Mechanism of Wnt Pathway on Cartilage Differentiation of Adipose Derived Stem

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)
Nanning Second Peoples Hospital

Research article

Keywords: Adipose-derived Stem Cells, Chondrogenic Differentiation, Wnt/β-catenin signaling pathway, Sox9,Collagen 2a

Posted Date: March 24th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-18465/v1

Abstract

Objective Exploring the interaction of Wnt signaling pathway and sox9 to elucidate the role of adipose-derived stem cells in chondrogenic differentiation. Methohs Through LiCl,DKK-1 stimulate ADSCs into cartilage differentiation, detection of middle-late late early differentiation into cartilage indicators such as collagen type II (Collagen2a), gather the proteoglycan (Aggrecan) and Wnt pathways key protein Sox9, BMP-2, beta serial protein (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 for stable expression, and the expression levels of relevant indicators were detected again. Results During the chondrogenic induction and differentiation of ADSCs, the Wnt pathway promoted the rapid proliferation of ADSCs in the early, middle and late stages and induced the expression of Collagen 2a Aggrecan to increase in the early stage but not significantly ($P<0.05$).At the later stage, the phenotype of mature cartilage was inhibited and cartilage hypertrophy and early cartilage ossification were promoted.Overexpression was fed back to inhibit the expression of Sox9, the activity of Wnt pathway, up-regulated in early, mid-stage and down-regulated in late stage ($P<0.01$). 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 adult healthy stem cells from the groin of Sprague Dawley and isolated and induced them[18]. We have found that during the process of chondrogenic induction of ADSCs, the early, middle and late stages of Wnt pathway promote its rapid appreciation and induce chondrogenic differentiation. The expressions of Collagen 2a and Aggrecan were not significantly increased in the early stage and significantly increased in the middle and late stages; later, they inhibited the mature cartilage phenotype and promoted cartilage hypertrophy and early cartilage ossification; overexpression inhibited Wnt pathway activity. Sox9 expression was up-regulated in the early and middle stages and down-regulated in the late stage. 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±25g, 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.

ADSCs extraction,culture and transfection

According to Van Harmelen et al.[19], collagenase digestion method extracts ADSCs from the superficial fat of abdominal skin. Routine adherent culture. 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 vectors mediate transfection of cells[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 passage were ADSCs.

Identification of ADSCs differentiation potential

The stabilized and purified ADSCs were induced by chondroblast induced differentiation solution and stained with specific alixin blue.

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: AACGACCCCTTCATTGACCTC, downstream primer: CCTTGACTGTGCCGTTGAACT; Sox9 upstream primer: GTGGGAGCGACAACTACTACC, downstream primer: GCGAGCACTTAGCAGAGGC; BMP-2 upstream primers: AGACCTTCTTGGTATCCC, downstream primers: CTCCAGCTTGTTTTTGTT; Collagen 2a upstream primers: CACCCAGAGTGGAAGAGCG, downstream primers: TCAGTGGAGTAGACGGAGGA; Aggrecan upstream primers; CAAACAGCAGAGCACACACATCA, 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 ± standard deviation (± s). Comparison between groups was analyzed by t test or one-way analysis of variance.

Results

Detection of ADSCs and identification of differentiation potential

The extracted adipocytes were cultured according to the improved method of Zuk and others. The growth conditions were observed under different microscopes at 4h, 48h, and 2d and the morphology changed with time and became a spindle-shaped distribution **(Fig.1 A)**.In order to further strengthen the cell components, CD44-FITC antibody was used for specific labeling. After fluorescent staining, red fluorescence was visible under the microscope, and the nuclei of DAPI counterstained cells were blue, suggesting that the surface antigen CD44 of ADSCs was positively expressed **(Fig.1 B)**.It was confirmed that the cells obtained from the culture were ADSCs.In order to verify the differentiation potential, Alisin blue was specifically stained after induced differentiation, showing a large number of blue matrix particles, indicating that ADSCs have good chondrogenic differentiation potential **(Fig. 1 E)**.

Effect of Wnt pathway activation on adipose stem cell proliferation

CCK-8 was used to detect the value-added of adipose stem cells at different times. The results showed that the experimental group stimulated the Wnt pathway with the agonist LiCl. The adipose stem cells entered exponential growth on the second day, and the adipose 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 expression of PCNA protein in the experimental group and the blank control group stimulated by LiCl was detected and recorded. The expression of PCNA in the experimental group was significantly higher than that in the control group **(Fig. 1 D and supporting information S1).**

Cartilage Indexes and β-catenin Expression in Different Stages of ADSCs Chondrogenic Induction

During the process of chondrogenic differentiation of ADSCs, the expression levels of mRNA and protein of Sox9, Collagen 2a, and Aggrecan chondrogenic differentiation were detected on day 0, 7, 14, and 21, and the results showed that Sox9 had a higher expression on day 7. On the 14th and 21st days, it slowly increased, but Collagen 2a and Aggrecan also showed high expression on the 7th day, and the expression was obvious on the 14th and 21st days **(Fig. 2 A)**.Western blot showed the same result **(Fig. 2 B and supporting information S2)**.Quantitative detection of GAG content in the culture medium, the results showed that GAG began to express a small amount on day 7, gradually increased on day 14, and significantly expressed on day 21 **(Fig. 2 C)**.

The expression of the important factor β-catenin protein in the Wnt pathway is initially large, but with the progress of chondrogenic differentiation, the expression gradually decreases. The protein is low on the 7th and 14th days, but by the 21st day The β-catenin expression rebounded, and the content was higher than the initial level **(supporting information S3)**.

ADSCs were at the late stage of chondrogenic differentiation. The mRNA expression levels of the cartilage differentiation markers Collagen 2a, Collagen 10, and RUNX were measured on days 21, 28, and

35. The results showed that the mRNA expression of Collagen 10 and RUNX 2 increased with chondrogenesis The induced differentiation increased over time, and Collagen 2a expression gradually decreased **(Fig. 2 D)**.Western blot results showed that the expression of Sox9 protein gradually decreased with the time of cartilage induction differentiation, but the expression of β-catenin protein gradually increased **(Fig. 2 E & F)**.

Role of Wnt pathway in ADSCs during chondrogenic differentiation

The agonist LiCl and the inhibitor DKK-1 interfere with the regulation of the Wnt pathway.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 supporting information S4)**.Compared with the blank control group, β-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 β-catenin protein in the LiCl-agonized group was significantly higher Compared with the latter, CyclinD protein and PCNA protein also increased significantly, while the DKK-1 inhibitory group showed the opposite result **(Fig. 3 B and supporting information S4)**.

In the late stage of induced differentiation, on day 21, it was observed that the expression of β-catenin was activated in the LiCl group, while the expression was inhibited in the DKK-1 inhibition group.Western blot analysis showed that both Collagen 2a and Sox9 increased in the LiCl agonist group, which increased significantly with Collagen 2a, while the expression was significantly suppressed in the DKK-1 inhibitory group **(Fig. 3 C and supporting information S4)**.Cartilage indicators Aggrecan, Collagen 2a, and Sox9 mRNA analysis showed that the cartilage indicators of the LiCl-excited group increased, while Aggrecan and Collagen 2a increased significantly, but the DKK-1 inhibitory group was significantly suppressed **(Fig. 3 D)**.Alixin blue staining **(Fig. 3 E)** and quantitative analysis **(Fig. 3 F)** showed that the chondroitin sulfate in the LiCl-agonized group was significantly higher than that in the cartilage-induced differentiation group, while the chondroitin sulfate in the DKK-1 inhibitory group was lower than that of Induced differentiation group.GAG quantitative analysis results also show the same trend **(Fig. 3 G)**.

In the late stage of induced differentiation, on the 28th day, quantitative and qualitative analysis by Western blot showed that LiCl group significantly continued to activate β-catenin protein expression and DKK-1 inhibited expression; compared with the cartilage-induced differentiation group, the expression of Sox9 protein in the LiCl agonized group was significantly reduced, and DKK -1 Inhibition group decreased slightly **(Fig.3 H & I)**.Cartilage indicators showed that compared with the cartilage-induced differentiation group, the expression of Collagen 2a was reduced, and the expression of Collagen 10 and RUNX 2 were increased in the LiCl-excited group; the expression of Collagen 2a was increased in the DKK-1 inhibitory group, and the expression of Collagen 10 and RUNX 2 was decreased **(Fig. 3 J & K)**.

Detection of Sox9 protein expression after ADSCs lentivirus transfection

ADSCs were transfected with the entire sequence of Sox9 lentivirus, and quantitative and qualitative analysis by Western blot revealed that Sox9 was highly expressed **(Fig. 4 A & B)**.

Cartilage Indexes and β-catenin Expression at Different Stages of Chondrogenic Induction and Differentiation of ADSCs After Lentivirus Transfection

During transfection-induced differentiation, Sox9 protein expression in the lentivirus transfection group was significant. Compared with the blank control group, the difference between the 7th day and the 14th day was particularly obvious, and β-catenin protein was lower than that of the blank control group in each period **(Fig. 4 C and supporting information S5)**.On days 0, 7, 14, and 21, the expressions of the chondrogenic differentiation markers Collagen 2a and Aggrecan mRNA were detected. 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 It appears higher **(Fig. 4 D & E)**.On the 7th and 14th days, 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 the 21st day **(Fig. 4 F)**.

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

On day 21 of transfection-induced differentiation, immunofluorescence detection showed that the expression of Collagen 2a protein was significantly higher in the Sox9 lentivirus transfection group than in the blank control group **(Fig. 4 G)**.Western blot was used to detect the expression of Wnt signaling pathway-related proteins. The expression of β-catenin and GSK-3β protein increased in the Sox9 lentivirus transfection group, but the total β-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.

In order to explore the role of Wnt signaling pathway and its mechanism during its differentiation, and its relationship with Sox9 expression, we recorded the expressions of β-catenin, Sox9, Aggrecan, Collagen 2a and so on in detail.Finding the most suitable external induction and culture conditions for differentiation, so as to more accurately control ADSCs into directional differentiation of cartilage and promote cartilage repair, thus providing ideas and possibilities for cartilage repair in osteoarthritis.

This experimental study proved that the expression of PCNA in the LiCl group was significantly enhanced, which further stimulated the proliferation ability of ADSCs, which is consistent with the findings of Miki TD et al. LiCl indirectly activates the Wnt signaling pathway by inhibiting the phosphorylation of GSK-3β[27].In the early stage of chondrogenic differentiation of ADSCs, LiCL not only promoted the expression of PCNA and Cyclin D, but also enhanced the expression of Sox9.However, even though Sox9 began to show high expression, Aggrecan and Collagen 2a did not change significantly, which proves that Sox9 gene plays a key role in the early stage of chondrogenic differentiation.In the middle stage of induced differentiation, β-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, which is the same as Yano's experimental results.In the late stage of induced differentiation, the Wnt pathway continued to be activated and opened, β-catenin expression continued to increase, Sox9, Aggrecan, and Collagen 2a expression increased significantly, and the cell GAG secretion content also increased, indicating that during the chondrogenic differentiation period, the Wnt pathway up-regulates β-catenin To promote ADSCs into cartilage differentiation.In the late stage of induced differentiation, β-catenin continued to be highly expressed, but the expression of Sox9 did not rise but decreased. At this time, early osteogenesis indicators Collagen 10 and RUNX2 were detected to increase, which proved that during the late stage of chondrogenic differentiation, continuous activation of the Wnt signaling pathway is not good for cartilage Phenotype maintenance, consistent with the dual effects reported in the Graneli C study[28].In order to further study the effect of Sox9 reverse on β-catenin of the Wnt signaling pathway, we introduced a lentiviral vector containing the full-length Sox9 gene into ADSCs.The experimental results showed that the expression of β-catenin protein in the Sox9 lentiviral transfection group was lower than that in the blank control group, indicating that Sox9 can feedback inhibit β-catenin expression. It is conjectured that by acting on GSK-3β, β-catenin phosphorylation can be enhanced Inhibits osteogenic differentiation of ADSCs[29–32].In summary, in the process of adipogenic differentiation of adipose-derived stem cells, we must accurately grasp the relationship between the Wnt pathway and Sox9, and grasp the dynamic balance between the two to better regulate the chondrogenic differentiation of ADSCs.

Conclusions

This experiment explored in detail the relationship between the activation status of Wnt / β-catenin signaling pathway and Sox9 at different stages of chondrogenic differentiation in ADSCs.The

experimental results show that in the first and middle stages, the Wnt pathway promotes the rapid proliferation of ADSCs, while up-regulating Sox9 promotes chondrogenic differentiation; in the later stage, the Wnt pathway down-regulates Sox9 expression, weakens the mature cartilage phenotype, promotes cartilage hypertrophy and early chondrogenesis.This study further examined the effect of Wnt pathway factor overexpression on induced differentiation, and found that overexpression of Sox9 inhibited Wnt pathway activity, maintained cartilage phenotype, delayed cartilage hypertrophy and early osteogenesis.

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 β-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.

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-3β:Glycogen synthase kinase-3β.

RUNX2:Core binding factor alphal 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 was the first author to collect and process experimental data and was a major contributor in writing the manuscript. ZB collected and processed the experimental data, and was the first author to write the manuscript. HZQ analyzed and explained the date of WB. SGL and YYJ analyzed and explained the types of adipose stem cells. CM and FZ transfected and validated adipose stem cells. LXY has designed and directed the experiment and sponsored the project funding, and is the corresponding author of this paper. 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, ea. 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, Katsuhiro 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 Nl. 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/β-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.

Figures

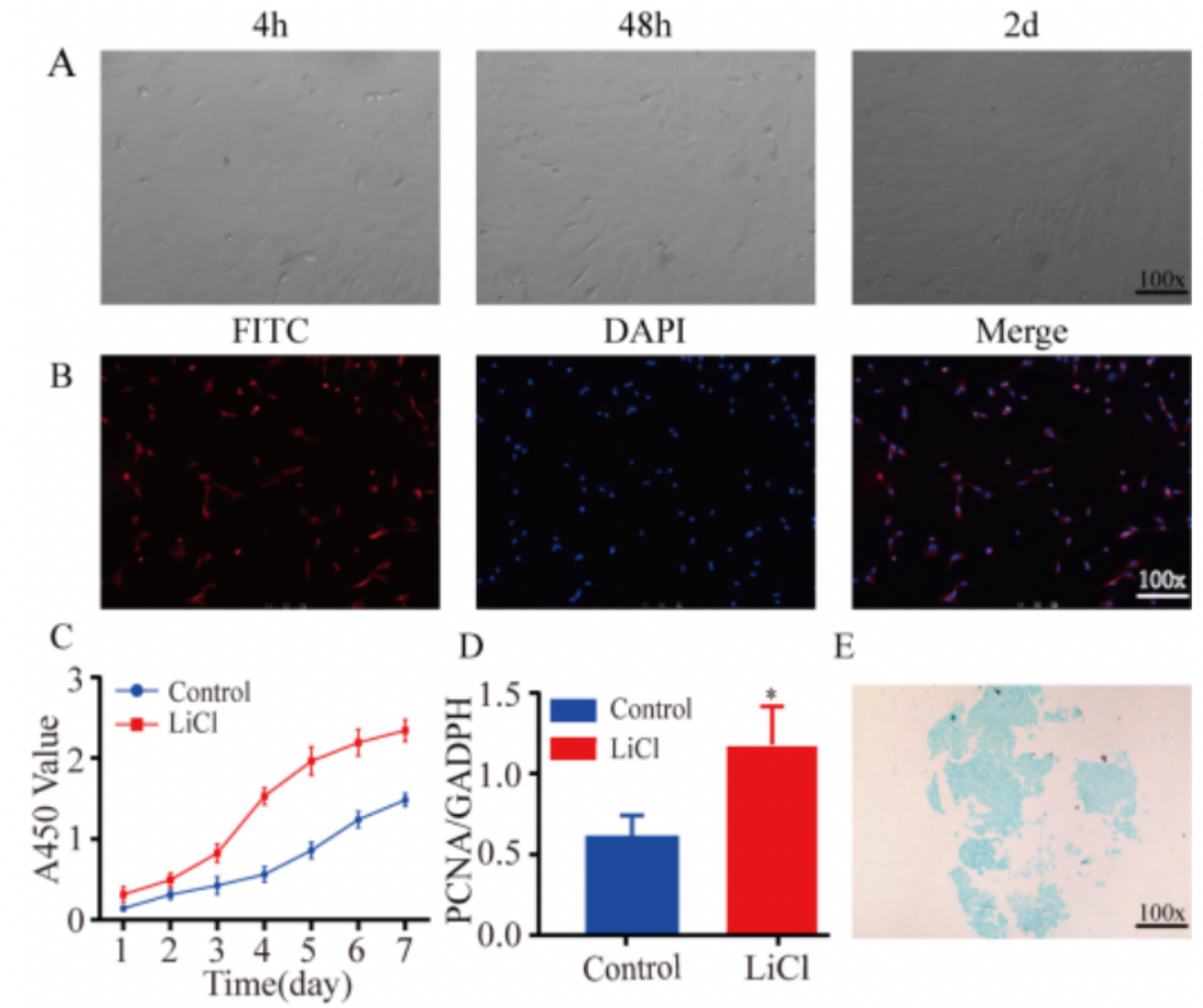

Figure 1

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.

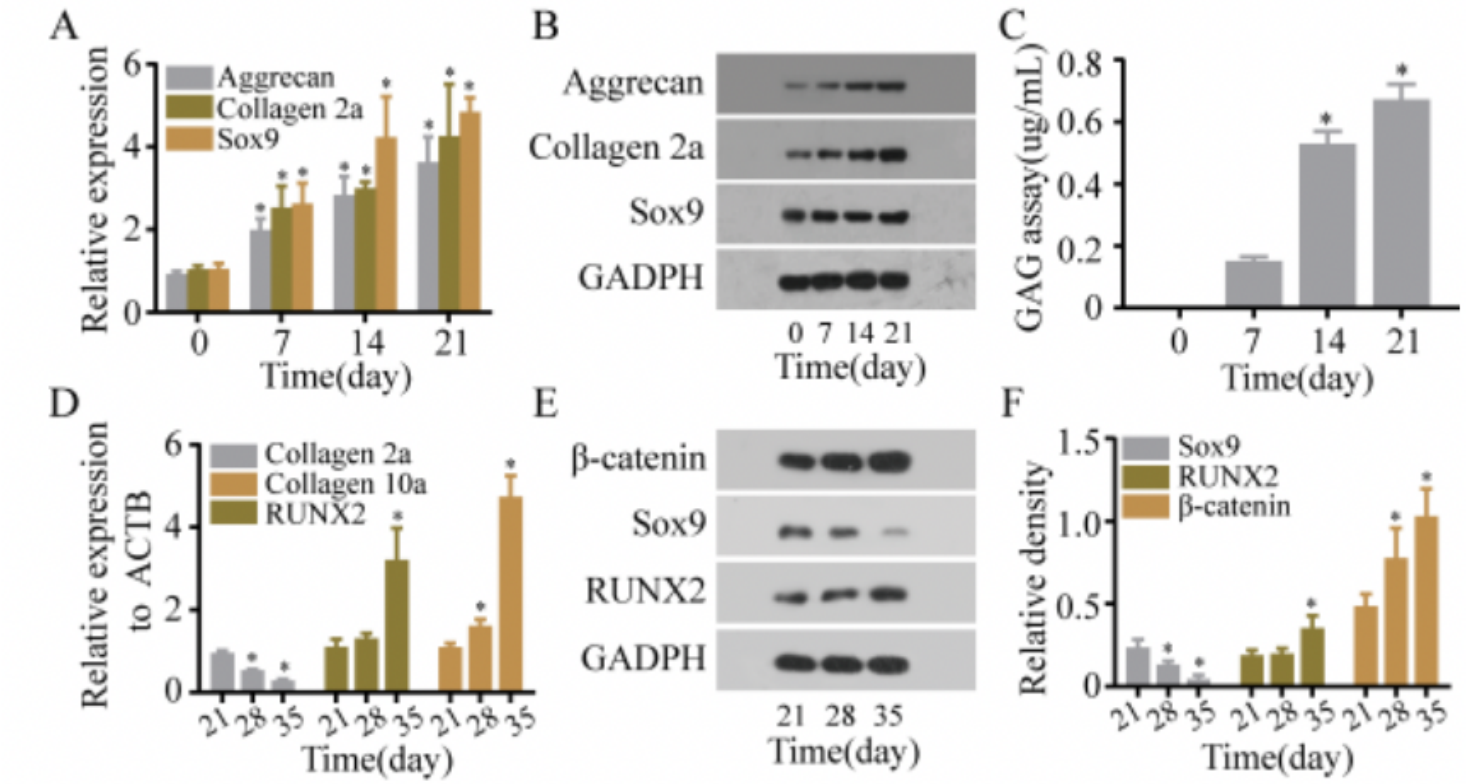

Figure 2

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).

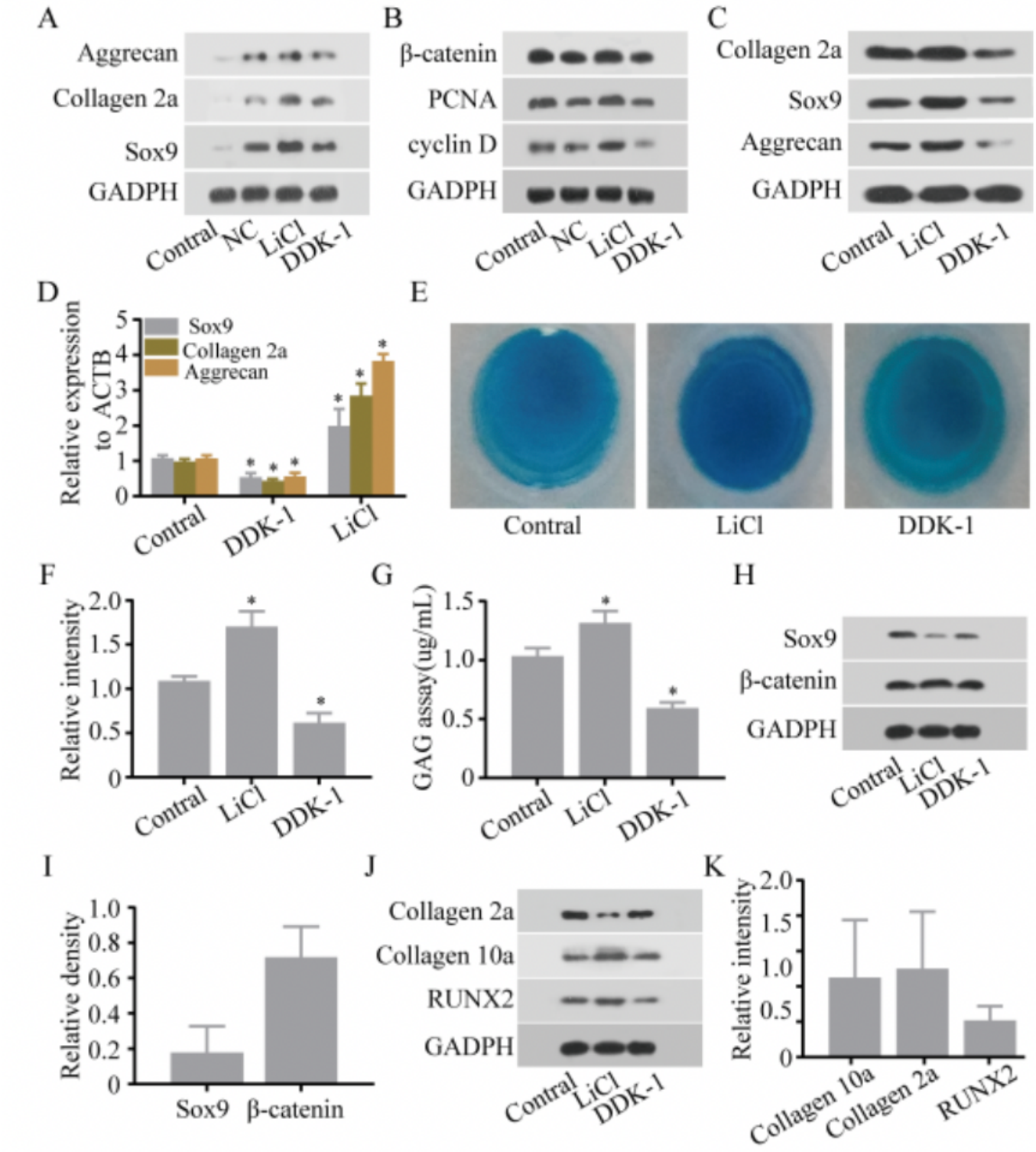

Figure 3

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.

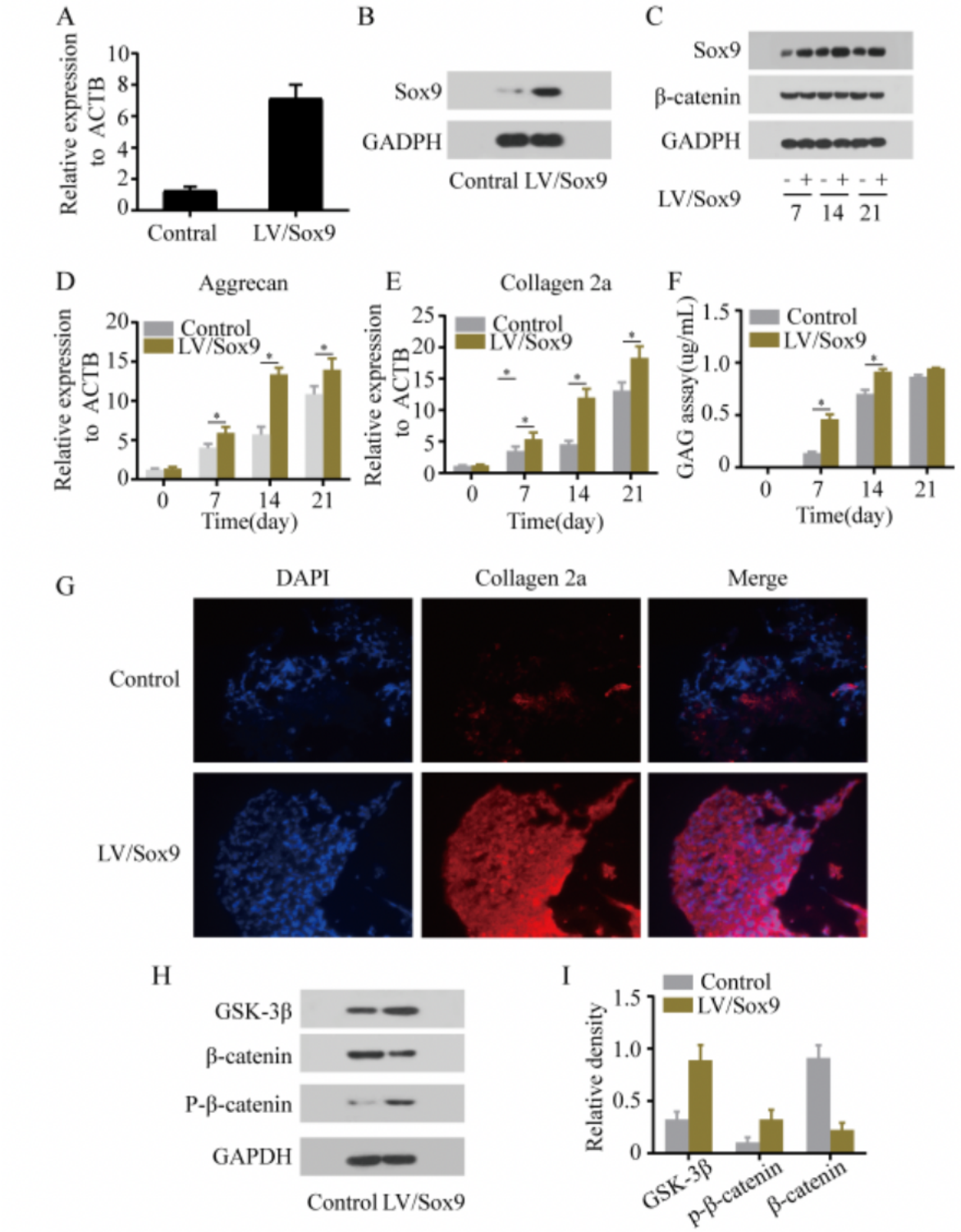

Figure 4

Induced differentiation of cartilage and expression of β-catenin in ADSCs after lentivirus transfection.Quantitative (a) and qualitative (b) expression of Sox9 protein after lentivirus transfection.(c)

Sox9 and β-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.docx