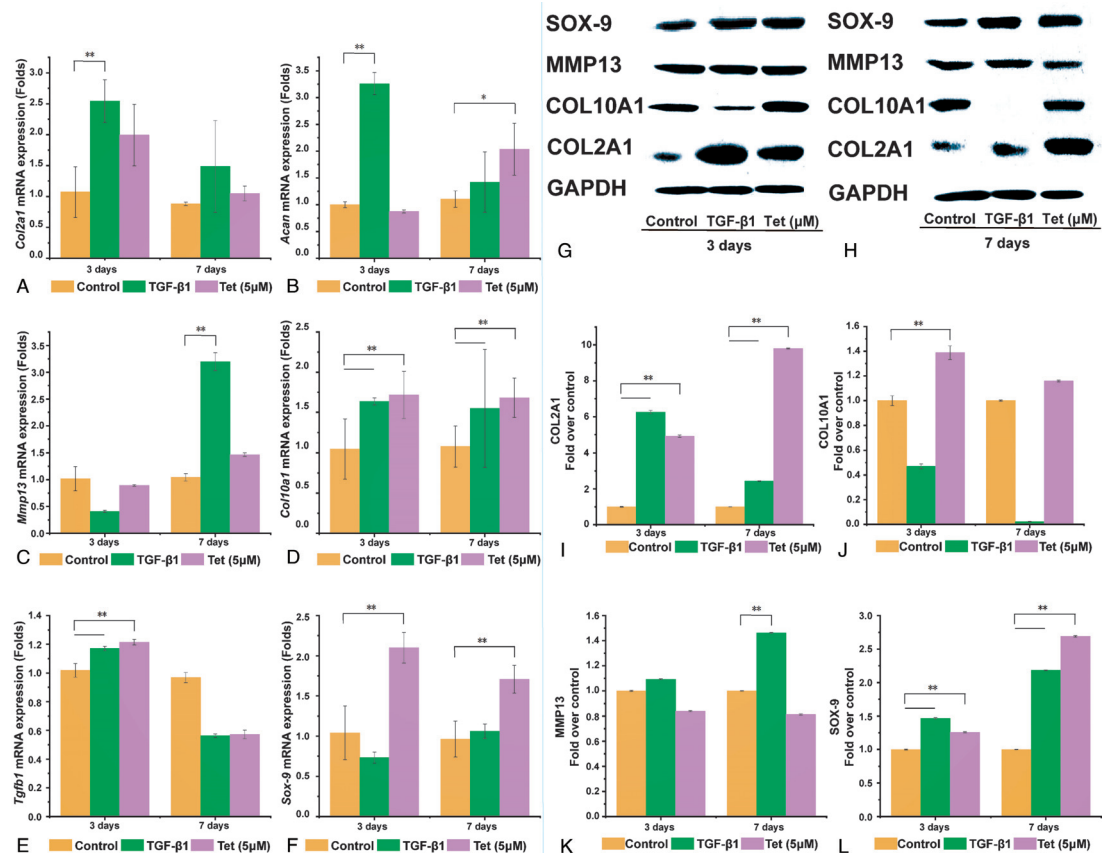


Figure 5. Effects of tetrandrine (Tet) after 3 days or 7 days of administration. (A–F) After 3 days or 7 days of Tet administration, the mRNA expression of *Col2a1*, *Acan*, *Mmp13*, *Col10a1*, *Tgfb1*, and *Sox-9* was tested. (G–L) After 3 days or 7 days of Tet administration, the protein expression of COL2A1, COL10A1, MMP13, and SOX-9 was tested. * $P < 0.05$ or ** $P < 0.01$ was considered to indicate a significant difference.

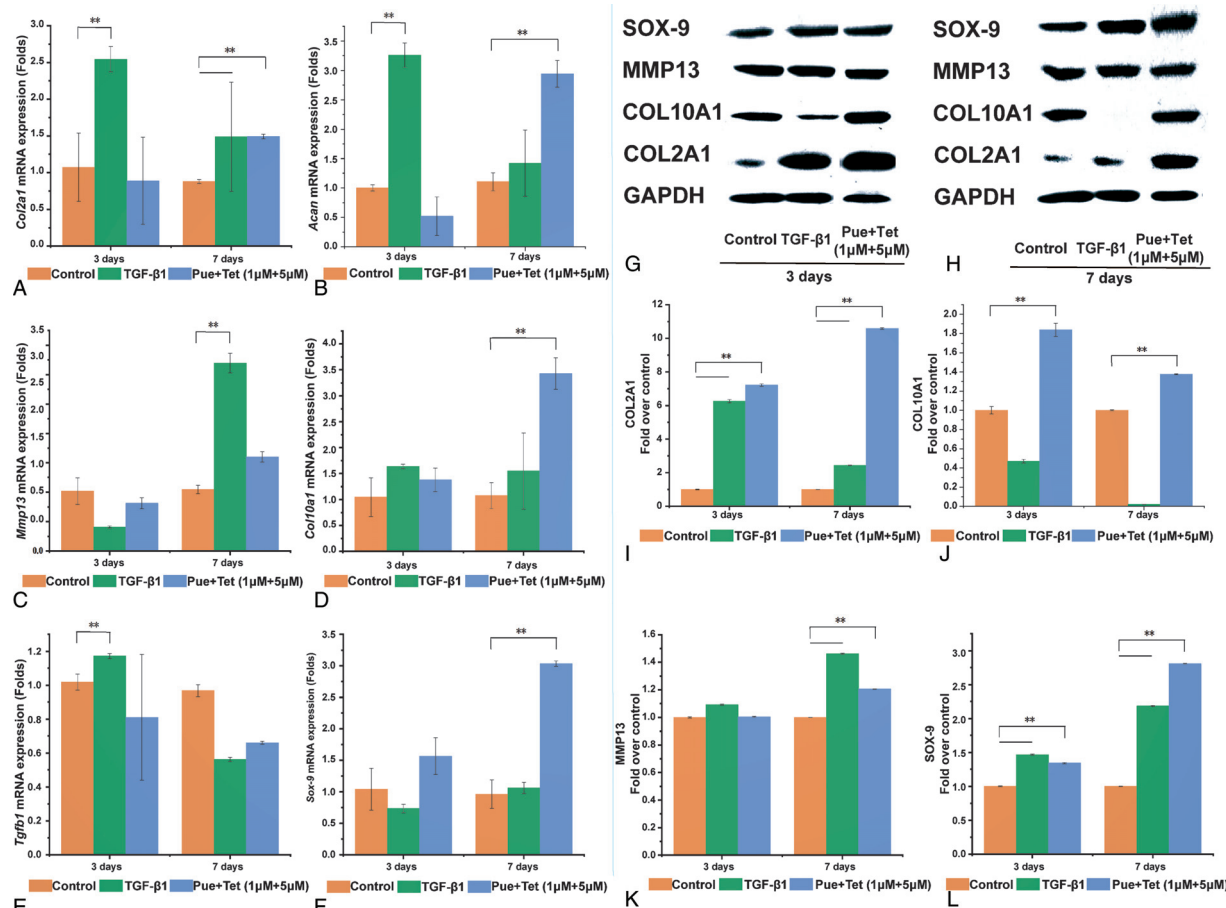


Figure 6. Effects of combined puerarin and tetrandrine (Pue+Tet) after 3 days or 7 days of administration. (A–F) After 3 days or 7 days of Pue+Tet administration, the mRNA expression of *Col2a1*, *Acan*, *Mmp13*, *Col10a1*, *Tgfb1*, and *Sox-9* was tested. (G–L) After 3 days or 7 days of Pue+Tet administration, the protein expression of COL2A1, COL10A1, MMP13, and SOX-9 was tested. * $P < 0.05$ or ** $P < 0.01$ was considered to indicate a significant difference.

part of the hypertrophic chondrocyte transforms into osteocytes, called osteogenic chondrocytes, and the other part becomes apoptotic. These two types of chondrocytes were terminally differentiated (Figure 2).

TGFB1 can promote the formation of the extracellular matrix of chondrocytes, especially the synthesis of ACAN, which was confirmed by the experimental results of this study. As a marker of cellular hypertrophy in the late stage of chondrogenic differentiation, the treatment effects on MMP13 proved that puerarin can promote the expression of related proteins in the late stage of cartilage differentiation of BMSCs better than that with tetrandrine, and results confirmed the compatibility of puerarin and tetrandrine. Tetrandrine promoted the expression of chondrogenic-related genes, such as *Sox-9*, and related proteins better than puerarin. In addition, the selection of detection time points had a significant effect on gene and protein expression.

Conclusion

Network pharmacology studies have demonstrated that puerarin and tetrandrine influence the chondrogenic differentiation of BMSCs, therefore, we performed experimental verification by inducing the chondrogenic differentiation of BMSCs using puerarin, tetrandrine, and a combination of the two. These findings revealed that

puerarin, tetrandrine, and their combination have inductive effects on the chondrogenic differentiation of BMSCs. Interestingly, the induction effects of these drugs on BMSCs were not completely consistent. Puerarin had a better catalytic effect on late chondrogenic differentiation, whereas tetrandrine and the combination of puerarin and tetrandrine had a better promoting effect on middle chondrogenic differentiation. Noticeably, among the genes and proteins related to cartilage formation, results of only a few genes and proteins showed that the combination of puerarin and tetrandrine was better than puerarin or tetrandrine alone. Compared with puerarin and tetrandrine alone, the combination of puerarin and tetrandrine did not significantly change this effect. These results might be related to the different effects of the drugs on protein expression. However, the current conclusions are only based on cell experiments, and more animal experiments and future clinical trials are needed to further verify our conclusions.

Conflict of interest statement

Yuan-Lu Cui is editorial board members of this journal and other authors declare no conflicts of interest.

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Author contributions

Yuan-Lu Cui and Ke-Feng Li designed the study. Xin-Ran Dong and Meng-Jiao Hu prepared the manuscript and analyzed the data. Hui-Xin Pan contributed to the experiments.

Ethical approval of studies and informed consent

Not applicable.

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None.

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