


SCIENTIFIC ARTICLE

Synergistic Effects of Kartogenin and Transforming Growth Factor- β 3 on Chondrogenesis of Human Umbilical Cord Mesenchymal Stem Cells *In Vitro*

Yanhong Zhao, MD^{1†}, Binhong Teng, MD^{1,2†}, Xun Sun, MD³, Yunsheng Dong, PhD⁴, Shufang Wang, PhD⁴, Yongcheng Hu, MD³, Zheng Wang, PhD⁵, Xinlong Ma, MD³, Qiang Yang, MD³ 

¹Stomatological Hospital of Tianjin Medical University, ³Department of Spine Surgery, Tianjin Hospital, Tianjin University and ⁴Key Laboratory of Bioactive Materials for Ministry of Education, College of Life Sciences, Nankai University, Tianjin and ²Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology, Peking University and ⁵Department of Orthopedics, No. 1 Medical Center of Chinese PLA General Hospital, Beijing, China

Objective: To explore the effect of kartogenin (KGN) on proliferation and chondrogenic differentiation of human umbilical cord mesenchymal stem cells (hUCMSC) *in vitro*, and the synergistic effects of KGN and transforming growth factor (TGF)- β 3 on hUCMSC.

Methods: Human umbilical cord mesenchymal stem cells were isolated and cultured. Then the differentiation properties were identified by flow cytometry analysis. hUCMSC were divided into four groups: control group, KGN group, TGF- β 3 group, and TK group (with TGF- β 3 and KGN added into the medium simultaneously). Cells in all groups were induced for 21 days using the suspension ball culture method. Hematoxylin and eosin, immunofluorescence, and Alcian blue staining were used to analyze chondrogenic differentiation. Real-time reverse transcriptase polymerase chain reaction was performed to investigate genes associated with chondrogenic differentiation.

Result: Hematoxylin and eosin staining showed that cells in the TGF- β 3 group and the TK group had formed cartilage-like tissue after 21 days of culture. The results of immunofluorescence and Alcian blue staining showed that compared with the control group, cells in the KGN and TGF- β 3 groups demonstrated increased secretion of aggrecan after 21 days of culture. In addition, cells in the group combining KGN with TGF- β 3 (5.587 ± 0.27 , $P < 0.01$) had more collagen II secretion than cells in the TGF- β 3 alone group (2.86 ± 0.141 , $P < 0.01$) or the KGN group (1.203 ± 0.215 , $P < 0.01$). The expression of aggrecan (2.468 ± 0.097 , $P < 0.05$) and SRY-Box 9 (4.08 ± 0.13 , $P < 0.05$) in cells in the group combining KGN with TGF- β 3 was significantly higher than those in the TGF- β 3 group (2.216 ± 0.09 , 3.02 ± 0.132 , $P < 0.05$).

Conclusion: The combination of KGN and TGF- β 3 had synergistic effects and induced hUCMSC chondrogenesis. This could represent a new approach for clinical application and studies on cartilage repair and regeneration.

Key words: Chondrogenesis; Kartogenin; Mesenchymal stem cells; Transforming growth factor- β 3

Introduction

Articular cartilage has poor regenerative capacity because of its aneural, avascular, and low cellular tissue¹. Traditional cell therapies primarily focus on implantation of

autologous chondrocytes. However, current problems observed with this clinical strategy include donor-site morbidity, limited body implants, and limited matrix production following cell expansion^{2,3}. The progress in cartilage tissue

Address for correspondence Qiang Yang, MD, Department of Spine Surgery, Tianjin Hospital, Tianjin University, 406 Jiefang Nan Road, Hexi District, Tianjin, 300211, China; Tel: +86 15102229608; Fax: +86 022-60910608; Email: yangqiang1980@126.com; Xinlong Ma, MD, Department of Spine Surgery, Tianjin Hospital, Tianjin University, 406 Jiefang Nan Road, Hexi District, Tianjin, 300211, China; Tel: +86 13512089385; Email: maxinlong8686@sina.com

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[†]Yanhong Zhao and Binhong Teng contributed equally to this work.

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engineering had led to new approaches for addressing these problems⁴⁻⁶. Increasingly, researchers are applying mesenchymal stem cells (MSC) to repair cartilage and, importantly, using chondrocyte-related growth factors and chemicals to promote chondrogenesis of MSC *in vitro* and *in vivo*⁷⁻⁹.

Kartogenin (KGN), a small molecular organic compound, was first studied by Johnson *et al.* in 2012². Given its ability to regulate expression of the transcription factor RUNX1, which facilitates transcription of cartilage-related proteins into activation, KGN showed great potential in promoting chondrogenesis of MSC and chondrocyte proliferation¹⁰. Compared with protein growth factors, KGN maintains stable physicochemical properties *in vitro* and *in vivo*. This could allow KGN to covalently bind to other compounds and to be loaded in nanoparticles or scaffolds without affecting its performance¹¹. Although KGN has been researched in recent years as a cartilage-promoting molecule, the effect of using KGN alone to induce chondrogenesis of MSC is still not ideal *in vitro*, and it has not been compared with an appropriate positive control group in many studies^{12,13}. Chondrocyte-related growth factors, such as transforming growth factor β 3 (TGF- β 3), have been attracting increased attention in cartilage tissue engineering¹⁴. A huge number of studies have reported that TGF- β 3 stimulates the expression of cartilage-related genes, accelerates the secretion of glycosaminoglycan (GAG) and collagen type II, and obviously enhances neo-cartilage regeneration *in vivo*^{7,15-17}. Moreover, other studies have found that TGF- β 3 could stimulate migration and recruitment of MSC^{17,18}. Because of its key role in chondrogenesis, in the present study, we use TGF- β 3 as a positive control group and further investigate whether KGN and TGF- β 3 have synergistic effects on MSC.

Related studies on chondrogenic differentiation of stem cells by KGN have mainly focused on BMSC^{2,11}, patellar tendon stem/progenitor cells (PTSC)^{10,19}, and human synovium-derived mesenchymal stem cells (SMSC)²⁰; other MSC have rarely been reported. Although bone marrow MSC (BMSC) as a source of stem cells is the focus in cartilage tissue engineering, collecting bone marrow is accompanied by inevitable pain and injury^{21,22}. In addition, the utilization rate of collected BMSC is quite low²³, and the capacity of proliferation and differentiation decrease with age^{24,25}. Therefore, it is reasonable to seek an alternative MSC as a source of stem cells. Because of its low immunogenicity and chondrogenesis potential, hUCMSC has been studied in cartilage tissue engineering and is a promising source of MSC²⁶.

Based on the above discussion, it is worthwhile studying the effect of KGN alone on hUCMSC chondrogenesis and examining whether KGN increases the potential of TGF- β 3-induced hUCMSC chondrogenesis. In the present study, isolation and culture of hUCMSC were observed by optical microscope (OM) and identified by flow cytometry. The effects of KGN and TGF- β 3 on cell growth were examined using a Cell Counting Kit-8 and on chondrogenic induction and differentiation using qualitative histology and

immune-fluorescence, as well as quantitative chondrogenic gene expression.

Materials and Methods

Isolation and Culture of Human Umbilical Cord Mesenchymal Stem Cells

The hUCMSC were isolated and cultivated as previously described²⁷. Human umbilical cords were collected from Tianjin Hospital with patients' informed consent, and in compliance with national legislation. All experimental protocols were approved by the Ethics Committee of Tianjin Hospital.

The umbilical cord samples were harvested and thoroughly washed several times with physiological salt solution. The umbilical cord vessels were moved and we cut Wharton's jelly into small pieces (2 to 3 mm³) with sterile equipment. These small pieces were digested with 0.2% Type II Gibco Collagenase (Gibco, Grand Island, NY, USA) dissolved in Hanks' balanced salt solution at 37°C for 2 h under gentle shaking. Next, Dulbecco's modified Eagle medium (DMEM) with 50% fetal bovine serum (FBS) was added and, to remove tissue debris, these materials were filtered through a 200- μ m pore size mesh. After cells were centrifuged, we further washed the pellets with supplemented DMEM containing 1% penicillin/streptomycin, and the cells were seeded accordingly. The resulting cells were resuspended in DMEM containing 20% FBS and 1% penicillin/streptomycin, then cultured in flasks in a 5% CO₂ humidified incubator at 37°C.

Identification of Human Umbilical Cord Mesenchymal Stem Cells

The hUCMSC were identified by flow cytometry analysis as described previously^{28,29}. The antibodies used were Alexa Fluor 430-conjugated CD73, allophycocyanin (APC)-conjugated CD105, phycoerythrin (PE)-conjugated CD13, CD90, fluorescein isothiocyanate (FITC)-conjugated CD34, and CD45 (all from eBioscience, Santiago, California, USA).

Proliferation Assay of Human Umbilical Cord Mesenchymal Stem Cells

To study the effects of KGN on cell proliferation, a density of 2000 cells per well of hUCMSC were seeded into a 96-well plate as described previously³⁰. KGN (1 μ M, MCE, Med-ChemExpress, USA) and TGF- β 3 (10 ng/mL, Peprotech, Rocky Hill, NJ, USA) were added into each well. Cell activities were analyzed by Cell Counting Kit-8 (CCK-8; Dojindo, Rockville, MD, USA) following the manufacturer's protocol.

Evaluation of Chondrogenic Differentiation of Human Umbilical Cord Mesenchymal Stem Cells

Chondrogenic differentiation of hUCMSC was performed as described²⁰. Briefly, 5 \times 10⁵ cells were placed and centrifuged in 15-mL polypropylene conical tubes. The chondrogenic medium contained 0.1 μ M dexamethasone, 50 μ g/mL L-ascorbic acid, 2% ITS (0.625 mg/mL insulin, 0.625 mg/mL

TABLE 1 The primer sequences

Gene	Primer sequences
GAPDH	5'-AGAAAAACCTGCCAAATATGATGAC-3' 5'-TGGGTGTCGCTGTTGAAGTC-3'
COL2A1	5'-GGCAATAGCAGGTTACAGTACA-3' 5'-CGATAACAGTCTTGCCCCACTT-3'
AGG	5'-TGCATTCCACGAAGCTAACCTT-3' 5'-GACGCCTCGCCTTCTTGA-3'
SOX9	5'-AGCGAACGCACATCAAGAC-3' 5'-GCTGTAGTGTGGGAGGTTGAA-3'
COL10A1	5'-CAAGGCACCATCTCCAGAA-3' 5'-AAAGGGTATTTGTGGCAGCATATT-3'
RUNX2	5'-AGAAGGCACAGACAGAAGCTTGA-3' 5'-AGGAATGCGCCCTAAATCACT-3'

transferrin, and 0.625 $\mu\text{g}/\text{mL}$ selenous acid, Cyagen, China), 100 $\mu\text{g}/\text{mL}$ sodium pyruvate, 40 $\mu\text{g}/\text{mL}$ L-proline, supplemented with TGF- β 3 (10 ng/mL), or KGN (1 μM), or the combination. Typically, TGF- β 3 plays a key role in chondrogenic differentiation and is widely used. In this study TGF- β 3 serves as a positive control. All the culture mediums were changed every 2 or 3 days. There were four groups: a DMSO group (DMSO), a KGN group (KGN), a TGF- β 3 group (TGF- β 3), and a group with TGF- β 3 and KGN (TK).

Histology and Immunofluorescence Analysis

After 21 days of culture, pellets were collected for hematoxylin and eosin (H&E, Solarbio, Beijing, China) staining, Alcian blue (AB, Solarbio, Beijing, China) staining, and

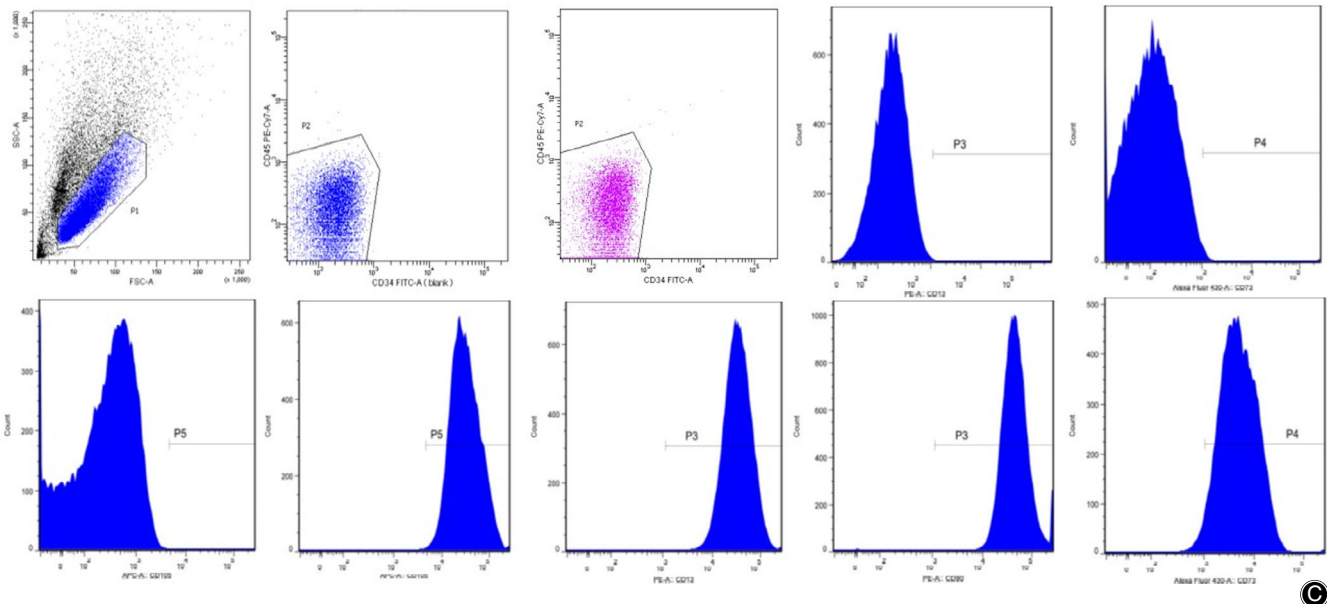
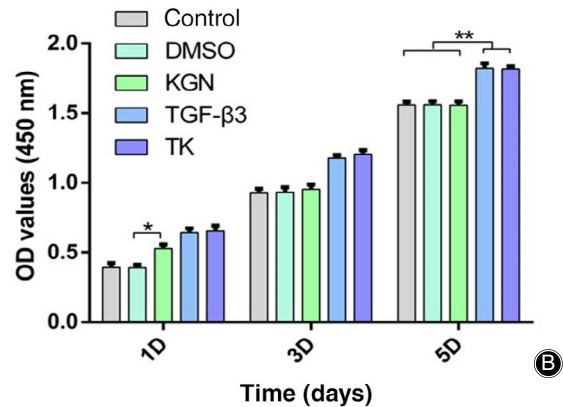
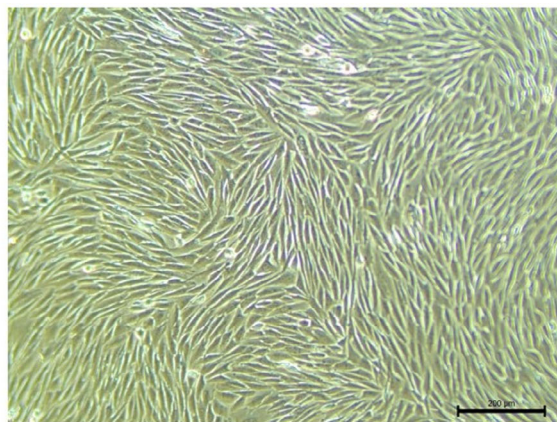


Fig. 1 (A) Morphology of human umbilical cord mesenchymal stem cells (hUCMSC). (B) Assay of proliferation of HUCMSC using a CCK-8 Kit. (C) Flow cytometric analysis of surface-marker expression on HUCMSC. Positive expression of CD13, CD44, CD73, and CD90 and negative expression of CD34 and CD45. $n = 4$.

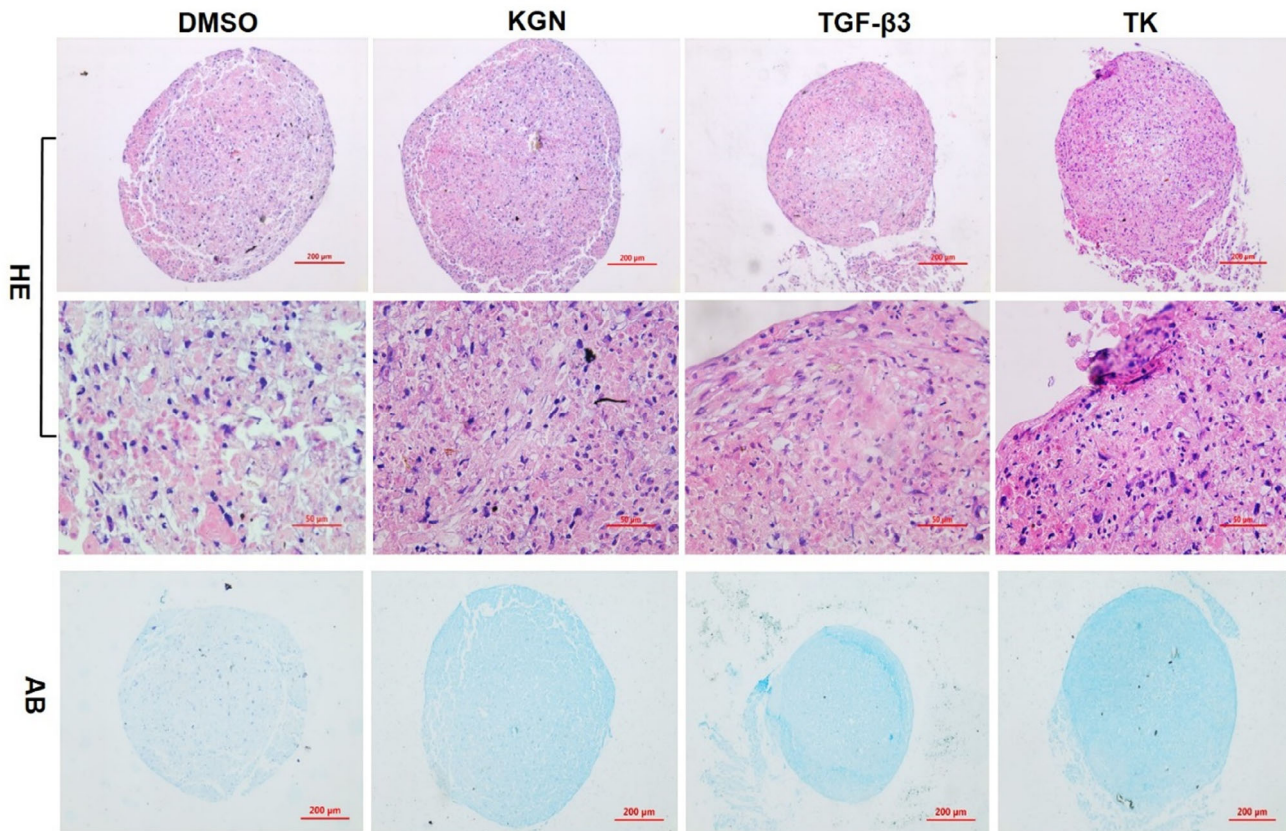


Fig. 2 Hematoxylin and eosin and Alcian blue staining of pellet sections after 3 weeks of cartilage differentiation.

immunofluorescence analysis of collagen II and aggrecan (AGG). Pellets were washed with phosphate buffered saline and fixed in 4% (w/v) paraformaldehyde overnight. After dehydration by graded ethanol, the pellets were embedded in paraffin and cut into 6- μ m-thick sections for staining and other tests. Sections were deparaffinized, rehydrated, and stained with H&E following the manufacturer's protocol, and then the staining status was observed under an inverted light microscopy (Leica, Germany). For immunofluorescence analyses, after deparaffinization, rehydration, and antigen retrieval, mouse-derived monoclonal antibodies against human aggrecan (1:200 dilution; Abcam, USA) and rabbit-derived polyclonal antibodies against human to collagen II (1:200 dilution; Abcam, USA) were applied to the sections, which were incubated overnight at 4°C. The next day, the sections were washed with secondary antibody, goat anti-mouse antibody (1:200 dilution; Abcam, USA), and goat anti-rabbit antibody (1:200 dilution; Abcam, USA), followed by 30-min incubation at room temperature. The sections were then washed three times with phosphate buffered saline. The sections were detected using a fluorescence microscope (Zeiss Axio Imager Z1, Germany).

Real-Time Reverse Transcriptase Polymerase Chain Reaction Assay of Chondrogenic-Related Gene Expression

The expression of chondrogenic-related genes superoxide dismutase 9 (SRY-Box 9), aggrecan (AGG) and collagen type II (COL2A1), and hypertrophy-related genes collagen type X (COL10A1) and runt-related transcription factor 2 (RUNX2) were analyzed by real-time reverse transcriptase polymerase chain reaction (RT-PCR). After 21 days of culture, total RNA of pellets was extracted using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. The total RNA concentration was quantified using a NanoDrop1000 (Thermo Scientific). Subsequently, 300 ng total RNA was reverse-transcribed using a First Strand cDNA Synthesis Kit (Fermentas Life Sciences). RT-PCR involved use of the SYBR Green system (Fermentas Life Sciences) at 95°C for 10 min, followed by 40 cycles at 94°C for 30 s, 56°C for 60 s, and 72°C for 40 s. Expression was calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Primer sequences are presented in Table 1.

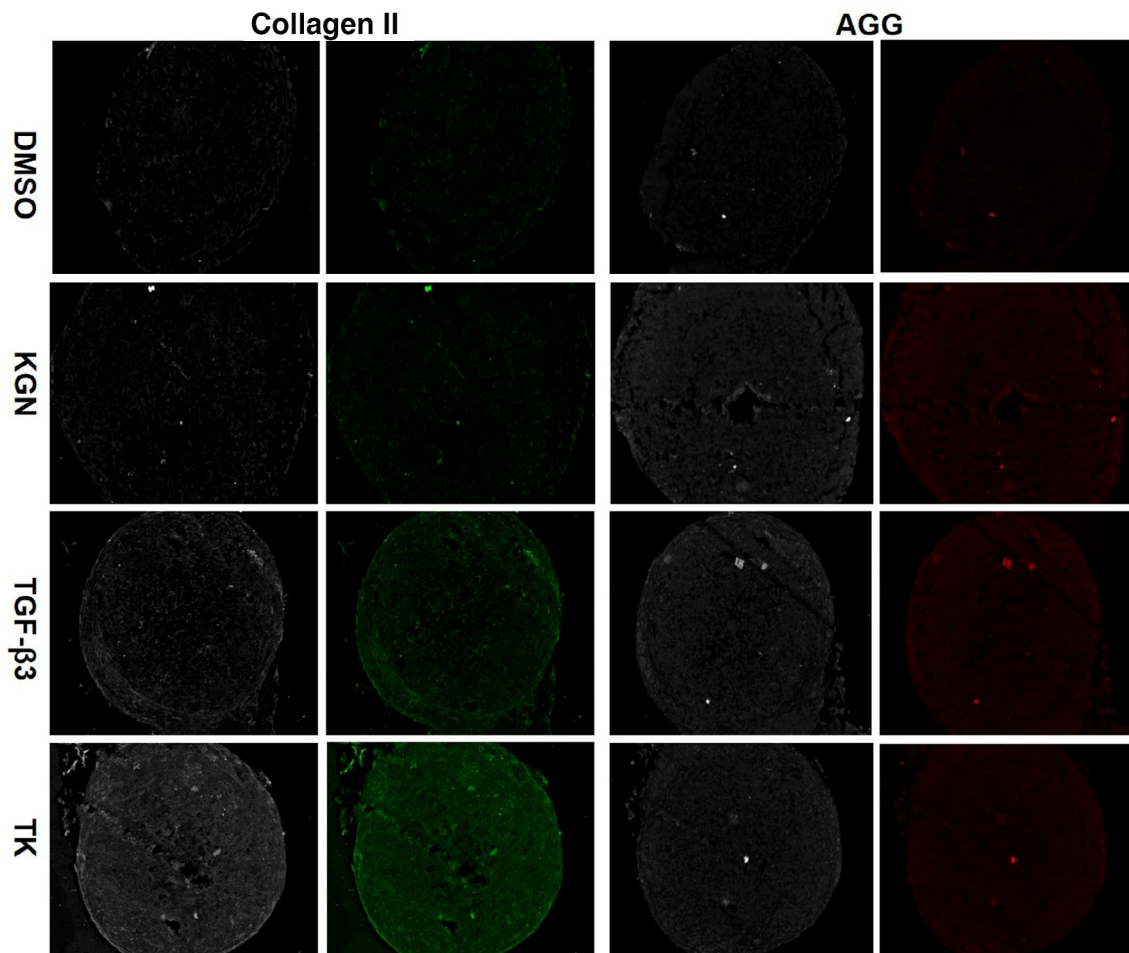


Fig. 3 Immunofluorescence staining for collagen type II and aggrecan on pellet sections after 3 weeks of cartilage differentiation.

Statistical Analysis

SPSS v19.0 (SPSS, Chicago, IL, USA) was used for data analysis. Data are presented as mean \pm SD and were compared by one-way ANOVA and Newman–Keuls comparison test. * $P < 0.05$ and ** $P < 0.01$ were considered statistically significant.

Results

Morphology and Identification of Human Umbilical Cord Mesenchymal Stem Cells

Before chondrogenic differentiation of hUCMSC, cells were observed under inverted light microscopy (Leica, Germany) and identified by flow cytometry. Figure 1A shows a uniformly spindle-shaped cellular morphology. The flow cytometric analysis of surface-marker expression on hUCMSC is shown on Fig. 1C. Flow cytometry revealed that hUCMSC showed negative expression of CD34 and CD45 (hematopoietic-related cell antigens). In contrast, high expression of CD13, CD44, CD73, and CD90 (mesenchymal

stem cell-related antigens) was observed. The results showed that hUCMSC derived from human mesenchymal stem cells. Figure 1B shows that TGF- β 3 has a promoting effect on the growth of hUCMSC and KGN dissolved in DMSO or DMSO alone did not affect the proliferation of cells. Although KGN enhanced the growth of cells after 1 day of culture in 96-well plates, there was no impact of proliferation by KGN after 5 days of culture.

Chondrogenic Differentiation and Human Umbilical Cord Mesenchymal Stem Cells

Compared to the other groups, the DMSO group did not form a compact smooth spherical matrix after 21 days of culture (Fig. 2). The KGN group secreted more extracellular matrix, and more lacuna-like structures existed in the DMSO group than in the KGN group. The TGF- β 3 and TK groups formed a smoother and more compact spherical structure than the KGN-alone group, and could form cartilage-like tissue. The pelleted cartilage-like tissue was of a larger quantity and a more uniform distribution in the TK and TGF- β 3

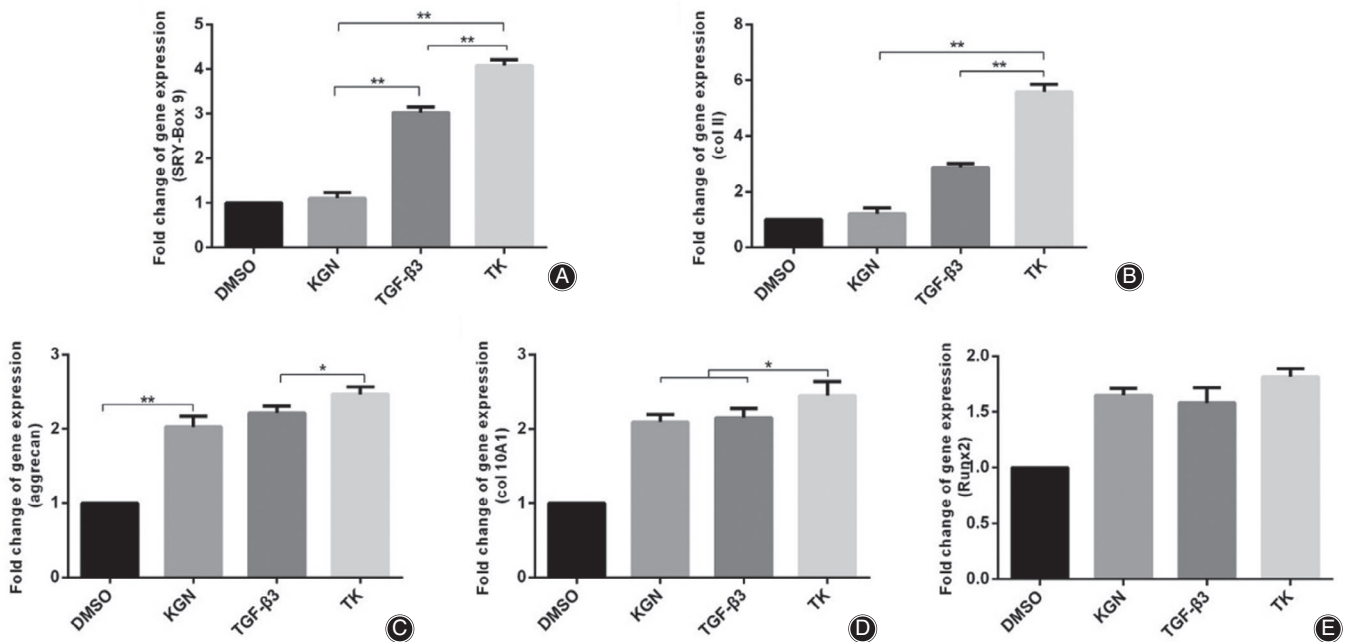


Fig. 4 Expression levels of chondrocyte marker genes and hypotrophy-related genes after 21 days of induction. Relative expression levels of (A) SRY-Box 9, (B) collagen type II, (C) aggrecan, (D) collagen type X, and (E) RUNX2. $n = 3$.

groups than the other groups. AB staining showed that from the DMSO group to the TK group, the secretion of GAG was accordingly increased; moreover, the secretions of GAG in TK and TGF- β 3 groups were most obvious, which concurs with immune-histochemical staining of AGG. Strong expression of collagen II in TK and TGF- β 3 based on immunofluorescence staining and more prominent collagen II secretion in the TK group than the TGF- β 3 group are observed in Fig. 3.

To further evaluate the induced chondrogenesis of KGN, we detected the expression of cartilage-related genes. Interestingly, adding KGN and TGF- β 3 into the chondrogenic medium distinctly upregulated the expression of collagen II, AGG, and SRY-Box 9 (Fig. 4A-C). As expected, the TK group possessed higher SRY-Box 9, AGG, and COL II expression than the other groups. Although the expressions of RUNX2 and collagen type X in KGN, TGF- β 3, and TK groups were higher than those of the DMSO group, there was no significant difference in expression among these groups (Fig. 4D and E). These results indicated that KGN alone can induce the secretion of cartilage-related GAG, but it cannot induce formation of cartilage-like tissue and expression of cartilage-related genes very well (see Fig. 3 and Fig. 4). Combined application of TGF- β 3 and KGN could enhance chondrogenesis of hUCMSC as determined through qualitative and quantitative detection (Figs 2–4).

Discussion

The application of chondrocytes or directed differentiation of stem cells in the treatment and repair of cartilage

defects and in cartilage tissue engineering has become a hot topic^{31,32}. A variety of bioactive factors are applied to induce stem cells to differentiate into cartilage, such as bone morphogenetic proteins³³, TGF- β ³⁴, and insulin-like growth factor³⁵. However, these bioactive protein-factors/substances easily degenerate when used indirectly or directly *in vivo* or *in vitro*. Safe and stable materials need to be developed to reduce the side effects of these factors and to promote chondrogenic differentiation^{2,31}. KGN is a stable and reliable chemical substance that provides a sustainable, safe state for inducing chondrogenesis of BMSC². In this study, KGN was added to the medium once by dissolving in DMSO. However, because of the instability and strict storage requirements of TGF- β 3, it was added to the medium each time when medium was replaced. Compared with the DMSO group, the addition of KGN did not have a negative effect on the proliferation of cells, which indicated that KGN could promote the proliferation of cells (Fig. 1). KGN enhancing growth and secretion of MSC concurs with previous studies.

Compared with previous studies, our research employed KGN to stimulate hUCMSC and explored their chondrogenesis. Johnson *et al.* discovered that KGN could promote the differentiation of human BMSC into cartilage and explored its mechanism². Shi *et al.* studied chondrogenesis of human synovial stem cells induced by KGN and found that KGN could enhance chondrogenic differentiation of cells *in vitro*²⁰. Other studies have focused on tendon stem/progenitor cells and have found that KGN could enhance the capacity to repair synovium and cartilage *in vivo*^{10,19}.

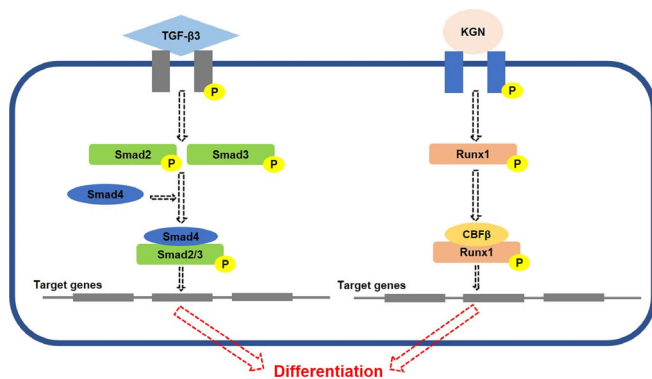


Fig. 5 Mechanism of transforming growth factor (TGF)- β 3 and kartinogenin (KGN) pathways that promote hUCMSC chondrogenic differentiation.

In this study, hUCMSC were first applied to study the effect of KGN on chondrogenesis of MSC. Although KGN could facilitate differentiation of hUCMSC and promote hUCMSC to secrete GAG, it failed to induce chondrogenesis of hUCMSC *in vitro*. This result was in accordance with former research by Shi *et al.*¹³, in which KGN alone failed to form cartilage-like pelleted structures after 21 days of culture. Ono *et al.*¹² also found a similar phenomenon of KGN alone failing to significantly enhance the formation of the extracellular matrix of cartilage cells. However, combining KGN with TGF- β 3 or BMP-2 could obviously induce chondrogenesis¹³. It is worth noting that Shi *et al.* (and other teams) did not regard TGF- β 3 alone as a positive group in their study. Therefore, to study the synergistic effects of KGN with other factors on chondrogenic differentiation, it was necessary to design a positive control group in our experiment. Our study demonstrated that TGF- β 3 alone could induce chondrogenesis of hUCMSC. Moreover, adding KGN into the medium of the TGF- β 3 group could strengthen the effect of chondrogenesis. This synergism

generated from different substances was responsible for their different mechanism of action on MSC. However, it remains unclear which signaling pathways or mechanisms plays a major role. It is possible that KGN activating the RUNX1 signaling pathway and TGF- β 3 activating the SMAD signaling pathway together contribute to chondrogenic differentiation (Fig. 5)^{2,36}. Further study of the inherent mechanism of the interaction effect produced by these two factors is necessary. Due to KGN having distinctly different physicochemical properties from TGF- β 3, there is potential to apply them together to induce chondrogenesis of MSC in a flexible way, or to combine them with other materials in different ways to induce chondrogenesis of MSC^{11,13}. The combination of KGN with TGF- β 3 will be an important means to regulate chondrogenesis of HCMSC or other MSC^{37,38}.

Conclusion

In this study, we investigated the effects of KGN and TGF on chondrogenic differentiation of mesenchymal stem cells. *In vitro* experiments demonstrated that KGN alone fails to obviously induce chondrogenic differentiation of hUCMSC. However, when we added KGN to TGF- β 3 medium, the effect of MSC on chondrogenic differentiation was enhanced. Based on these results, we conclude that KGN and TGF- β 3 can synergistically promote chondrocyte differentiation. The interaction of KGN and TGF- β 3 with chondrogenesis of hUCMSC provides a new method for regeneration and repair of cartilage.

Acknowledgments

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