

# Preliminary Study on the Differentiation of Bone Marrow Mesenchymal Stem Cells into Chondrocytes Induced by TGF- $\beta$ 1, Yougui Yin Drug-Containing Serum and Mechanical Stimulation

Zengping Lin<sup>1\*</sup>, Kanghua Zheng<sup>2</sup>, Darong Nie<sup>1</sup>, Huoquan Ye<sup>2</sup>, Hongjie Zhang<sup>1</sup>, Linfeng Tang<sup>2</sup>, Zhong Dong<sup>1</sup>

<sup>1</sup>Department of Orthopaedics, Fujian Provincial 2nd People's Hospital, Affiliated Hospital of Fujian University of Traditional Chinese Medicine, Fuzhou, China

<sup>2</sup>Fujian Provincial 2nd People's Hospital, Affiliated Hospital of Fujian University of Traditional Chinese Medicine, Fuzhou, China  
Email: \*510374867@qq.com

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## Abstract

**Objective:** Bone marrow mesenchymal stem cells (BMSCs) have the potential to differentiate into chondrocytes, it is the ideal seed cells for treating cartilage injury. The purpose of this study was to investigate the inducible factors that stimulate the differentiation of bone marrow mesenchymal stem cells into chondrocytes. **Methods:** Rat BMSCs were isolated and subcultured, and then inoculated on PLGA scaffolds after successfully identifying of BMSCs. They were divided into 5 groups. Blank serum group, TGF- $\beta$ 1 + Yougui Yin drug-containing serum induction group, TGF- $\beta$ 1 + mechanical stimulation induction group, Yougui Yin drug-containing serum + mechanical stimulation induction group, TGF- $\beta$ 1 + Yougui Yin drug-containing serum + mechanical stimulation induction group, 4 weeks later, the adhesion status of cells and scaffolds was observed by Scanning Electron Microscopy, and then chondrocyte induction was performed on different treatment groups. After induction, the expression of Aggrecan and Collagen II was detected by immunofluorescence, and the mRNA expression of Aggrecan and Collagen II was detected by qPCR. **Results:** BMSCs were identified as rat bone marrow mesenchymal stem cells by immunofluorescence CD34 and CD44 cells. Scanning Electron Microscopy observed that BMSCs grew well on PLGA. Compared with PLGA group, the scaffold space of BMSCS-PLGA composite was significantly reduced, and the growth of cell adhesion scaffold was significantly increased.

Compared with the blank serum group, the expression of Aggrecan in TGF- $\beta$ 1 + Yougui Yin drug-containing serum group and Yougui Yin drug-containing serum + mechanical stimulation group was significantly increased. The expression of Collagen II in TGF- $\beta$ 1 + Yougui Yin medicated serum group, Yougui Yin medicated serum + mechanical stimulation group, TGF- $\beta$ 1 + mechanical stimulation group and TGF- $\beta$ 1 + Yougui Yin medicated serum + mechanical stimulation group were significantly increased; PCR results showed that compared with blank serum group, the expression of Aggrecan in other groups was significantly increased, except for Collagen II expression in Yougui Yin drug-containing serum + mechanical stimulation group. **Conclusion:** BMSCs can differentiate into chondrocytes on PLGA scaffold. Compared with blank serum group, BMSCs can be induced to differentiate into chondrocytes in all induction groups, among all induction groups, TGF- $\beta$ 1 + Yougui-yin drug-containing serum group and TGF- $\beta$ 1 + mechanical stimulation group were more significant, and the difference was statistically significant.

### Keywords

BMSCs, Yougui Yin Drug-Containing Serum, TGF- $\beta$ 1, Mechanical Stimulation, PLGA, Chondrocyt

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## 1. Introduction

Cartilage degeneration, injury and defect are common clinical diseases. With the aging of society, the prevalence of diseases affecting cartilage is still increasing. According to epidemiology, more than 50% of middle-aged and elderly people over 65 years old have cartilage-related diseases [1], but the traditional treatment methods (such as joint grinding and shaping, subchondral bone trepanation, arthroscopic debridement, microfracture and osteotomy) for cartilage diseases are not effective. The treatment of cartilage defect and injury with microfracture can relieve pain and improve function to a certain extent in the medium term, but the long-term effect is weakened [2] [3]. Most importantly, because the traditional surgical treatment cannot produce articular cartilage with mechanical function [4]. Cartilage tissue engineering has been proposed in recent years as a new method to treat cartilage injury. Bone marrow mesenchymal stem cells (BMSCs) have the multidirection differentiation potential to differentiate into bone, cartilage, tendon, fat and other tissues, and are regarded as ideal seed cells for cartilage tissue engineering [5]. In my previous studies, I found that mechanical stimulation can promote the differentiation of bone marrow mesenchymal stem cells into chondrocytes. However, during the differentiation of bone marrow mesenchymal stem cells into chondrocytes, in addition to mechanical stimulation, they are also affected by growth factors, traditional Chinese medicine, etc. Therefore, the purpose of this study is to explore various factors that contribute to the differentiation of bone marrow mesenchymal stem cells into chondrocytes. In order to find the

most suitable cartilage or chondrocyte for clinical application, it has a broad application prospect.

## 2. Materials and Methods

### 2.1. Experimental Materials

Male SD rat (SCXK(Beijing) Biotechnology Co., Ltd., SCXK (Beijing) 2019-0010), DMEM/F12 (10565018, Gibco), TGF- $\beta$ 1 (HY-P70648, MCE), Yougui Yin Prescription (Pharmacy of Traditional Chinese Medicine, The Second People's Hospital Affiliated to Fujian University of Chinese Medicine), CD34 antibody (GB121693, Servicebio, 1/500), CD44 (DF6392, Affinity, 1/200), Aggrecan antibody (DF7561, Affinity, 1/200), Collagen II antibody (AF0135, Collagen II antibody) Affinity, 1/200), Trizol reagent (CW0580S, CWBIO) RNA Kit (CW0581M, CWBIO), RNA reverse transcription reagent (R223-01, Vazyme). This study was completed in the corresponding laboratory from 2023.6-2024.7.

### 2.2. Experimental Methods

1) Preparation of Yougui Yin drug-containing serum and blank serum: Yougui Yin drug-containing serum of healthy SPF male SD rat was prepared according to the method described in the literature [6], and was filtered with 0.22  $\mu$ m microporous filter membrane to remove bacteria, and stored in the refrigerator at -20°C for later use. The blank serum group was given 1 mL/100g normal saline intragastric administration, and the method was the same as that of Yougui Yin group.

2) Obtaining bone marrow mesenchymal stem cells (BMSCs) from SD rats: The third generation of high purity BMSCs were obtained by density gradient centrifugation, adherent culture and proliferation.

3) Identification of 3rd generation bone marrow mesenchymal stem cells: Flow cytometry was used to identify the bone marrow mesenchymal stem cells by expressing CD44 and CD44 antigen, = combined with the biological characteristics of bone marrow mesenchymal stem cells.

4) BMSCs-PLGA Composite: The 3rd generation BMSCs were uniformly planted on polylactic acid-polyglycolic acid copolymer (PLGA), and cultured in a sterile centrifuge tube. The growing and adhering of BMSCs on PLGA was observed under Scanning Electron Microscope.

5) Groups were divided into 5 groups and different induction factors were applied: Blank serum group, TGF- $\beta$ 1 induction + Yougui drink drug-containing serum induction group, TGF- $\beta$ 1 induction + mechanical stimulation induction group, Yougui drink drug-containing serum + mechanical stimulation induction group, TGF- $\beta$ 1 + Yougui drink drug-containing serum + mechanical stimulation induction group, induced for 4 weeks.

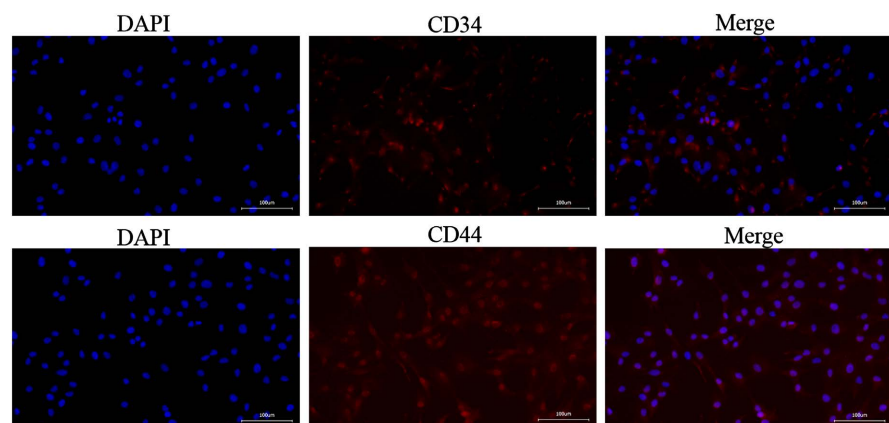
6) The expression of Aggrecan and Collagen II were detected by immunofluorescence.

7) The mRNA expression of Aggrecan and Collagen II was detected by qPCR.

8) Statistical methods: Graphpad Prism9.0 software was used for graph drawing and statistical analysis. All experiments were repeated 3 times, and the quantitative results were expressed as mean  $\pm$  standard deviation ( $X \pm S$ ). One-way analysis of variance was used for quantitative comparison among multiple groups, and S-N-K method was used for pairwise comparison.  $P < 0.05$  indicated significant difference.

### 3. Result

1) BMSCs identification by immunofluorescence: BMSCs isolated from the femur of SD rats were selected for immunofluorescence identification of the expressions of CD34 and CD44. The results were shown in **Figure 1**. The expression of CD34 and CD44 in bone marrow mesenchymal stem cells could be identified as bone marrow mesenchymal stem cells by immunofluorescence identification.



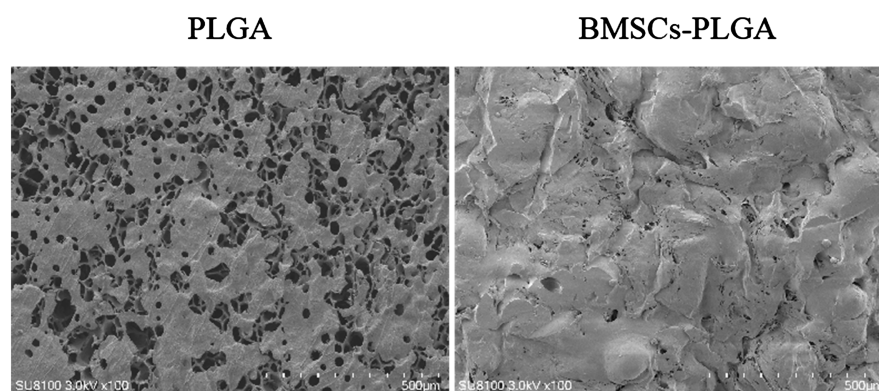
**Figure 1.** Immunofluorescence identification of CD34 and CD44 expression in bone marrow mesenchymal stem cells.

2) Scanning Electron Microscopy (SEM) was used to observe the growth status of cells on the PLGA before and after the cells which were inoculated on the PLGA scaffold. The results were shown in **Figure 2**. Compared with the PLGA group, the scaffold space in the BMSCs-PLGA group significantly reduced, and the cells which adhered on the scaffold grew significantly.

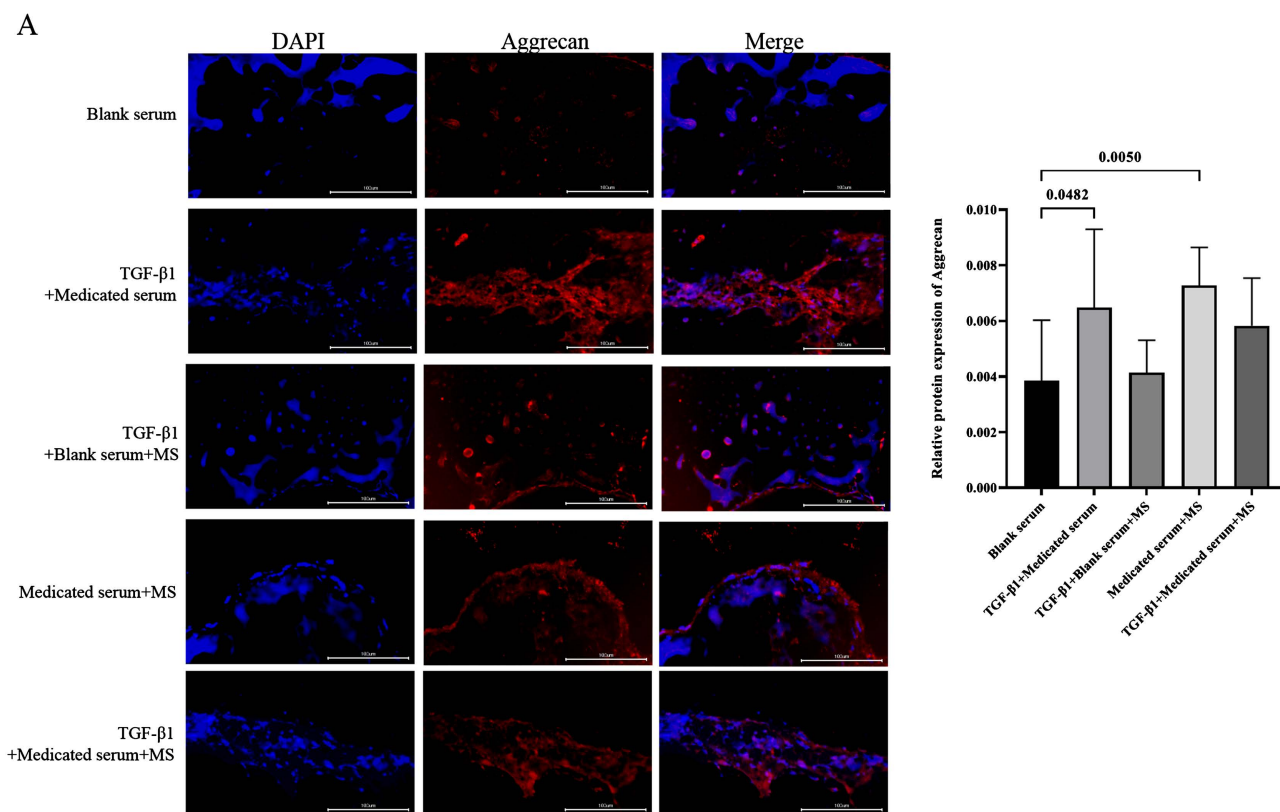
3) The expression of Aggrecan and Collagen II was detected by immunofluorescence: Immunofluorescence was used to detect the expression of Aggrecan and Collagen II in each group, as shown in **Figure 3(A)** and **Figure 3(B)**. Compared with Blank serum group, The expression of Aggrecan was significantly increased in TGF- $\beta$ 1 + Medicated serum group and Medicated serum + MS group. The expression of Aggrecan was increased in TGF- $\beta$ 1 + Blank serum + MS group and TGF- $\beta$ 1 + Medicated serum + MS group, but there was no significant difference. Compared to the Blank serum group, Collagen II expression was significantly increased in TGF- $\beta$ 1 + Medicated serum + MS, Medicated serum + MS, TGF- $\beta$ 1 +

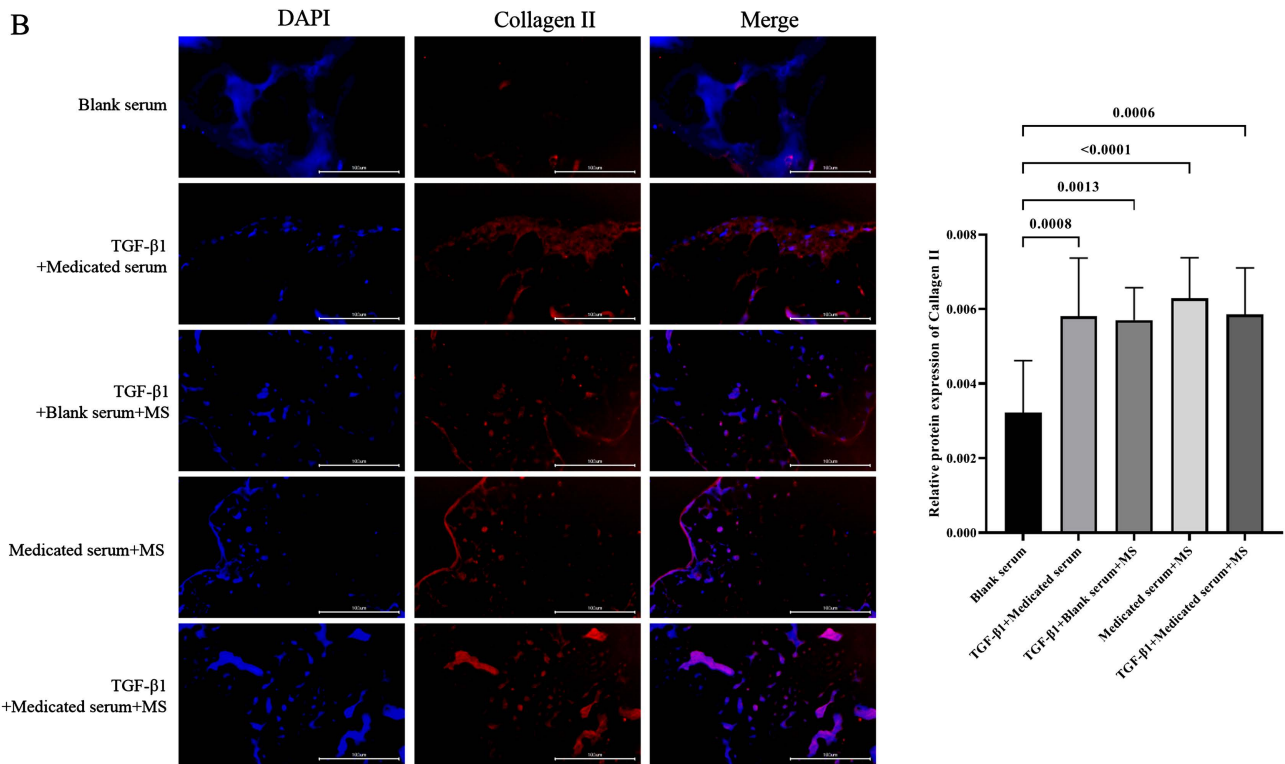
Blank serum + MS and TGF- $\beta$ 1 + Medicated serum + MS groups.

4) The mRNA expression of Aggrecan and Collagen II was detected by qPCR: qPCR was used to detect the expression of Aggrecan and Collagen II in each group, as shown in **Figure 4(A)** and **Figure 4(B)**. Compared with Blank serum group, The expression of Aggrecan in TGF- $\beta$ 1 + Medicated serum + MS, TGF- $\beta$ 1 + Blank serum + MS, Medicated serum + MS and TGF- $\beta$ 1 + Medicated serum + MS groups was significantly increased. All P-values are less than 0.0001. The expression of Collagen II in TGF- $\beta$ 1 + Medicated serum, TGF- $\beta$ 1 + Blank serum + MS and TGF- $\beta$ 1 + Medicated serum + MS groups was significantly increased, all P-values are less than 0.0001.

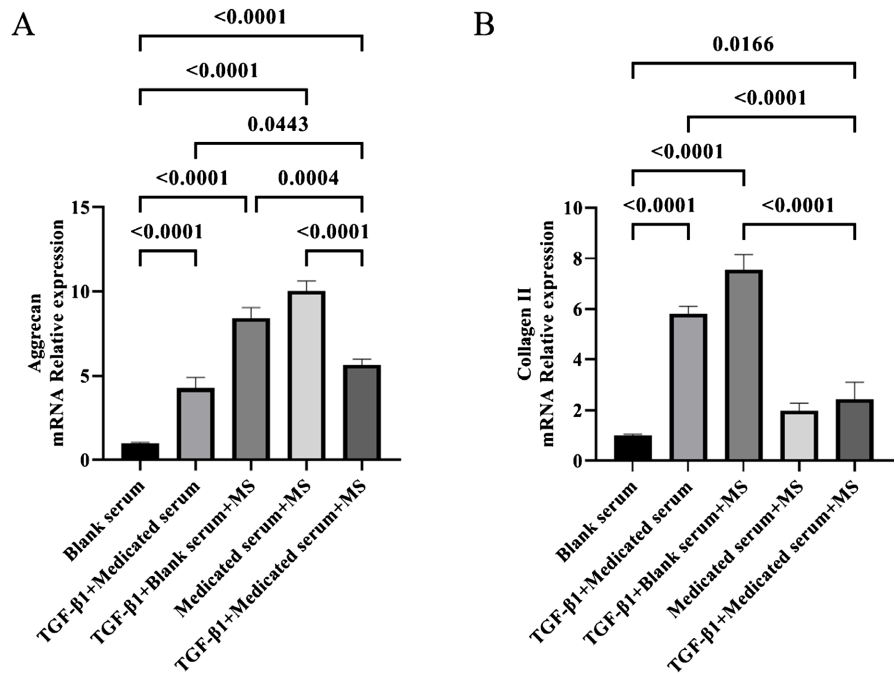


**Figure 2.** SEM image.





**Figure 3.** Detection of Aggrecan by immunofluorescence.



**Figure 4.** PCR detecting mRNA expression of Aggrecan (A) and Collagen II (B).

### 4. Discussion

Cartilage degeneration and injury are common clinical diseases, but cartilage repairing is a difficult problem. Traditional surgical methods (such as arthroplasty,

subchondral trepanation, arthroscopic debridement, microfracture and osteotomy) are not satisfactory. The treatment of cartilage defect and injury with microfracture can relieve pain and improve function to a certain extent in the medium term, but the long-term effect is weakened [2] [3]. The most important is that conventional surgical treatment cannot produce mechanically functional articular cartilage [4]. Therefore, it is greatly scientific significance to find an economical, effective and early repairing method for damaged cartilage.

With the development of materialogy and bioscience, cartilage tissue engineering has become a new method to treat cartilage injury [7]. The three main elements of cartilage tissue engineering are seed cells, factors that induce seed cells to differentiate into chondrocytes, and scaffold materials. BMSCs are ideal seed cells for cartilage tissue engineering [5]. However, BMSCs can differentiate into chondrocytes only under certain induced conditions. Shuai Liu *et al.* [8] explored the effects of  $\gamma$ -PGD hydrogel scaffolds on cartilage differentiation of human bone marrow mesenchymal stem cells and found that the expressions of agglutinoglycan and type II collagen in  $\gamma$ -PGD hydrogel scaffolds containing growth factors were higher than those without growth factors, indicating that in cartilage tissue engineering, Growth factors should be used to induce the differentiation of BMSCs into chondrocytes. At present, the main growth factors were commonly used to induce bone marrow mesenchymal stem cells to differentiate into chondrocytes including transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), which induces bone marrow mesenchymal stem cells to differentiate into chondrocytes by down-regulating gene expression related to MAPK signaling pathway [9]. In recent years, Chinese medicine is effective and safe in the treatment of cartilage degeneration or traumatic arthritis. Studies have shown that TCM decoction, TCM monomer and its effective ingredients can promote the differentiation of bone marrow mesenchymal stem cells into chondrocytes [10]. Peijian Tong *et al.* [6] showed that Yougui-yin drug-containing serum can promote the differentiation of bone marrow mesenchymal stem cells into chondrocytes and secrete more type II collagen and agglutinoglycan. Weidong Wang *et al.* [11] showed that, Yougui Yin has a certain effect on articular cartilage degeneration by inhibiting SDF-1 expression throughout the whole process. Luwei Xiao *et al.* [12] showed that Yougui Yin drug-containing serum can better promote chondrogenic differentiation of BMSCs, and Yougui Yin may play an important role in blocking MAPK signaling pathway by up-regulating miR-24-3p. Tan Yu *et al.* [13] showed that Youguyin can induce chondrogenic differentiation of rabbit BMSCs, and the BMSCs/fibrin glue complex induced by Youguyin can promote the repair of rabbit knee cartilage defects. Therefore, Yougui Yin or its drug-containing serum can induce the differentiation of BMSCs into chondrocytes. At the same time, in nature, chondrocytes are not only affected by the surrounding matrix, but also by various biomechanical effects, such as centrifugal force, static pressure, etc. Mechanical factors are one of the physical factors necessary for chondrocyte metabolism and proliferation. Studies have shown [14] that the co-culture of bone marrow mesenchymal stem cells and

chondrocytes can differentiate bone marrow mesenchymal stem cells into chondrocytes, indicating that the metabolic environment of chondrocytes is conducive to promoting the differentiation of BMSCs into chondrocytes. Therefore, *in vitro* induction experiments, the closer the physiological environment of chondrocytes is to that of chondrocytes, the more favorable it is to promote the differentiation of bone marrow mesenchymal stem cells into chondrocytes, that is, mechanical stimulation is conducive to the differentiation of bone marrow mesenchymal stem cells into chondrocytes. Ouyang X *et al.* [15] suggest that co-culture of mesenchymal stem cells with chondrocytes and appropriate mechanical stimulation may be an appropriate way to rapidly expand cells with improved chondrogenic phenotype in cartilage tissue engineering. Kim *et al.* [16] showed that mechanical stimulation promoted the differentiation of stem cells into chondrocytes by inhibiting TGF- $\beta$ 1-induced actin remodeling and activating mitogen-activated protein kinase (MAPK). Chen *et al.* [17] also found that long-term mechanical stimulation such as dynamic compression enhancement and TGF- $\beta$ 3 induced chondrogenesis of bovine stem cells. MAPK and other signal pathways may play a key role in dynamic compression enhancement. Zengping Lin *et al.* [18] studied different mechanical stimuli on the differentiation of bone marrow mesenchymal stem cells into chondrocytes and found that the application of appropriate centrifugal force in TGF- $\beta$ 1 induction solution was more conducive to the differentiation of BMSCs into chondrocytes and the secretion of more type II collagen and aggrecan, and the difference was statistically significant compared with the group using inducers alone. Therefore, previous relevant studies have shown that growth factors, mechanics, Chinese medicine or Chinese medicine serum can effectively induce BMSCs to differentiate into chondrocytes, which is consistent with the results of this study. How to optimize the combination of the above three induction factors to better induce the differentiation of BMSCs into chondrocytes is the focus of current research. Through this experimental study, BMSCs can adhere on PLGA and multiply differentiate into chondrocytes on PLGA scaffolds. TGF- $\beta$ 1, Yougui Yin drug-containing serum and mechanical stimulation can induce the differentiation of BMSCs into chondrocytes. Among them, TGF- $\beta$ 1 + Yougui Yin drug-containing serum group and TGF- $\beta$ 1 + mechanical stimulation group were more significant, and the difference was statistically significant. Meanwhile, this experimental study also showed that in the process of inducing BMSCs to differentiate into chondrocytes, TGF- $\beta$ 1 + Yougui Yin drug-containing serum + mechanical stimulation group was not as good as TGF- $\beta$ 1 + Yougui Yin drug-containing serum group and TGF- $\beta$ 1 + mechanical stimulation group, it is indicating that it is not true of the more combination of inductive factors, the more conducive to the differentiation of BMSCs into chondrocytes, and its mechanism needs further study.

In summary, cartilage tissue engineering is a new method to treat cartilage injury, BMSCs are ideal seed cells for cartilage tissue engineering, PLGA can be used as a scaffold for BMSCs, TGF- $\beta$ 1 + Yougui Yin drug-containing serum group and

TGF- $\beta$ 1 + mechanical stimulation group are superior to other groups, and the difference is statistically significant. Due to the limited sample size of this experiment, it is only a preliminary study. At the same time, why TGF- $\beta$ 1 + Yougui Yin drug-containing serum group and TGF- $\beta$ 1 + mechanical stimulation group are better than TGF- $\beta$ 1 + Yougui Yin drug-containing serum + mechanical stimulation group, and its mechanism needs to be further studied.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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