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Modification of Insulin-Like Growth Factor-1 Gene Induces Differentiation of Adipose-derived Stem Cells into Chondrocytes

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KEYWORDS Adipose-derived Stem Cell. Chondrocyte. Differentiation. Insulin-like Growth Factor-1

ABSTRACT The researchers aimed to assess the effects of modification by insulin-like growth factor-1 (IGF-1) gene on the differentiation of adipose-derived stem cells (ADSCs) into chondrocytes. Rat ADSCs were divided into normal, control and experiment groups. Experiment and control groups were transfected with pcDNA3.1-IGF-1 and pcDNA3.1-IGF-1-NC, respectively. Their osteogenic, adipogenic and chondrogenic differentiation capacities were determined using alizarin red staining, oil red O staining and toluidine blue staining, respectively. The expressions of type I collagen (ColI) and ColII were detected by immunofluorescence assay, and the protein expressions of ColI, ColII and IGF-1 were measured by Western blotting. Compared with the normal group, the experiment group had significantly enhanced osteogenic, adipogenic and chondrogenic differentiation capacities and increased protein expressions of ColI, ColII and IGF-1 ($P<0.05$), whereas normal and control groups had similar results ($P>0.05$). Modification by IGF-1 gene obviously contributes to the differentiation of rats ADSCs into chondrocytes.

INTRODUCTION

Articular cartilage injury is a common orthopedic disease, and satisfactory outcomes cannot be achieved by traditional treatment approaches due to limited self-repair ability (Simon et al. 2018). Cartilage tissue engineering combining cell biology with material science has now become the most promising technique for repairing articular cartilage injury (Yang et al. 2017). The foundation of cartilage tissue engineering is to select appropriate seed cells (Armiento et al. 2018). Adipose-derived stem cells (ADSCs) have been highlighted in cartilage tissue engineering, since they have advantages such as wide sources, rapid proliferation, multi-directional differentiation potential and mild secondary trauma (Uz et al. 2019). ADSCs have good damage-repairing and self-renewal abilities and low immunogenicity as well as potential of differentiation into osteocytes, myocytes, hepatocytes and tendon cells, so they have been extensively applied in the regenerative medicine of bone and muscle, bringing hope for the reconstruction, repair and wound healing in patients with bone defects (Naderi et al. 2017). However, the differentiation of ADSCs into chondrocytes has seldom been referred. Numerous growth factors, such as basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β), can promote the proliferation of seed cells and provide a microenvironment for stabilizing cell phenotypes in the differentiation of seed cells (Grimm et al. 2018). The addition of exogenous insulin-like growth factor-1 (IGF-1) can induce the differentiation potential of human umbilical cord-derived mesenchymal stem cells (Cheng et al. 2021), but it is difficult to apply exogenous cytokines because they have low activity and are prone to loss. Growth factor genes are transfected into seed cells using genetic techniques (Wang et al. 2017) owing to self-expression, thereby avoiding the immunogenicity of exogenous proteins and promoting the induction of differentiation of ADSCs.

Objectives

In this study, the role of IGF-1 in the differentiation of ADSCs into chondrocytes by genetic

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transfection and modification of ADSCs in rats was investigated, aiming to provide novel insights into the development of cartilage tissue engineering.

METHODOLOGY

Experimental Animals, Reagents and Apparatus

Three SPF Sprague-Dawley rats aged 8-10 weeks old and weighing 180-200 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China, license No. SCXK (Beijing) 2012-0001). They were adaptively fed with standard diets and freely available water in an SPF environment for 1 week according to the management methods of the Laboratory Animal Center in the researchers' hospital prior to experiments.

PcDNA3.1-IGF-1 and its negative control (NC) pcDNA3.1-IGF-1-NC were designed by Shanghai GenePharma Co., Ltd. (China). The osteogenic, adipogenic and chondrogenic induction media of ADSCs were purchased from Cyagen (USA), and transfection reagent Lipofectamine 3000 and cell basal medium were provided by Gibco (USA). Rabbit anti-human type I collagen (ColI) and ColII and IGF-1 antibodies and horseradish peroxidase (HRP)-labeled secondary antibody were purchased from Abcam (USA). The kits for alizarin red staining, oil red O staining, immunofluorescence assay, Western blotting and toluidine blue staining were bought from Sigma (USA), and phosphate-buffered solution (PBS) was offered by Nanjing Jiancheng Bioengineering Institute (China).

CO_2 cell incubator was purchased from Beckman Coulter (USA), and UV-D6.0 gel imager was provided by Shimadzu Corporation (Japan). Tissue microtome was bought from Shel Lab (USA), and LIOO S600T fluorescence microscope was obtained from Nikon (Japan). High-speed refrigerated centrifuge was provided by Beijing Liuyi Biotechnology Co., Ltd. (China).

Isolation, Culture and Identification of ADSCs

SD rats were sacrificed by cervical dislocation, and the adipose tissues around the omentum, perirenal fat capsule and testis were collected (Luo et al. 2015). Then the connective tissues were resected, and red blood cells were washed off. The resulting tissues were digested by 0.1 percent type I collagenase for 1 h in warm water bath, added high-glucose medium containing 15 percent fetal bovine serum (FBS) and centrifuged at 1,500 rpm. After the supernatant was discarded, the pellets were diluted with high-glucose medium and centrifuged. The cells were then harvested, inoculated in a culture flask, and cultured with 5 percent CO_2 at 37°C. When the density reached 85 percent, the cells were digested by 0.25 percent trypsin and passaged.

After the concentration was adjusted, ADSCs were seeded in a 6-well plate at 2×10^4/well, fixed in paraformaldehyde, washed with PBS, added dropwise rabbit anti-rat cluster of differentiation 14 (CD14), CD29, CD34 or CD44 primary antibodies (1:100) and incubated overnight. On the next day, the cells were washed with PBS and incubated with secondary antibodies (1:100) at room temperature for 2 h. Finally, the expressions of marker molecules on the surface of cells were observed under the fluorescent microscope.

Cell Transfection and Experimental Grouping

The extracted cells were first seeded in a culture flask with RPIM 1640 and 20 percent FBS and cultured with 5 percent CO_2 at 37°C. When the density reached 90 percent, the cells were digested by 0.25 percent trypsin and those in the logarithmic growth phase were taken for transfection. One hour before transfection, the cells were kept attached and transfected with 100 nmol/L pcDNA3.1-IGF-1 or pcDNA3.1-IGF-1-NC by the cationic liposome-mediated method using Lipofectamine 3000 (Chen et al. 2015). Afterwards, the cells were cultured with 5 percent CO_2 at 37°C for 48 h for subsequent experiments. They were divided into normal group (ADSCs cultured with medium only containing Lipofectamine 3000 reagent), control group (ADSCs transfected with pcDNA3.1-IGF-1-NC) and experiment group (ADSCs transfected with pcDNA3.1-IGF-1).

Observation of ADSC Morphology

The concentration of ADSCs was first adjusted, and they were then plated in a 24-well plate at a concentration of 1×10^6/well and grouped in the same way as mentioned above. The cells were further cultured in a 5 percent CO_2 incubator at 37°C for 14 d, during which the medium was changed every two days. Each group of cells was harvested

after centrifugation at 800 rpm, and they were then prepared into 200 μL cell suspension using PBS. Finally, the morphological changes of cells were observed under the microscope.

Observation of Differentiation of ADSCs into Osteoblasts by Alizarin Red Staining

After the concentration was adjusted, ADSCs were seeded in a 6-well plate at 5×10^5/well, grouped as mentioned above, mixed gently with the osteogenic differentiation medium and conventionally cultivated in the 5 percent CO_2 incubator at 37°C for 14 d. The supernatant was then removed, and the cells were fixed with 4 percent formaldehyde for 30 min, washed by PBS twice (5 min/time) and stained with alizarin red. Finally, the formation of mineralized nodules in cells was observed under the microscope (Khademi et al. 2019).

Observation of Differentiation of ADSCs into Lipoblasts by Oil Red O Staining

Following cell concentration adjustment, ADSCs were inoculated in a 6-well plate at 3×10^4/well and grouped as mentioned above. After being gently mixed with the adipogenic induction medium, the cells were cultured in the 5 percent CO_2 incubator at 37°C for 14 d. Subsequently, they were fixed in 4 percent formaldehyde for 30 min and rinsed using PBS twice (5 min/time), followed by oil red O staining. After sterile water washing, the formation of lipid droplets in cells was observed under the microscope (Zhou et al. 2020).

Observation of Differentiation of ADSCs into Chondroblasts by Toluidine Blue Staining

After the concentration was adjusted, ADSCs were seeded in a 6-well plate at 3×10^4/well, grouped as mentioned above, mixed gently with the chondrogenic induction medium and cultured in the 5 percent CO_2 incubator at 37°C for 14 d. The resulting cells were fixed in 4 percent formaldehyde for 30 min, embedded into paraffin and sectioned. Afterwards, the sections were washed by PBS twice (5 min/time) and stained with toluidine blue dye. After the sections were washed by distilled water, the formation of proteoglycan in the cartilage matrix of cells was observed under the microscope (Fujii et al. 2020).

Detection of ColI and ColII Expressions by Immunofluorescence Assay

After the concentration was adjusted, ADSCs were plated in a 24-well plate at 5×10^5/well and grouped as mentioned above. They were then mixed gently with the osteogenic differentiation medium and cultured in the 5 percent CO_2 incubator at 37°C for 14 d, followed by washing with PBS. The resulting cells were fixed in paraformaldehyde, mounted, and incubated with primary antibodies and with secondary antibodies on the second day. Subsequently, the cells were prepared into ultra-thin frozen sections and observed under a laser scanning confocal microscope.

Detection of ColI, ColII and IGF-1 Protein Expressions by Western Blot

The concentration of ADSCs was adjusted. They were then inoculated in a 24-well plate at 1×10^4 /well and grouped as mentioned above. Afterwards, the cells were cultured with the osteogenic differentiation medium in the 5 percent CO2 biochemical incubator at 37°C for 14 d and harvested. Following protein concentration assay, 50 μg of proteins were subjected to SDS-PAGE and transferred onto a PVDF membrane that was then blocked with 10 percent skimmed milk for 3 h. Afterwards, the membrane was incubated with primary antibodies (diluted at 1:1,500) at 4°C overnight, washed and incubated with HRP-labeled secondary antibody for 3 h, followed by ECL color development for 30 min, exposure, image development and fixation. Finally, the expression levels of proteins were determined with GAPDH as an internal reference.

Statistical Analysis

All data were statistically analyzed by SPSS16.0 software and plotted with Graphpad5.01 software. Multigroup comparisons were conducted by one-way analysis of variance, and pairwise comparisons were performed with the t test. $P<0.05$ was considered statistically significant.

RESULTS

Identification of ADSCs

According to the immunofluorescence assay results (Fig. 1), the cells were positive for CD29

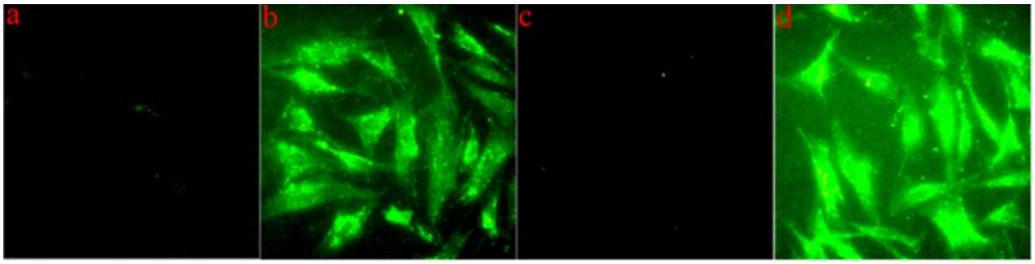

Fig. 1. Identification of ADSCs by immunofluorescence assay. A; CD14; b: CD29; c: CD34; d: CD44

and CD44 epitopes, but negative for CD14 and CD34 epitopes, suggesting that the extracted cells were ADSCs.

Morphological Changes of ADSCs

The adherent cells in normal and control groups grew well and had clear morphology and larger bodies, and they were mostly long-fusiform or angular and tightly attached to each other through processes (Fig. 2). In the experiment group, the adherent cells had clear morphology and grew in a swirl shape, most of which were long-fusiform. Compared with the normal group, the cells proliferated faster, with more central body processes and a tendency to form nodules.

Differentiation Capacities of ADSCs into Osteoblasts

The alizarin red staining results showed that the observation group had significantly more mineralized nodules than those of the normal group ($P<0.05$), without statistically significant difference between normal and control groups ($P>0.05$) (Fig. 3).

Adipogenic Differentiation Capacities of ADSCs

Based on the oil red O staining results (Fig. 4), the experiment group had significantly more lipid droplets and oil red O-positive cells than those of the normal group ($P<0.05$), while no statistically significant differences were observed between normal and control groups ($P>0.05$).

Chondrogenic Differentiation Capacities of ADSCs

As shown in toluidine blue staining, the observation group had significantly more toluidine blue-positive cells than those of the normal group ($P<0.05$), but there was no statistically significant difference between normal and control groups ($P>0.05$) (Fig. 5).

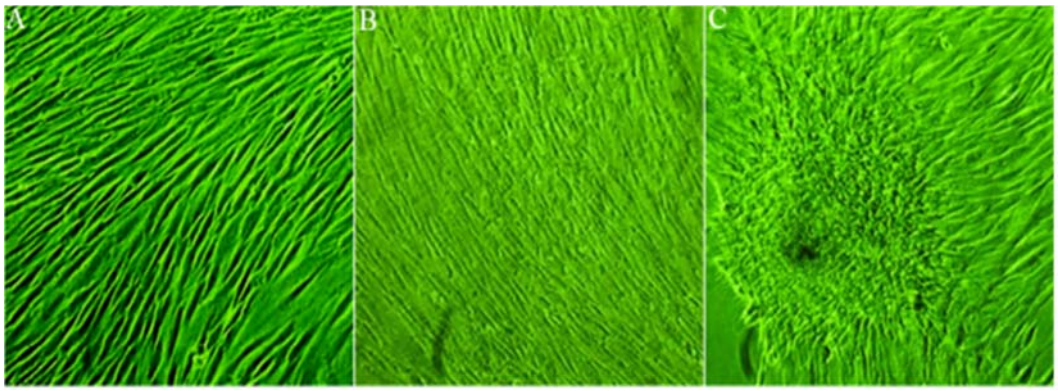

Fig. 2. Morphological changes of ADSCs observed by microscopy (SP ×200). A: Normal group; B: control group; C: experimental group

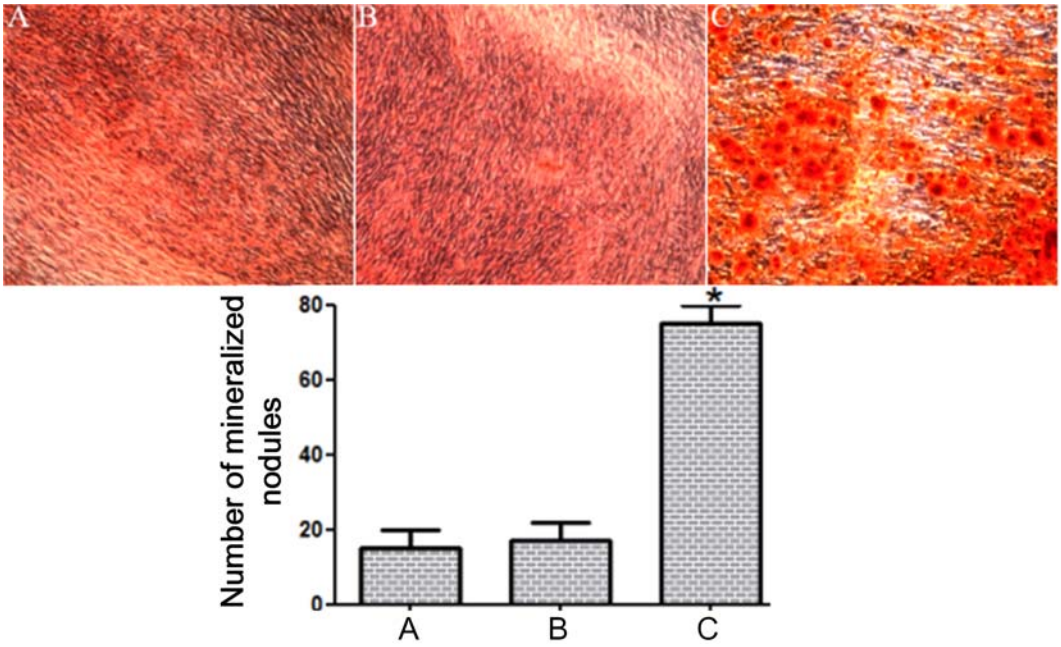

Fig. 3. Differentiation capacities of ADSCs into osteoblasts observed by alizarin red staining (SP ×200). A: Normal group; B: control group; C: experimental group. Compared with normal group, *P<0.05

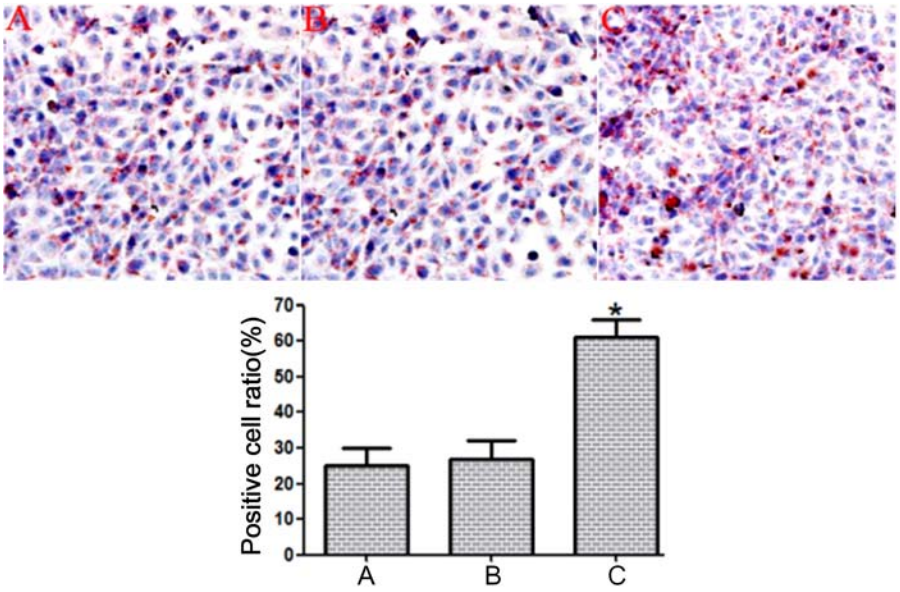

Fig. 4. Adipogenic differentiation capacities of ADSCs observed by oil red O staining (SP ×200). A: Normal group; B: control group; C: experimental group. Compared with normal group, *P<0.05

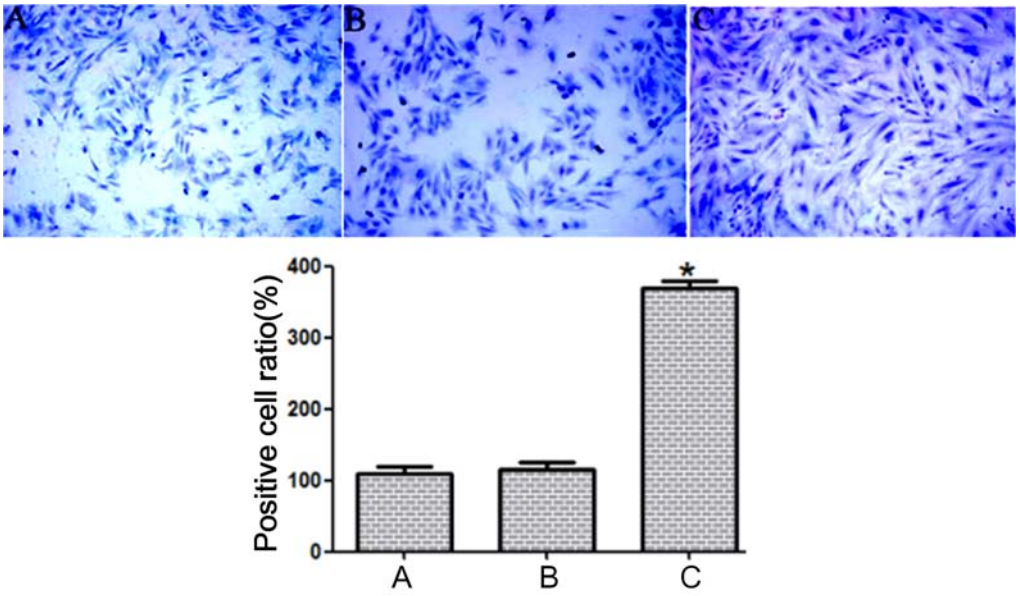

Fig. 5. Chondrogenic differentiation capacities of ADSCs observed by toluidine blue staining (SP ×200). A: Normal group; B: control group; C: experimental group. Compared with normal group, *P<0.05

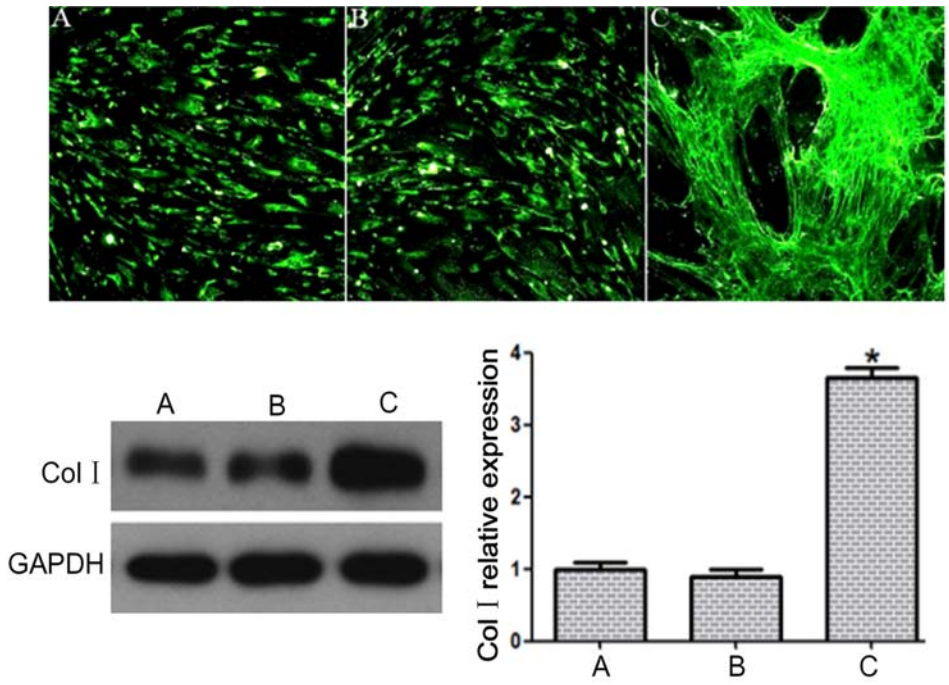

Fig. 6. ColI expressions in ADSCs. A: Normal group; B: control group; C: experimental group. Compared with normal group, *P<0.05

ColI and ColII Expressions in ADSCs

In comparison with the normal group, the expressions of ColI and ColII were raised in the experiment group ($P<0.05$), but they showed no statistically significant differences between normal and control groups ($P>0.05$) (Figs. 6 and 7).

IGF-1 Protein Expressions in ADSCs

According to the results of Western blotting, the expression level of IGF-1 in the experiment group was significantly higher than that of the normal group ($P<0.05$), but there was no statistically significant difference between control and normal groups ($P>0.05$) (Fig. 8).

DISCUSSION

Post-injury repair is among the challenging issues in the field of regenerative medicine, because articular cartilage has a limited regenerative capacity. Traditional therapies, such as massage and acupuncture, cannot completely regenerate or re-

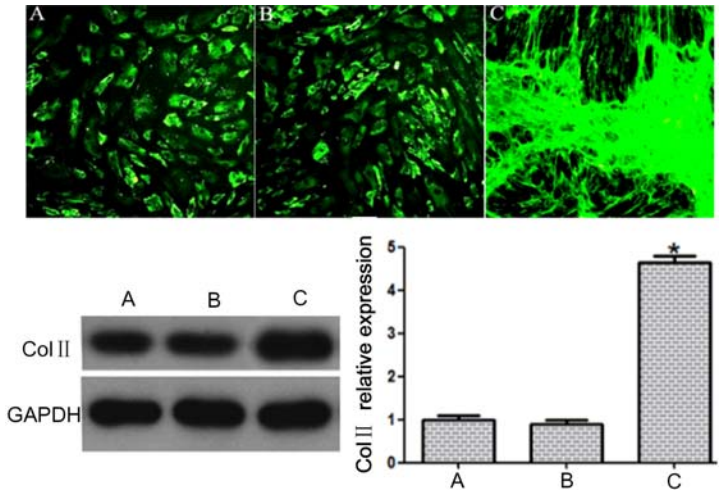

Fig. 7. ColII expressions in ADSCs. A: Normal group; B: control group; C: experimental group. Compared with normal group, *P<0.05

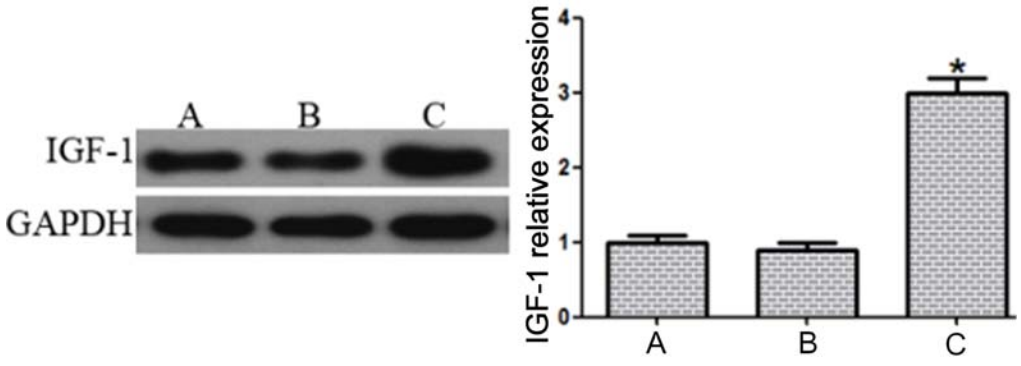

Fig. 8. IGF-1 protein expressions in ADSCs detected by Western blot. A: Normal group; B: control group; C: experimental group. Compared with normal group, *P<0.05

pair injured joints. Therefore, cartilage tissue engineering has become a research hotspot for effectively treating joint injuries.

Selecting appropriate seed cells is most important for cartilage tissue engineering (Ma et al. 2018). ADSCs, a class of adult stem cells, have been excellent stem cell donors for bone injury and repair tissue engineering. They have the advantages of minimally invasive acquisition and rapid proliferation in bone injury tissue engineering (Xie et al. 2017). With strong differentiation ability after induction, ADSCs promote wound healing in the burn wound healing model of rats (Li et al. 2018). Perikamana et al. reported that low immunogenicity facilitates the induced differentiation of ADSCs into tendon cells, thereby providing reliable seed cells for tendon-bone surface tissue engineering (Perikamana et al. 2018). However, the differentiation of ADSCs into chondrocytes remains largely unknown. In this study, the surface antigen identification results of ADSCs extracted from rats revealed that they were positive for CD29 and CD44, but negative for CD14 and CD34, suggesting that the cultured cells were ADSCs. They had stable and uniform morphology and proliferated rapidly, and were still fibroblast-like after long-term culture (14 d), with a stronger ability to differentiate into chondrocytes after addition of specific inducers.

The induced differentiation of ADSCs cultured *in vitro* is affected by multiple factors, such as cell density, spatial organization, basal medium composition, mechanical actions, cytokines and autoimmunity (Ikemoto et al. 2019). For example, cell induction factors play a pivotal role in the directed differentiation of ADSCs into chondrocytes (Song et al. 2017). Nevertheless, the promoting effect of exogenous addition on induced differentiation is far from potent. The transfection of cells with inducer genes can promote the differentiation of ADSCs more effectively. The genetic modification by TGF-β1 is able to promote the differentiation of mesenchymal stem cells into articular chondrocytes (Velot et al. 2022). Currently, there are more studies on the genetic modification with TGF-β1 in the differentiation of ADSCs than those using IGF-1. IGF-1, a mitogen-like small molecule protein, can promote mitosis and enhance induced differentiation in the growth and development of cells. The eukaryotic expression plasmid of IGF-1 is stably expressed in the umbilical cord mesenchymal stem cells of Tibetan miniature pigs and contributes to their differentiation (Tian et al. 2018). The genetic modification and transfection with IGF-1 can markedly promote the directed differentiation of rabbit bone marrow mesenchymal stem cells into chondrocytes (Gugjoo et al. 2017). In this study, ADSCs were genetically modified by IGF-1, and their growth and osteogenic, adipogenic and chondrogenic differentiation as well as the expressions of ColI and ColII were facilitated, suggesting that the modification by IGF-1 gene can obviously enhance the chondrogenic differentiation capacity of ADSCs.

CONCLUSION

In conclusion, the modification by IGF-1 gene can obviously contribute to the differentiation of ADSCs into chondrocytes.

RECOMMENDATIONS

It is recommended to further quantify the IGF-1 gene modification and to apply it into the clinical treatment of articular cartilage injuries.

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