

# Mechanism of Wnt Pathway on Cartilage Differentiation of Adipose-Derived Stem cells

**Wanwu Dai**

Nanning Second Peoples Hospital

**Bo Zhang**

Nanning Second Peoples Hospital

**Zuquan Huang**

Nanning Second Peoples Hospital

**Guili Sun**

Nanning Second Peoples Hospital

**Yejing Yang**

Nanning Second Peoples Hospital

**Min Cai**

Nanning Second Peoples Hospital

**Zhe Feng**

Nanning Second Peoples Hospital

**Xingyan Li** (✉ [448445226@qq.com](mailto:448445226@qq.com))

<https://orcid.org/0000-0002-7831-7169>

---

## Research article

**Keywords:** Adipose-derived Stem Cells, Chondrogenic Differentiation, Wnt/ $\beta$ -catenin signaling pathway, Sox9, Collagen 2a.

**Posted Date:** April 10th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-18465/v2>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

**Background:** Adipose-derived stem cells (ADSCs) therapy is considered as a promising alternative to treat osteoarthritis (OA). Considerable evidence has shown that the Wnt signaling pathway is involved in chondrogenic differentiation in various stem cells. In the present study, we explored in detail the mechanism of Wnt signaling pathway in the regulation of adipose-derived stem cell chondrogenic differentiation.

**Methods:** Adipose-derived stem cells were extracted from Sprague-Dawley groin area, isolated, cultured and verified. Then LiCl and DKK-1 used to induce ADSCs cartilage differentiation to detect indicators of cartilage at different stages, such as collagen type II (Collagen2a), aggrecan (Aggrecan) and Wnt pathway key proteins Sox9,  $\beta$ -catenin, Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) expression. Subsequently, lentivirus was used as the vector, the full-length Sox9 gene was transfected into ADSCs and the expression levels of relevant indicators were detected again after stable expression.

**Results:** In the process of inducing differentiation, the Wnt pathway promoted the rapid proliferation of ADSCs in the early, middle and late stages, and up-regulates Sox9 expression. Cartilage indicators Collagen 2a and Aggrecan expression levels were not significantly increased in the early stage, but significantly increased in the middle and late stages ( $P < 0.05$ ). At the later stage, however, the Wnt pathway down-regulated Sox9 expression, weakened the mature cartilage phenotype, promoted cartilage hypertrophy and early cartilage ossification. Finally, the overexpression of Sox9 feedback inhibits the Wnt pathway activity, down-regulates  $\beta$ -catenin expression, maintains cartilage phenotype, delays cartilage hypertrophy and early osteogenesis.

**Conclusion:** The Wnt pathway regulates chondrogenic differentiation of ADSCs by regulating Sox9, but its regulatory mechanism is significantly different at different stages of induced differentiation.

## Introduction

Osteoarthritis (osteoarthritis, OA) is the most common chronic bone and joint disease in the elderly[1]. It has seriously threatened the health and quality of life of the elderly[2]. Now it has become an important public health issue facing the whole society, and one of the serious problems facing medical systems around the world[3].

Studies have shown that in the course of osteoarthritis, articular cartilage degeneration, subchondral bone plate and synovial tissue hyperplasia are the main pathological changes. It first occurred in articular cartilage, gradually invaded the subchondral bone surface, joint capsule, and surrounding tissues, causing bone surface and synovial hyperplasia, deformity, osteophyte, resulting in narrowing of the joint space and biological stress balance[4, 5]. The main clinical treatment goals of osteoarthritis are: reduce pain symptoms, correct joint deformities, and improve joint mobility[6]. The treatment methods are mainly divided into: drug treatment, traditional Chinese medicine treatment, physical therapy, and surgical treatment. However, there is no clinically effective method to completely cure osteoarthritis[7]. With the

rapid development of medicine and related fields in recent years, regenerative medicine and biological tissue engineering have been born. It uses stem cells and cytokines to exert therapeutic effects and provides new possibilities for clinical treatment of osteoarthritis.

Adipose-derived stem cells (ADSCs) are pluripotent stem cells developed from the mesoderm and possess multipotential differentiation potential[8]. It can differentiate into chondrocytes[9], adipocytes, cardiomyocytes[10], odontoblasts[11, 12], and nerve cells[13] under different induction culture conditions. It has many advantages such as easy access, fast proliferation, low immunogenicity, and stable biological performance[14]. The use of ADSCs to treat osteoarthritis has become a new hotspot in clinical research in recent years, and also provides new possibilities for clinical cure of OA. Recent studies have shown that ADSCs can be successfully induced into chondrocytes, and have shown initial efficacy in autologous fat stem cell transplantation for traumatic cartilage defects[15, 16]. However, the process of chondrogenic differentiation of ADSCs is affected by a variety of signaling pathways and cytokines. Current studies have shown that the Wnt signaling pathway plays a significant role in the formation and repair of chondrogenesis. However, the specific regulatory mechanism of Wnt pathway in chondrogenic differentiation of ADSCs remains unclear[17].

In this study, we extracted adipose-derived stem cells from the groin of adult healthy Sprague Dawley rats, and isolated, cultured, identified, induced[18]. We eventually found that during the process of chondrogenic induction, the Wnt pathway promotes the rapid appreciation of ADSCs in the early, middle and late stages and induces chondrogenic differentiation by up-regulating Sox9. The expression of cartilage indicators Collagen 2a and Aggrecan did not increase significantly in the early stage, but increased significantly in the middle and late stages. In the later period, the Wnt pathway down-regulates the expression of Sox9, weakens the mature cartilage phenotype, promotes cartilage hypertrophy and early cartilage ossification. Finally, to clarify the effect of Sox9 overexpression in turn on the Wnt pathway, we transferred lentiviral vectors containing the full-length Sox9 gene into ADSCs. Overexpression of Sox9 will feedback inhibit Wnt pathway activity, down-regulate  $\beta$ -catenin, maintain cartilage phenotype, delay cartilage hypertrophy and early osteogenesis. In general, the Wnt pathway regulates ADSCs into chondrogenic differentiation by regulating Sox9, and its regulatory mechanism is significantly different at different stages of inducing differentiation.

## Materials And Methods

### Materials

Healthy adult Spargue-Dawley male rats, weighing  $175\pm 25$ g, purchased from Animal Experiment Center of Guangxi Medical University [Certificate No. SCXK (Gui) 2014-0002]; DMEM / F12 was purchased from Hyclone Corporation in the United States; Gibco fetuses Bovine serum was purchased from Hangzhou Sijiqing Company; induction differentiation medium was purchased from American Cyagen Biosciences; LiCl, DKK-1 cell counting kit was purchased from American SIGMA company; Sox9, BMP-2 full-length

gene sequence lentiviral vector and empty vector virus were purchased From Wuhan Google Biological Co., Ltd.

### **Extraction, cultivation and grouping of ADSC**

According to Van Harmelen et al.[19], collagenase digestion method extracts ADSCs from the superficial fat of abdominal skin. DMEM / F12 medium containing 10% FBS was cultured adherently in an incubator at 37 °C and 5% CO<sub>2</sub>. The cells were divided into LiCl stimulation group, DKK-1 stimulation group, Sox9 lentivirus transfection group, BMP-2 lentivirus transfection group, empty lentivirus transfection group, negative control group (normal chondrogenic differentiation medium), blank Control group (10% fetal bovine serum DMEM / F12 medium).

### **Lentiviral transfection**

Select well-grown ADSCs for digestion and inoculate  $5 \times 10^4$  cells per well plate. When the cells are attached, replace the medium with Sox9 lentiviral vector and empty lentiviral vector. After incubating in an incubator at 37 °C and 5% CO<sub>2</sub> for 8 hours, the medium was aspirated and replaced with a new medium. Then 48 hours of incubation again, the expression of EGFP (enhanced green fluorescent protein) was observed using a fluorescence microscope[8, 20].

### **ADSCs detection**

According to the announcement by the International Association for Fat Application Technology in 2013, the phenotypes of ASCs cultured in vitro are CD31-, CD44 +, CD45-, etc[8]. Because CD44 is specific in stem cells, it was selected as a surface marker for detecting ADSCs[21]. DAPI fluorescence staining confirmed that the cells obtained from the culture were ADSCs.

### **Identification of ADSCs differentiation potential**

Digest and centrifuge cells with stable shape and good growth, and inoculate at  $1.6 \times 10^5$  cells per well. Add chondrogenic induction medium, and then culture in an incubator at 37 °C and 5% CO<sub>2</sub>. The chondrogenic induction medium was replaced every 48 hours. When the cell mass was about 2 mm in diameter, it was fixed, paraffin-embedded, and stained with alixin blue after sectioning. The staining was observed under a microscope.

### **Cell proliferation analysis**

The cells were digested, counted, and seeded into a 96-well plate according to the concentration gradient. Then place it in an incubator at 37 °C and 5% CO<sub>2</sub> for a period of time and add 10 µl of CCK-8 reagent. After 4 hours of incubation, the OD was measured, a standard curve was drawn, and the number of cells was calculated based on the standard curve.

### **Immunofluorescence detection**

Prepare cell slides, sterilize and place the cells in a well plate for cell culture, fix the cells with pre-cold fixative for 20 minutes, rinse 3 times with PBS, incubate for 30 minutes at 0.05% Triton-100 at room temperature, and rinse 3 times with PBS. Anti-incubation, fluorescent labeling on glass slides, observation under a microscope.

### **RT-qPCR detection**

RNA was extracted by TRIzol method, and reverse-transcribed into cDNA for RT-qPCR detection. The primer sequence (5'→3') was as follows: GAPDH: upstream primer: ACGACCCCTTCATTGACCTC, downstream primer: CCTTGACTGTGCCGTTGAACT; Sox9 upstream primer: GTGGGAGCGACA ACTACTACC, downstream primer: GCGAGCACTTAGCAGAGGC; BMP-2 upstream primers: AGACCTTCTTGGTATCCC, downstream primers: CTCCAGCTTGTTTTTGT; Collagen 2a upstream primers: CACCCAGAGTGGAAGAGCG, downstream primers: TCAGTGGAGTAGACGGAGGA; Aggrecan upstream primers; CAAACAGCAGAGCACACATCA, downstream primers: GAAGGCTGAGAGCGACCGAGTGAGGTACGAG The relative expression was calculated using the  $2^{-\Delta\Delta Ct}$  method and repeated 3 times.

### **Western blot detection**

After the protein lysate is extracted, the sample is prepared for gelation, electrophoresis, transfer, blocking, incubation of primary antibodies, membrane washing, incubation of secondary antibodies, membrane washing, ECL color development, acquisition of bands and image analysis: gel imaging system band scanning It comes with software for analysis and comparison.

### **Statistical methods**

SPSS 20.0 statistical software was used for analysis. The measurement data were expressed as mean  $\pm$  standard deviation ( $\pm s$ ). Comparison between groups was analyzed by t test or one-way analysis of variance.

## **Results**

### **Detection of ADSCs and identification of differentiation potential**

Extracted adipocytes were isolated and cultured in accordance with improved methods by Zuk et al. At different time points 4h, 48h, and 2d (**Fig. 1 A**), the growth condition was observed under a microscope, and it can be seen that the morphology changed into a spindle-shaped distribution with time. In order to identify the cell type, CD44-FITC antibody was used for specific labeling and fluorescence staining. Red fluorescence was visible under the microscope, and the nuclei of DAPI counterstained were blue (**Fig. 1 B**), suggesting that the surface antigen CD44 of ADSCs was positively expressed. It was confirmed that the cells obtained from the culture were ADSCs. To further verify the differentiation potential, Alisin blue was

specifically stained after induced differentiation, showing a large number of blue matrix particles (**Fig. 1 E**), indicating that ADSCs have good chondrogenic differentiation potential.

### **Effect of Wnt pathway activation on adipose stem cell proliferation**

CCK-8 was used to detect the increase of fat stem cells at different times. The results showed that the agonist LiCl stimulated the Wnt pathway in the experimental group, and adipose-derived stem cells entered exponential growth on the second day, but the adipose-derived stem cells of the blank control group began to enter the exponential growth state on the third day (**Fig. 1 C**). During the observation period, the proliferation rate of the agonist LiCl stimulation group was significantly higher than that of the blank control group. On the seventh day of the experiment, the experimental group and the blank control group stimulated by LiCl were detected and recorded. The expression of proliferating cell nuclear antigen (PCNA) protein was significantly higher in the experimental group than in the control group (**Fig. 1 D and Additional file 1**).

### **Cartilage Indexes and $\beta$ -catenin Expression in Different Stages of ADSCs Chondrogenic Induction**

During the cartilage-induced differentiation of ADSCs, the mRNA and protein expression levels of Sox9, Collagen 2a, and Aggrecan chondrogenic differentiation indicators were measured on days 0, 7, 14, and 21. The results showed that Sox9 showed a higher expression on day 7, and slowly increased on day 14 and 21. At the same time, Collagen 2a and Aggrecan also showed high expression on the 7th day, and were significantly expressed on day 14 and 21 (**Fig. 2 A**). Western blot showed the same results (**Fig. 2 B and Additional file 2**). Quantitative detection of the content of GAG in the culture medium showed that GAG began to express a small amount on day 7, gradually increased on day 14, and was obviously expressed on day 21 (**Fig. 2 C**).

Expression of  $\beta$ -catenin protein, an important factor in the Wnt pathway, at the beginning, the expression level was large, but with the progress of chondrogenic induction differentiation, the expression level gradually decreased, and the protein was low on day 7 and 14. However, by day 21,  $\beta$ -catenin expression rebounded and its content was higher than the initial level (**Additional file 3**).

In the late stage of chondrogenic induction of ADSCs, The mRNA expression levels of the cartilage differentiation markers Collagen 2a, Collagen 10 and RUNX2 were measured on day 21, 28, and 35, The results show: the mRNA expression of Collagen 10 and RUNX 2 increased with the time of cartilage-induced differentiation, and the expression of Collagen 2a gradually decreased (**Fig. 2 D**). Western blot results showed: Sox9 protein expression gradually decreased with cartilage-induced differentiation time, but  $\beta$ -catenin protein expression gradually increased (**Fig. 2 E & F**).

### **Role of Wnt pathway in ADSCs during chondrogenic differentiation**

The Wnt pathway is regulated by the agonist LiCl and the inhibitor DKK-1. In the early stage of induced differentiation, on day 7, the expression of Sox9 in the LiCl-excited group was higher than that in the cartilage-induced differentiated group, whereas the DKK-1 inhibited group was the opposite. The changes

of the corresponding cartilage indicators Aggrecan and Collagen 2a protein expression were not significant compared with the cartilage-induced differentiation group (**Fig. 3 A and Additional file**). Compared with the blank control group,  $\beta$ -catenin protein was weakened in the cartilage-induced differentiation group, and various indicators of cell proliferation such as CyclinD protein and PCNA protein were weakened. Compared with the cartilage-induced differentiation group, the ratio of  $\beta$ -catenin protein in LiCl agonized group was significantly higher than that in the latter group, and CyclinD protein and PCNA protein were also significantly increased. However, the DKK-1 inhibition group had the opposite result (**Fig. 3 B and Additional file**).

In the late stage of induced differentiation, LiCl group activated  $\beta$ -catenin protein expression, while DKK-1 inhibited group suppressed expression. Western blot analysis showed that in the LiCl agonist group, both Collagen 2a and Sox9 increased, especially, Collagen 2a increasing significantly, and the DKK-1 inhibitory group significantly inhibiting expression (**Fig. 3 C Additional file**). The cartilage indicators Aggrecan, Collagen 2a, and Sox9 mRNA analysis showed that the cartilage indicators Aggrecan and Collagen 2a in the LiCl-excited group were significantly increased, but the DKK-1-inhibited group was significantly inhibited (**Fig. 3 D**). Alixin blue staining (**Fig. 3 E**) and quantitative analysis (**Fig. 3 F**) show that chondroitin sulfate in the LiCl agonizing group was significantly higher than that in the chondrogenic differentiation group, while chondroitin sulfate in the DKK-1 inhibitory group was lower than that in the chondrogenic differentiation group. GAG quantitative analysis results also show the same trend (**Fig. 3 G**).

In the late stage of induced differentiation, quantitative and qualitative analysis by Western blot showed that LiCl group obviously continued to activate  $\beta$ -catenin protein expression, and DKK-1 inhibited expression. Compared with the cartilage-induced differentiation group, the expression of Sox9 protein in the LiCl agonist group was significantly reduced, while the DKK-1 inhibitory group was slightly decreased (**Fig. 3 H & I**). Cartilage indicators show that compared with the cartilage-induced differentiation group, the expression of Collagen 2a is reduced, and the expression of Collagen 10 and RUNX 2 is increased in the LiCl-excited group. In the DKK-1 inhibitory group, the expression of Collagen 2a increased, and the expression of Collagen 10 and RUNX 2 decreased (**Fig. 3 J & K**).

### **Detection of Sox9 protein expression after ADSCs lentivirus transfection**

ADSCs were transfected with a lentivirus containing the full sequence Sox9 gene. Quantitative and qualitative analysis of Western blot revealed that Sox9 was abundantly expressed (**Fig. 4 A & B**), indicating that genes were successfully integrated into ADSCs and expressed.

### **Cartilage Indexes and $\beta$ -catenin Expression at Different Stages of Chondrogenic Induction and Differentiation of ADSCs After Lentivirus Transfection**

During transfection-induced differentiation, Sox9 protein expression was significant in the lentiviral transfection group. Compared with the blank control group, the difference between the day 7 and 14 was particularly obvious, and  $\beta$ -catenin protein was lower than that of the blank control group in each period

(Fig. 4 C Additional file 5). On days 0, 7, 14, and 21, the expression of the chondrogenic differentiation markers Collagen 2a and Aggrecan mRNA were detected, respectively. The PCR results showed that compared with the blank control group, the mRNA expression of Collagen 2a and Aggrecan in the Sox9 lentivirus transfection group appeared to be higher (Fig. 4 D & E). On the day 7 and 14, the quantitative results of GAG content showed that the Sox9 lentivirus transfection group was significantly higher, but the results of the two groups were not significantly different when monitoring on day 21 (Fig. 4 F).

### Effects of Sox9 overexpression on Wnt pathway in cartilage-induced differentiation

On day 21 of transfection-induced differentiation, immunofluorescence tests show that compared with the blank control group, the expression of Collagen 2a protein was significantly higher in the Sox9 lentivirus transfection group (Fig. 4 G). Western blot was used to detect the expression of Wnt signaling pathway-related proteins. The expression of  $\beta$ -catenin and GSK-3 $\beta$  protein increased in the Sox9 lentivirus transfection group, but the total  $\beta$ -catenin protein was significantly reduced (Fig. 4 H & I).

## Discussion

In recent years, mesenchymal stem cells (MSCs) have been used to repair full-thickness articular cartilage because of their regenerative potential, anti-inflammatory effects, and low invasiveness. In-depth clinical trials have found that BMSCs are effective and safe for treating cartilage defects method[22, 23]. However, mesenchymal stem cell have the disadvantages of low yield and long in vitro expansion time[24]. Adipose stem cells have become a better choice because of their high yield, rapid proliferation, and low immunity[25]. At present, ADSCs have been widely used to promote the recovery of early osteoarthritis. Studies have shown that injecting ADSCs into the knee joint of a mouse model of early osteoarthritis can significantly inhibit thickening of synovial joints, bone hyperplasia at ligament attachment points, and articular cartilage destruction[26]. Clinical studies have shown that ADSCs are injected into knee osteoarthritis patients after joint cavity lavage. The two-year follow-up results show that most patients share functions (87.5%), cartilage status has been maintained or improved, and pain has been significantly reduced[26]. However, there are still questions about the clinical application of ADSCs, because the molecular mechanisms of their biological behavior lack in-depth research, such as ADSCs proliferation and fate selection. Therefore, it is critical to clarify the molecular mechanism of ADSCs in the process of chondrogenic differentiation. To clarify the mechanism of ADSCs between Wnt /  $\beta$ -catenin signaling pathway and Sox9 in different stages of chondrogenic differentiation. We recorded the expression of cartilage indicators Aggrecan, Collagen 2 $\alpha$ , and key proteins of Wnt signaling pathway  $\beta$ -catenin, Sox9, etc. in detail, and further discussed the effect of Sox9 overexpression on Wnt signaling pathway. Exploring the most suitable external induction and culture conditions in the differentiation process. Therefore, it can more accurately control the directional differentiation of ADSCs into cartilage, promote cartilage repair, and provide ideas and possibilities for cartilage repair This experimental study found that the PCNA expression level in the LiCl group was significantly enhanced, and further stimulated the proliferation ability of ADSCs, which is consistent with the results of the study by Miki TD et al[27]. In the early stage of chondrogenic differentiation, LiCL not only promoted the expression of PCNA and

Cyclin D, but also enhanced the expression of Sox9 in ADSCs. However, even though Sox9 began to show high expression, Aggrecan and Collagen 2a did not change significantly. This demonstrates the key role of the Sox9 gene in the early stages of chondrogenic differentiation. In the middle stage of induced differentiation,  $\beta$ -catenin expression was at a low level, but the cartilage indicators Aggrecan and Collagen 2a maintained a steady increase, indicating that the Wnt signaling pathway is not unique during the process of chondrogenic differentiation. In the late stage of induced differentiation, the Wnt pathway continues to activate,  $\beta$ -catenin expression levels continue to increase, Sox9, Aggrecan, and Collagen 2a expressions increase significantly, and GAG secretion content of cells also increases. This shows that during the chondrogenic differentiation period, the Wnt pathway promotes chondrogenic differentiation of ADSCs by upregulating  $\beta$ -catenin. In the late stage of induced differentiation,  $\beta$ -catenin continued to be highly expressed, but the expression of Sox9 did not rise but decreased. At this time, early osteogenesis indicators (Collagen 10, RUNX2) were detected to increase. It is proved that during the late stage of chondrogenic differentiation, the continued activation of the Wnt signaling pathway is not conducive to the maintenance of phenotype of cartilage, which is consistent with the dual effects reported by Graneli[28]. To further study the effect of Sox9 reverse on  $\beta$ -catenin of the Wnt signaling pathway, we transferred lentiviral vectors containing the full-length Sox9 gene into ADSCs. The experimental results showed that the expression of Sox9 protein was significantly increased after lentivirus transfection, which proved that Sox9 gene was successfully transfected into ADSCs and expressed. In addition, the expression of  $\beta$ -catenin protein in the lentiviral transfection group was lower than the blank control group in each period, indicating that the overexpressed Sox9 can feedback inhibit  $\beta$ -catenin expression. It is conjectured that by acting on GSK-3 $\beta$ , it strengthens  $\beta$ -catenin phosphorylation and degradation, inhibits osteogenic differentiation of ADSCs, and maintains the cartilage phenotype[29-32].

## Conclusions

This experiment finally elaborated the relationship between the activation status of Wnt /  $\beta$ -catenin signaling pathway and Sox9 in different stages of chondrogenic differentiation in ADSCs. The experimental results show that in the early stage, the Wnt pathway promotes rapid proliferation and chondrogenic differentiation of ADSCs by up-regulating Sox9; In the later period, the Wnt pathway promotes cartilage hypertrophy and early cartilage ossification by down-regulating Sox9 expression. However, the overexpression of Sox9 also inhibited the Wnt pathway, down-regulated the expression of  $\beta$ -catenin, and maintained the phenotype of cartilage. Therefore, accurately grasping the dynamic balance between the Wnt pathway and Sox9 can better regulate the chondrogenic differentiation of ADSCs and thus better serve various fields of cartilage repair engineering.

## Abbreviations

Wnt: Wingless-type MMTV integration site family.

ADSCs: Adipose Derived Stem.

Sox9:SRY-related high mobility group-box gene 9.

DKK-1:Dickkopf related protein 1.

GSK-3 $\beta$ :Glycogen synthase kinase-3 $\beta$ .

RUNX2:Core binding factor alpha 1.

BMP-2:Bone morphogenetic protein 2.

## **Declarations**

### **Competing interests**

There is no competition for all financial and non-financial interests in this article.

### **Funding**

National Natural Science Foundation of China [81760389] Nanning Science and Technology Bureau [20173018-2].

### **Availability of data and materials**

The datasets analyzed in the current study are available from the corresponding author on reasonable request.

### **Authors' contributions**

DWW and ZB participated in the research design, data analysis and drafting of manuscripts, are equal in this article. GLS and YYJ assisted with experiments, data analysis, and interpretation of manuscripts. CM and FZ contributed to data collection and interpretation. LXY conceived the research, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

### **Ethics approval and consent to participate**

All animal experiment research protocols have been approved by the Ethics Review Committee of the Animal Experiment Center of Guangxi Medical University (SCXK (Gui) 2014-0002). Disposal of animals during the experiment follows the Guiding Opinions on the Treatment of Laboratory Animals issued by the Ministry of Science and Technology of the People's Republic of China and the Laboratory Animal-Guideline for Ethical Review of Animal Welfare issued by the National Standard GB/T35892-2018 of the People's Republic of China.

### **Consent for publication**

Not applicable.

## Acknowledgements

This work was supported by the Basic Science Research Program of the National Natural Science Foundation of China (NRF) (grant number: 81760389). This research was also funded by the Nanning Science and Technology Bureau (grant number: 20173018-2).

## References

1. Jones DG, Gray AR, Hutton LR, Stout KM, Abbott JH. Outcomes and factors influencing response to an individualised multidisciplinary chronic disease management programme for hip and knee osteoarthritis. *J Arthroplasty*. 2018; 33:2780-2786.
2. Meng Z, Huang, Rongzhong. Topical treatment of degenerative knee osteoarthritis. *Am J Med Sci*. 2017; 355:6-12.
3. Bijlsma JW BF, Lafeber FP. Osteoarthritis: An update with relevance for clinical practice. *Lancet*. 2011; 377:2115-2126.
4. Sniekers YH, Weinans H, Bierma-Zeinstra SM, Leeuwen JPTMv, Osch GJVMv. Animal models for osteoarthritis: the effect of ovariectomy and estrogen treatment – a systematic approach. *Osteoarthritis Cartilage*. 2008; 16:533-541.
5. Woolf AD, Pfleger B. Burden of major musculoskeletal conditions. *Bull World Health Organ* 2003; 81:646-656.
6. Lawrence RC, Felson DT, Helmick CG, Arnold LM, Wolfe F. Estimates of the Prevalence of Arthritis and Other Rheumatic Conditions in the United States, Part II. *Arthritis Rheum* 2008; 58:26-35.
7. Jevsevar DS, Brown GA, Jones DL, Matzkin EG, Manner PA, et al. The American Academy of Orthopaedic Surgeons evidence-based guideline on: treatment of osteoarthritis of the knee, 2nd edition. *J Bone Joint Surg Am*. 2013; 95:1885-1886.
8. Erickson GR GJ, Franklin DM, Rice HE, Awad H,, et al. Chondrogenic Potential of Adipose Tissue-Derived Stromal Cells in Vitro and in Vivo. *Biochem Biophys Res Commun*. 2001; 290:763-769.
9. Kazunari Kuroda TK, Katsuhiko Hayashi, Toru Maeda, Yoshitomo Kajino, Shintaro Iwai, Kenji Fujita, Kazuhiro Hasegawa, Daisuke Inoue, Naotoshi Sugimoto & Hiroyuki Tsuchiya. The paracrine effect of adipose-derived stem cells inhibits osteoarthritis progression. *BMC Musculoskelet Disord* 2015; 16:236.
10. Ma T, Sun J, Zhao Z, Lei W, Chen Y. A brief review: adipose-derived stem cells and their therapeutic potential in cardiovascular diseases. *Stem Cell Res Ther*. 2017; 8:124.
11. Jing W, Wu L, Lin Y, Liu L, Tian W. Odontogenic differentiation of adipose-derived stem cells for tooth regeneration: necessity, possibility, and strategy. *Med Hypotheses* 2008; 70:540-542.
12. Wu L, Zhu F, Wu Y, Lin Y, Nie X, Jing W, Qiao J, Liu L, Tang W, Zheng X. Dentin Sialophosphoprotein-Promoted Mineralization and Expression of Odontogenic Genes in Adipose-Derived Stromal Cells.

- Cells Tissues Organs. 2008; 187:103-112.
13. Zhu WD, Xu Y-m, Feng C, Fu Q, Song L-j, Cui L. Bladder reconstruction with adipose-derived stem cell-seeded bladder acellular matrix grafts improve morphology composition. *World J Urol.* 2010; 28:493-498.
  14. Chen L, Peng EJ, Zeng XY, Zhuang QY, Ye ZQ. Comparison of the Proliferation, Viability, and Differentiation Capacity of Adipose-Derived Stem Cells from Different Anatomic Sites in Rabbits. *Cells Tissues Organs.* 2011; 196:13-22.
  15. Ter Huurne M, Schelbergen R, Blattes R, Blom A, de Munter W, Grevers LC, Jeanson J, No?l D, Casteilla L, Jorgensen C. Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. *Arthritis Rheum.* 2012; 64:3604-3613.
  16. Toghraie F, Razmkhah M, Gholipour MA, Faghih Z, Ghaderi A. Scaffold-free Adipose-derived Stem Cells (ASCs) Improve Experimentally Induced Osteoarthritis in Rabbits. *Arch Iran Med* 2012; 15:495-499.
  17. Liu H, Cheng B. Related Issues in Clinical Translation Application of Adipose-derived Stem Cells. *Chinese Journal of Reparative and Reconstructive Surgery.* 2012; 26:1242-1246.
  18. Liu G CX. Isolating and Characterizing Adipose-Derived Stem Cells. *Methods Mol Biol.* 2018; 1842:193-201.
  19. Van Harmelen V, Skurk T, R?hrig K, Lee Y-M, Halbleib M, Aprath-Husmann I, Hauner H. Effect of BMI and age on adipose tissue cellularity and differentiation capacity in women. *Int J Obes Relat Metab Disord* 2003; 27:889-895.
  20. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006; 8:315-317.
  21. S.E.P, New, C., Alvarez-Gonzalez, B., Vagaska, S.G., Gomez, N.W., Bulstrode. A matter of identity – Phenotype and differentiation potential of human somatic stem cells. *Stem Cell Res* 2015; 15:1-13.
  22. Topoluk N, Steckbeck K, Siatkowski S, Burnikel B, Tokish J, ea. Amniotic mesenchymal stem cells mitigate osteoarthritis progression in a synovial macrophage-mediated in vitro explant coculture model. *J Tissue Eng Regen Med.* 2018; 12:1097-1110.
  23. Stella C, Karine T, Marie M, Patricia L-C, Olivier B-B, Christian J, Danièle NI. Mesenchymal stem cells-derived exosomes are more immunosuppressive than microparticles in inflammatory arthritis. *Theranostics.* 2018; 8:1399-1410.
  24. Tocci AF, L. Mesenchymal stem cell: use and perspectives. *Hematol J.* 2003; 4: 92–96.
  25. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, et al. Multilineage Cells from Human Adipose Tissue: Implications for Cell-Based Therapies. *TissueEng.* 2001; 7:211-228.
  26. Rosenberger RE, Gomoll AH, Bryant T, Minas T. Repair of Large Chondral Defects of the Knee With Autologous Chondrocyte Implantation in Patients 45 Years or Older. *Am J Sports Med.* 2003; 36:2336-2344.

27. Miki T, Yasuda S-y, Kahn M. Wnt/ $\beta$ -catenin Signaling in Embryonic Stem Cell Self-renewal and Somatic Cell Reprogramming. *Stem Cell Rev Rep*. 2011; 7:836-846.
28. Cecilia Granéli AT, Ulla Ruetschi, Helena Brisby, Peter Thomsen, Anders Lindahl, Camilla Karlsson. Novel markers of osteogenic and adipogenic differentiation of human bone marrow stromal cells identified using a quantitative proteomics approach. *Stem Cell Res*. 2014; 12:153-165.
29. Xu H, Xu B, Qiang Y, Li X, Li C. Experimental study on chondrogenic differentiation of adipose-derived stem cells co-cultured with chondrocytes. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi*. 2013; 27:193-198.
30. Mardani M, Roshankhah S, Hashemibeni B, Salahshoor M, Esfandiari E. Induction of chondrogenic differentiation of human adipose-derived stem cells by low frequency electric field. *Adv Biomed Res*. 2016; 5:97.
31. Pakfar A, Irani S, Hanaee-Ahvaz H. Expressions of pathologic markers in PRP based chondrogenic differentiation of human adipose derived stem cells. *Tissue & Cell*. 2016; 49:122-130.
32. Phuc Van Pham KH-TB, Dat Quoc Ngo, Ngoc Bich Vu, Nhung Hai Truong, et al. Activated platelet-rich plasma improves adipose-derived stem cell transplantation efficiency in injured articular cartilage. *Stem Cell Res Ther*. 2013; 4:91.

## Supplementary Information

**Additional file 1:** The Wnt pathway promotes ADSCs proliferation under LiCl activator conditions.

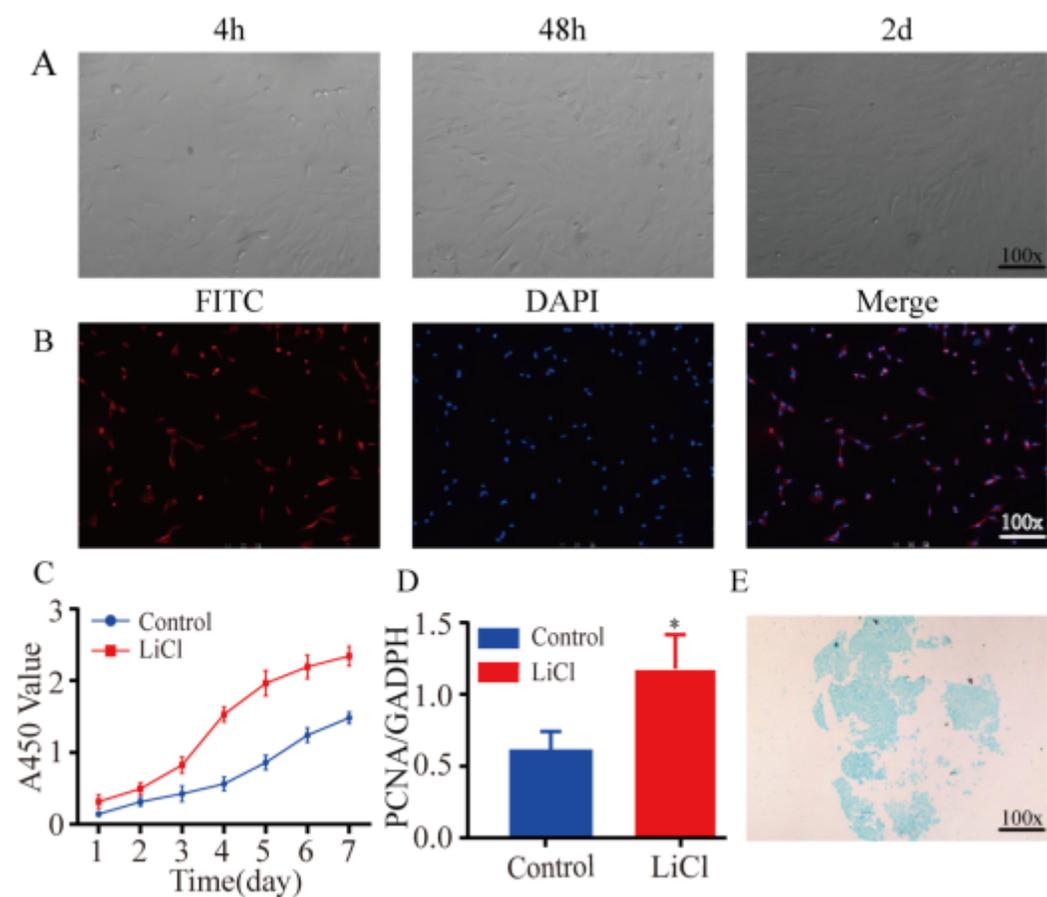
**Additional file 2:** Western blot expression of cartilage index during the induction and differentiation of ADSCs cartilage.

**Additional file 3:** Expression of  $\beta$ -catenin, an important factor in Wnt pathway.

**Additional file 4:** Quantitative analysis of the expression of key proteins during chondrogenic differentiation of ADSCs.

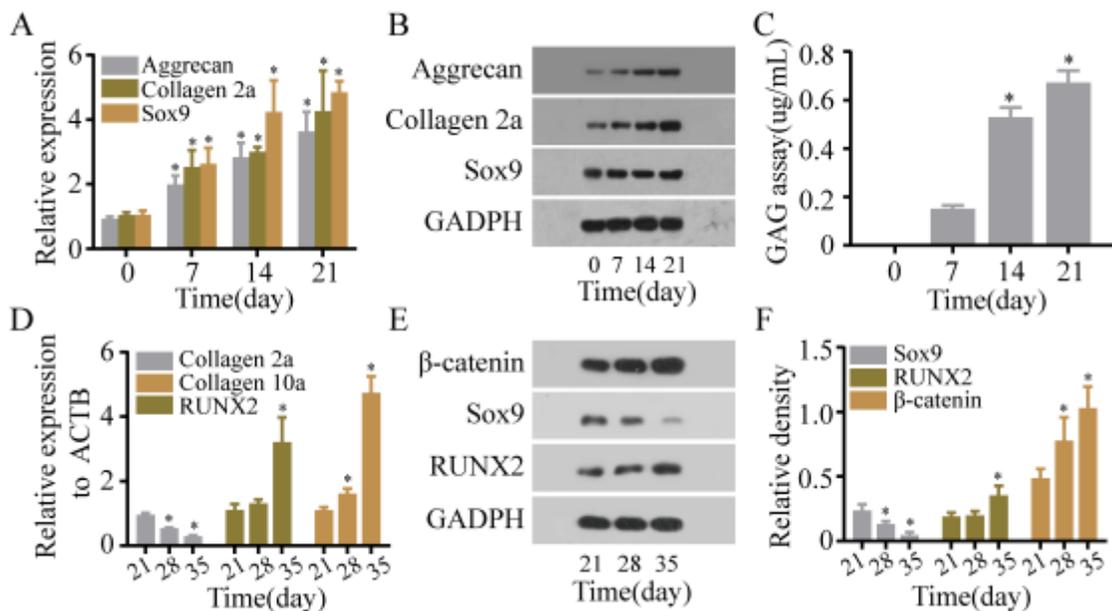
**Additional file 5:** Quantitative expression analysis of Sox9 -catenin protein after ADSCs induction and differentiation by lentivirus transfection.

## Figures



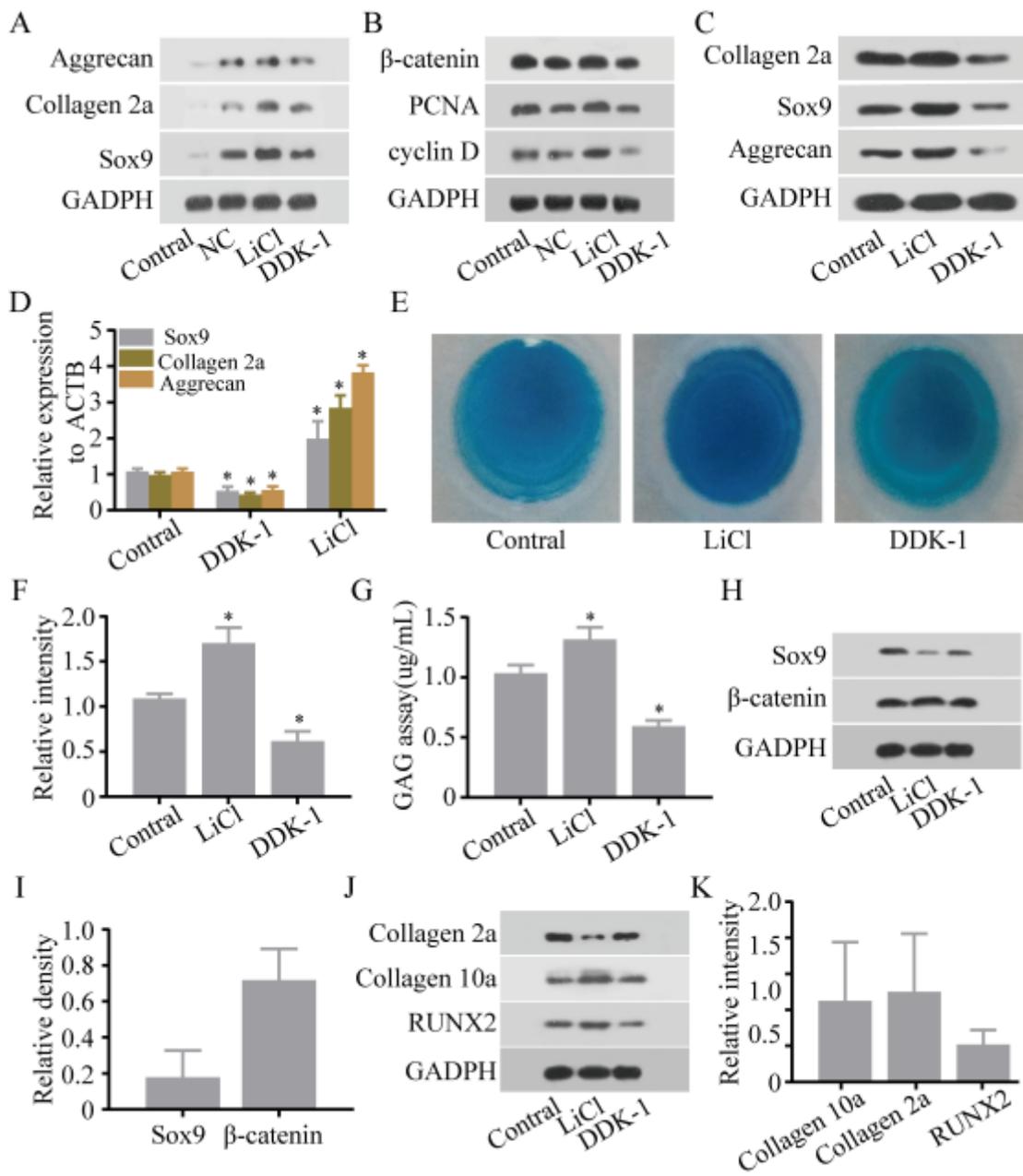
**Figure 1**

Detection of ADSCs, identification of proliferation and differentiation potential.(a) ADSCs growth status at different times, (b) CD44-FITC antibody specific staining (red), The nuclei were counterstained with DAPI (blue). Scale bar, 100 um.(c) Wnt pathway activation was used to detect the proliferation of adipose stem cells. On day 7 (d) Western blot was used to detect PCNA protein expression. (e) Detection of chondrogenic differentiation performance. Scale bar, 100 um.



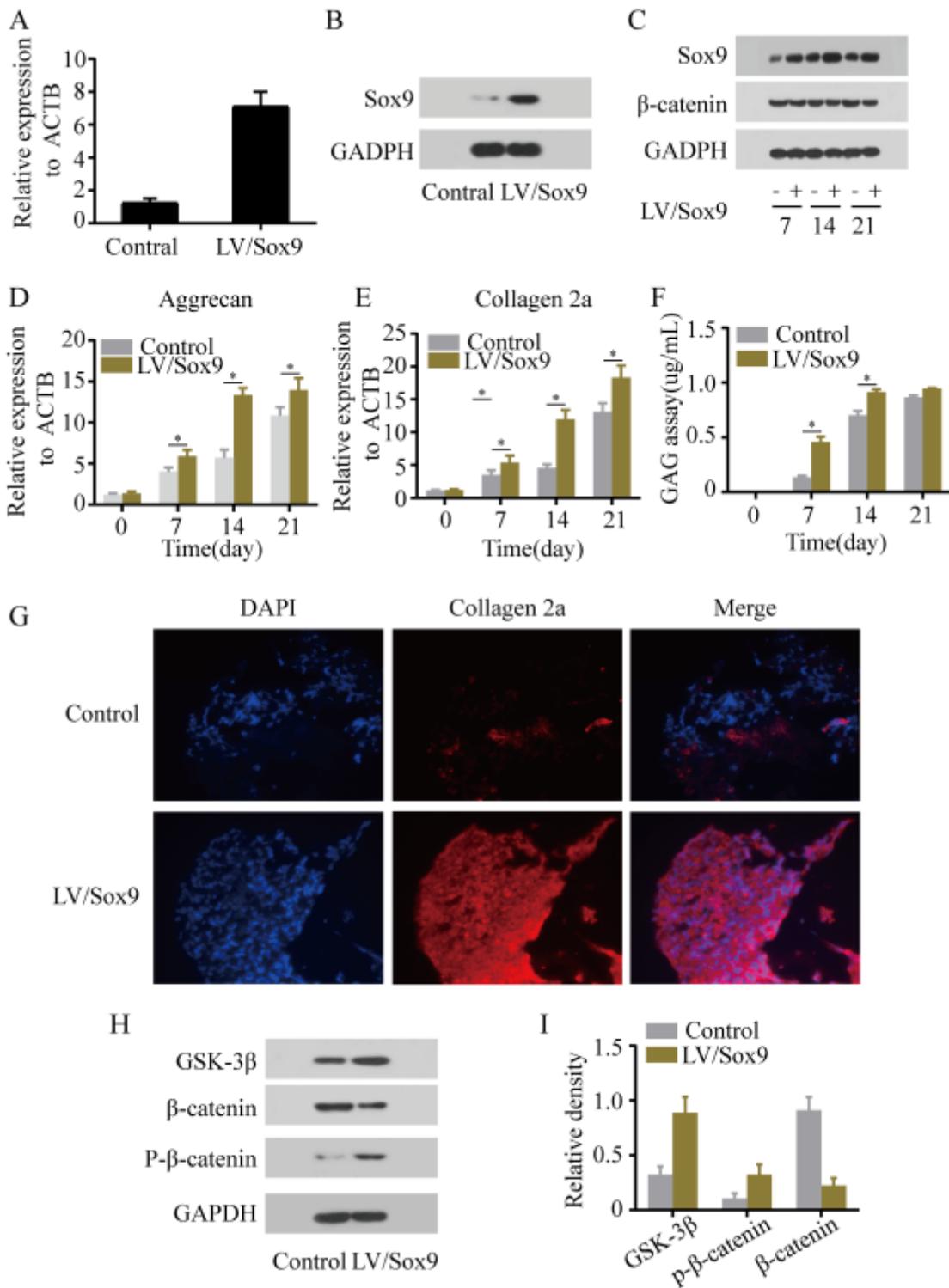
**Figure 2**

Cartilage Indexes in Different Stages of Adipose Stem Cell Chondrogenic Induction.(a) Early mRNA expression of Aggrecan, Collagen 2a, Sox9 and (b) protein expression, (\* P <0.05);And (c) determination of GAG content in the medium;(d) Late-stage expression of Collagen 10, Collagen 2a, RUNX 2 mRNA and (e) Sox9, β-catenin, and RUNX2 protein expression and (f) quantitative analysis (\* P <0.05).



**Figure 3**

The role of Wnt pathway in the process of chondrogenic differentiation of ADSCs. On day 7 of induced differentiation, (a) the expression of various cartilage index proteins; (b) the expression of β-catenin and CyclinD and PCNA proteins. On day 21 of induced differentiation, (c) cartilage formation index and β-catenin protein expression; (d) cartilage formation index mRNA expression; (e) Chondroitin sulfate alixin blue staining and (f) quantitative analysis, (g) quantitative analysis of GAG, (\* P < 0.05). On the 28th day of induced differentiation, the quantitative (h) and qualitative (i) expressions of Sox9 and β-catenin proteins; the quantitative (j) and qualitative (k) expressions of Collagen 10, Collagen 2a, and RUNX 2 proteins.



**Figure 4**

Induced differentiation of cartilage and expression of  $\beta$ -catenin in ADSCs after lentivirus transfection. Quantitative (a) and qualitative (b) expression of Sox9 protein after lentivirus transfection. (c) Sox9 and  $\beta$ -catenin protein expression; chondrogenic differentiation markers Aggrecan (d) and Collagen 2a (e) mRNA expression; (f) GAG content quantification; (g) Collagen 2a immunofluorescence detection; Wnt Qualitative (h) and quantitative (i) expression of signaling pathway related proteins.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supportinginformation.pdf](#)