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Original Article

Primary culture and adenoviral infection of adult mouse knee articular chondrocytes: a new tool for investigating cartilage

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Abstract: Object: To establish a method for primary culture and gene manipulation with adenoviral infection of chondrocytes from knee articular cartilage of adult mice (C57BL/6J). Significant findings: Knee articular chondrocytes were dissected from tibial plateaus and femoral condyles of four-month old C57BL/6J mice for primary culture. A range of morphological and biochemical features of the chondrocytes of different passages were carefully determined to find the very passage suitable for further deep investigation. Moreover, adenovirus was employed as powerful gene transfer vector system for gene silencing of SREBP-2 in cultured mouse chondrocytes. We found that cells from P0 and P1 harbored the typical chondrocytes morphology, with a rounded or polygonal shape, but cells of P2 and P3 seemingly underwent dedifferentiation with the shape transforming into that of fibroblasts. Alcian-Blue staining and immunofluorescence showed that chondrocytes from P1 express both proteoglycans and collagen II. Real time-PCR analysis showed that the level of collagen II and aggrecan decreased with the increment of cell passages. The expression of collagen I manifested an opposite manner to that of collagen II and aggrecan, indicating that chondrocytes of elder passage transformed into the fibroblast-like cell types and were not suitable for deep investigation. Gene delivery via adenoviral infection was highly efficient and reproducible at 50 MOI (multiplicity of infection) for 48 h. Conclusions: We have developed a practical and reliable protocol to isolate and culture chondrocytes from knee articular of adult mice and to implement acute gene knock-down in these chondrocytes via adenoviral infection. The culture and adenoviral infection of adult mouse knee articular chondrocyte described here was supposed to provide a powerful tool for deeper researches concerning knee articular chondrocytes.

Keywords: Cartilage, chondrocyte, SREBP-2, Col2A1, aggrecan

Introduction

The articular cartilage is composed of chondrocytes, extracellular matrix (ECM) and other non-collagen glycoproteins. The chondrocytes take only 1-2% of all of the components of the articular cartilage, and are responsible for producing, sustaining, and degrading the ECM [1]. Proteoglycan aggrecan and collagen II are two main macromolecules in articular cartilage ECM that were produced and secreted by chondrocytes, as a result, gene expression of aggrecan and collagen II was considered to be the molecular marker of chondrocytes [2].

Chondrocytes culture is one of the most powerful tools for basic and biomedical studies especially for researches associated with chondrocyte differentiation and activation, the deviation of which process underlies the pathophysiology of osteoarthritis (OA), one of the most prevalent disorders affecting the elderly [3]. Differentiation analysis and comparison of the gene expression at the mRNA or protein level between the chondrocyte from OA patients and normal counterparts was supposed to help providing molecular markers, therapeutic targets for diagnosis and treatment of OA [4].

Mice are of great use for cellular and molecular studies, in particular, rapid development of transgenic and gene-targeted mice and acute genetic manipulation via gene transfer vector system constitute powerful instruments for basic and biomedical studies [5]. The ability to isolate primary chondrocytes from wild-type

and genetically altered mice has provided tremendous advances in the understanding of signaling networks that regulate chondrocytes in health and OA diseases [6].

C. Salvat et al have described a new protocol for primary culture of immature murine articular chondrocyte, which was widely adopted, and has greatly promoted the studies in the studies associated with chondrocytes [7]. However, protocol concerning primary culture of adult mouse knee articular chondrocyte was not yet established. Our goal was to establish a method about primary culture and adenoviral infection of mature articular chondrocytes which are supposed to facilitate the pathophysiological studies for joint diseases such as osteoarthritis.

Materials and methods

Mice

All animal studies were conducted in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care and Use Committee. Male C57BL/6J mice were purchased from the experimental animal center of the Peking University People's Hospital. The mice were housed four per cage supplied with a normal diet in a pathogen-free facility with 12-h light, 12-h dark cycle. 16-weeks old mice were subjected to primary chondrocyte isolation.

Isolation of adult mouse chondrocyte

The mice were anaesthetized with 10% chloral hydrate, and sacrificed. The isolation of chondrocytes was carried out in a Class II biological safety cabinet using sterile technique. Douse the body of the sacrificed mice into the mixture of iodine and 70% Ethanol for sterilization. Remove the skin and soft tissues from the knee of hindlimbs with no destruction of joint capsule. Primary mouse articular chondrocytes were isolated by dissection of the tibial plateaus and femoral condyles and carefully peeled off with a blunt-ended forceps. A careful removal of the synovial layer and muscle around the knee was carried out under a dissecting microscope. The cartilage was crushed the and then put into 50 ml conical tubes containing cold 1×PBS (Phosphate-buffered saline) on ice. Carefully decant the PBS and wash the cartilage three times with additional 1×PBS. The cartilage was subsequently digested with 0.1% collagenase D (Roche) at 37°C for 12 h at a sterile petri dish. The contents in the petri dish were pipetted up and down several times to literate cells from the cartilage pieces, and were dispensed over a 70 μm cell strainer (BD, Falcon) positioned over a 15 ml conical tube. The cells were centrifuged at 1300 rpm/m for 5 minutes, and the supernatants containing collagenase D were removed. Primary chondrocytes were cultured in 60 mm plates with Hyclone Dulbecco's modified Eagle's medium (Thermol) supplemented with 15% fetal calf serum (Invitrogen), 1% glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, 0.05 mg/ml ascorbic acid maintained and proliferated at 37°C in the presence of 5% CO_2 for 7 days till 70-80% confluence as passage 0. 0.2% trypsogen-EDTA was employed for digestion of the chondrocytes for next passages.

Alcian blue staining of proteoglycans

Chondrocytes were washed with PBS for 3 times, and then fixed in 4% paraformaldehyde for 30 min at room temperature, washed in 0.1 N HCL for 5 min, and finally stained in 1% Alcian Blue 8GX in 0.1 N HCL (pH 1) overnight. After three times of brief wash with 0.1 N HCL, the cells were allowed to detection.

Immunofluorescence

Chondrocytes were seeded directly onto Bottom Dish at a density of 2×10^4 cells/dish. At 70-80% confluence, the cells were rinsed twice with (PBS), then fixed with 4% paraformaldehyde for 10 min at room temperature, methanol punched, 0.8% bovine serum albumin (BSA) blocked and incubated with the primary antibody overnight at 4°C (collagen II anti-body from Scant Cruz). After two washes with 0.8% BSA, the cells were incubated with the fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Abcam) diluted 1/100 in 0.8% BSA, dishes were observed with a Leica microscope equipped with a mercury lamp.

Real-time polymerase chain reaction assays

Total RNA was extracted using the Trizol regent (Invitrogen) according to the manufacture's protocol. Reverse-transcription was carried out

Table 1. Primer sequences

Primer	Sequences
Collagen I	
Sense	AAC GAG ATC GAG CTC AGA GG
Antisense	GAG TGT CTT GCC CCA AGT TC
Collagen II	
Sense	CAG GTG AAC CTG GAC GAG AG
Antisense	ACC ACG ATC TCC CTT GAC TC
Aggrecan	
Sense	GTT GGT TAC TTC GCC TCC AG
Antisense	GTC CTC CAA GCT CTG TGA CC
GAPDH	
Sense	CCA TCA CCA TCT TCC A
Antisense	CCT TCT CCA TGG TGG T

using Rever Tra Ace Qrna RT Master Mix with gDNA Remover (TOYOBO), where 0.5 μg of total RNA was include in a final volume of 10 μl containing 2 μl 4×DN Master Mix, 2 μl 5× RT Master Mix II, according to the manufacturer's instructions. The PCR reactions were performed in final volumes of 10 μl mixture containing 1 μl complementary DNA (cDNA), 400 ng of specific primers, and 5 μl SYBR Green Realtime PCR Master Mix. Sample were denatured for 30 sec at 95°C then amplified for 40 cycles as follows: denaturation at 95°C for 5 sec, annealing at 55°C for 10 sec and extension at 72°C for 15 sec. The Ct values of the products were normalized to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control and the expression levels of the genes of interest were calculated with the $2^{-\Delta\Delta CT}$ method. Primer details were shown in **Table 1**.

Design of hairpin siRNA template oligonucleotide

The target sequence of SREBP-2 siRNA is 5'-GGACAACACACAATATCAT-3' according to our previous research. Sense template: 5'-CCGGGCGGACAACACACAATATCATCTCGAGATGATATTGTGTGTTGTCCGCTTTTTG-3', antisense template: 5'-AATTCAAAAAGCGGACAACACACAATATCATCTCGAGATGATATTGTGTGTTGTCCGC-3'. Two template oligonucleotides were synthesized by Genechem Co., Ltd. (Genechem, Shanghai, PR China), the annealed siRNA template were inserted into the CV-120 vector between the polyclonal sites of AgeI, and EcoRI. Successful cloning was confirmed by DNA sequence analysis. The adenoviral PBHG plasmid and the CV120 vector containing siRNA template were linearized and co-transfected into human embryonic kidney cell line HEK-293 using a calcium phosphate method to produce recombinant adenovirus. After adenovirus was expanded in HEK-293 cells, viral particles were purified with BD Adeno-X™ Virus Purification Kits (BD Biosciences, Mountain View, CA). Viral titer was determined by End point dilution method, the silencing effect of Ad-siRNA was tested in chondrocytes with reverse transcription polymerase chain reaction (RT-PCR), and the recombinant adenovirus was stored at -80°C until used in experiment. The empty recombinant adenovirus was used as control vectors throughout the study. All endonucleases were purchased from New England Biolabs (NEB).

Western blot

Cells were washed twice in cold PBS and lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100 and 0.1% SDS) plus protease inhibitor cocktail (Roche, Mannheim, Germany). Protein lysates were subjected to SDS-PAGE, transferred to PVDF membrane (Millipore) and detected with appropriate primary antibodies coupled with HRP-conjugated secondary antibodies by ECL reagent (GE Healthcare, Buckinghamshire, UK). Primary antibodies against the following proteins were used: collagen I (ab-6308), collagen II (ab-34712), aggrecan (sc-36-861), SREBP-2 (ab-30682) (Abcam, Cambridge, USA). Secondary antibodies used were: HRP-goat anti-mouse, HRP-goat anti-rabbit, and HRP-rabbit anti-goat (Zhongshanjinqiao Biotech, Beijing, China).

Statistical analysis

Statistical analysis was performed with SPSS 20.0 (IBM SPSS, Chicago, IL USA). The results are presented as mean ± standard deviation from three independent experiments, using Student's t-test and/or one-way analysis of variance to perform appropriate data comparisons. *P* values less than 0.05 were considered statistically significant.

Result

Characterization of the chondrocytes phenotype

Chondrocytes were seeded on a 6 mm culture dish and reached a confluence of 70-80% by

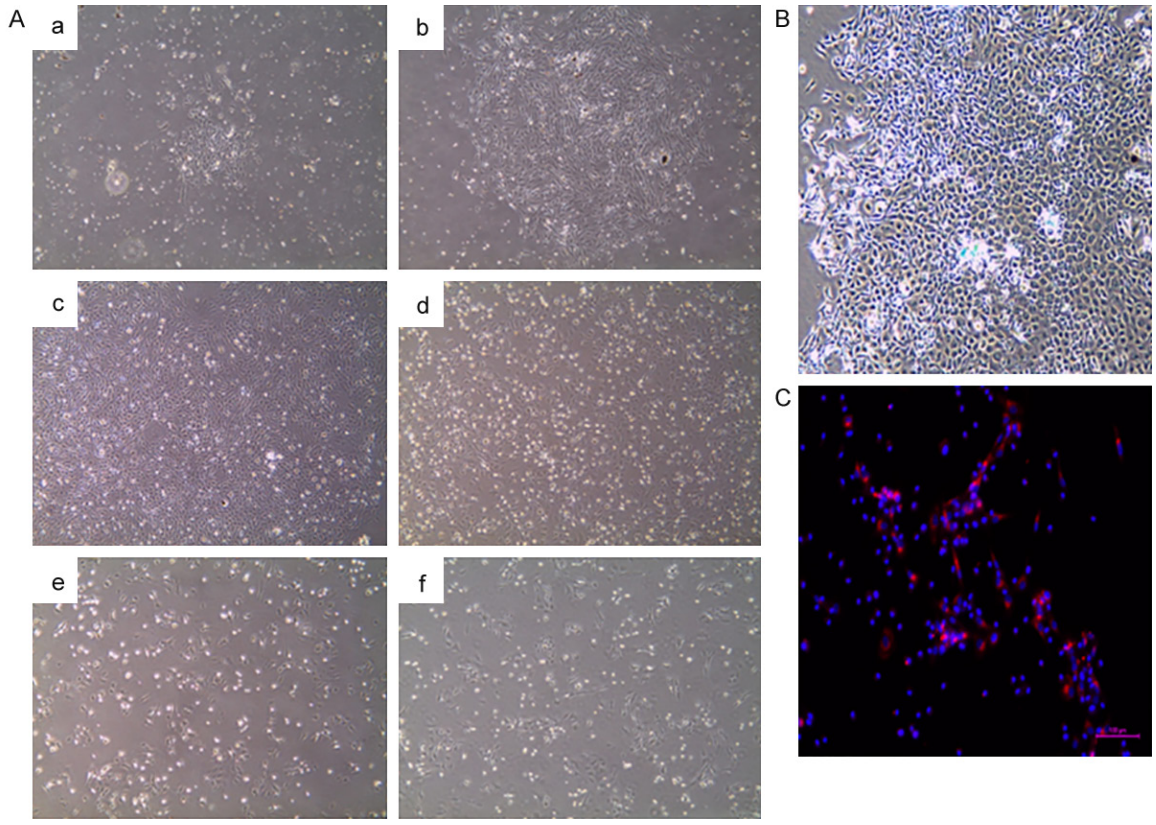

Figure 1. Characterization of the chondrocytes phenotype. A. Cells at these stages exhibit the typical chondrocyte morphology, with a rounded or polygonal shape and slab stone-like cytoplasm (a-d). As the passages went on, the chondrocytes become dedifferentiation with the shape changed as fibroblast-like morphology (e, f) (Bars = 20 μm). B. Staining with Alcian Blue. C. Immunofluorescence staining with an anti-Collagen II antibody.

day 7-8. Cells at this stage exhibited typical chondrocyte morphology, with a rounded or polygonal shape and slabstone-like cytoplasm. As the passages went on, the chondrocytes became dedifferentiation with the shape changing into fibroblast-like morphology (**Figure 1A**).

Alcian-Blue assay indicated that chondrocytes from P1 express proteoglycans (**Figure 1B**), and immunofluorescence staining showed that collagen II was highly expressed in this passage of chondrocytes (**Figure 1C**), both of which were considered as evidence of functional chondrocytes differentiation.

Dedifferentiation of passaged chondrocytes

Real-time PCR was used to quantitate the relative expression of collagen II, collagen I, and aggrecan at the mRNA level from both primary and passaged chondrocytes. Collagen II and aggrecan are mainly characteristic of the chondrocytes phenotype and is downregulated when chondrocytes begin to dedifferentiate. In contrast to that of collagen II, the level of collagen I is hardly detectable in P0, and increases up to nearly ten-fold in the P3 (**Figure 2A**). Furthermore, western blot was conducted to detect the protein level of collagen II, collagen I, and aggrecan, and the data was consistent with that of Real-time PCR (**Figure 2B**), which was accordance with the progression of dedifferentiation.

Adenoviral infection of chondrocytes

Chondrocytes were seeded into Costar 96-well plates at a final density of 5×10^3 cells/well and cultured for 12 hours until confluence reached 40-50%. Experiments were performed with the P1 cells. The chondrocytes were infected with recombinant adenovirus at the MOI of 0, 50, 100 for 12, 48, 72 h and 96 h, and MOI 50 was determined as the final concentration used in the following experiments due to the high effi-

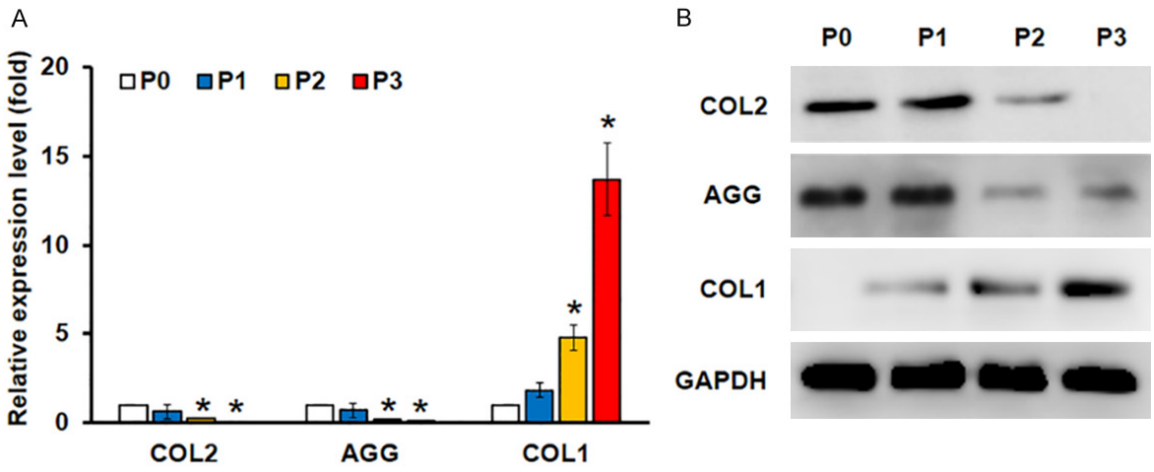

Figure 2. Dedifferentiation of passaged chondrocytes. A. Relative expression of collagen II (COL2), collagen I (COL1), and aggrecan (AGG) in RNA from both primary and passaged chondrocytes, as determined using real-time PCR. The mRNA level from P0 was used as the control, *, P<0.05 compared with control. B. Relative expression of COL2, COL1, and AGG in protein from both primary and passaged chondrocytes, as determined using western blot, with GAPDH as the control.

ciency of transfection and less toxic (**Figure 3A** and data not shown). As shown by fluorescence microscopy, 94% of chondrocytes were positively stained at MOI 50 after 48 h (**Figure 3A**). According to RT-PCR (**Figure 3B**), western blot (**Figure 3C**) analysis, we found that the expression of SREBP-2 was evidently reduced at both mRNA and protein level, indicating that Ad-siRNA had silencing effect in chondrocytes. In addition, we found that further improvement of knockdown effect was not observed at 48 h post infection (**Figure 3D**, **3E**), instead, the apoptotic or necrotic cell morphology appeared from 72 h, which also verified the stability of transfection.

Gene expression with SREBP-2 knockdown

In many chondrocytes diseases, the expression of collagen II, collagen I, and aggrecan was dysregulated. In passaged chondrocytes, when we knockdown the endogenous expression of SREBP-2 in chondrocytes, we found that the expression of collagen II and aggrecan were significantly upregulated for nearly four folds. On the other hand, the expression of collagen I was greatly decreased at both mRNA (**Figure 4A**) and protein level (**Figure 4B**). In addition, immunofluorescence assay was also used to detect the expression of key factor collagen II, we found the same change trend for the expression of collagen II, indicating that SREBP-2 could reverse the expression of collagen II (**Figure 4C**). Accordingly, the chondrocyte morphology was reversed from fibroblast-like morphology to that of typical chondrocyte (**Figure 4D**), which confirmed the function of SREBP-2 in chondrocyte differentiation.

Discussion

To obtain the most meaningful insights into human OA, it is essential to use skeletally adult animals whenever possible [8]. Thus isolation of chondrocyte from adult mice knee articular provides an easy and economical way to study this disease. However, how to obtain articular chondrocytes of large quantity and high density of mouse was still unsolved. Our results confirmed what Salvat [7] had described that as the passage goes the chondrocytes from mature mouse knee articular underwent dedifferentiation, as evidenced by the morphological as well as molecular marker changes.

With the development of methods of the genetic manipulation, a plethora of transgenic mice came under research, the most of which have the characteristics of osteoarthritis with the cartilage degeneration and formation of osteophyte. However, previous researches have alwasys focused on the disease with the method of immunohistology [9], even with the culture of chondrocyte from new-born mice to avoid the dedifferenation and hypotrophy of chondrocytes during passages, Gartland et al

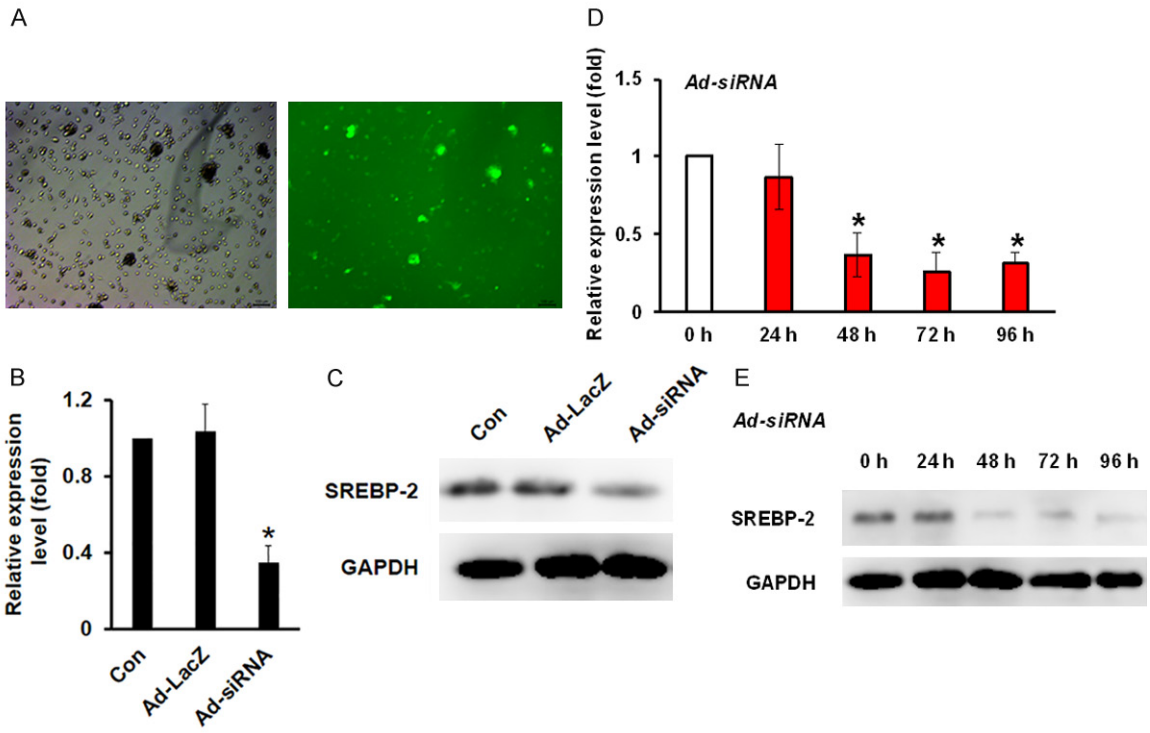

Figure 3. Adenoviral infection of chondrocytes. A. Efficiency of gene transduction was assessed from microscopic observation of the cells that stained green. B. Relative expression of SREBP-2 in RNA from chondrocytes infected with Ad-LacZ and Ad-siRNA, as determined using real-time PCR. The mRNA level from normal chondrocytes was used as the control, *, P<0.05 compared with control. C. Relative expression of SREBP-2 in protein from chondrocytes infected with Ad-LacZ and Ad-siRNA, as determined using western blot, with GAPDH as the control. D. Relative expression of SREBP-2 at the mRNA level of chondrocytes infected with Ad-siRNA at different time points, as determined using real-time PCR. *, P<0.05 compared with 0 h. E. Relative expression of SREBP-2 at the protein levle from chondrocytes infected with Ad-siRNA at different time points, with GAPDH as the internal control.

[10] found costochondral chondrocytes could be employed as a source of chondrocyte, but suggested that the source of cartilage could influence the differentiation of chondrocytes. Lamplot et al [11] found a new method to separate and culture reversibly immortalized mouse articular chondrocytes with immature CD1 chondrocytes which acquire long term proliferative capability while retaining the chondrogenic phenotype. Articular cartilage immortalized cell lines, C-28/I2 [12] have been described for many years, but also showed decreased ability of producing two characteristic molecules of ECM, collagen II and aggrecan during passages. Articular chondrocyte cell line such as ATDC5 [13] was derived from teratocarcinoma cells, which might probably limit its application in the study of degenerative disease concerning chondrocytes. Mouse models of OA, together with our method of dissecting and culture of adult mice chondrocytes, might overcome the defects in using chondrocytes from of new-born mice, as well as immortalized cells, which was supposed to better reflecting the nature of this troublesome pathophysilogical condition.

Adenoviral vectors mediated siRNA transfection are thought to be transiently expressed in cells and with high efficiency in certain types of cells, especially in postmitotic [14]. We next explored the feasibility of adenoviral vector-mediated gene silencing in adult mouse chondrocyte. Ad-siRNA on SREBP-2 was used for infecting chondrocytes. As shown by fluorescence microscopy, 94% of chondrocytes were positively transfected at MOI 50 after 48 h. These results indicated that adenovirus-mediated gene knock-down in adult mouse chondrocyte was highly efficient. The susceptibility of chondrocytes to adenoviral vector transduction has been broadly documented in vitro using reporter genes, with efficiencies reaching up to 100% [15]. Since cell selection usually requires

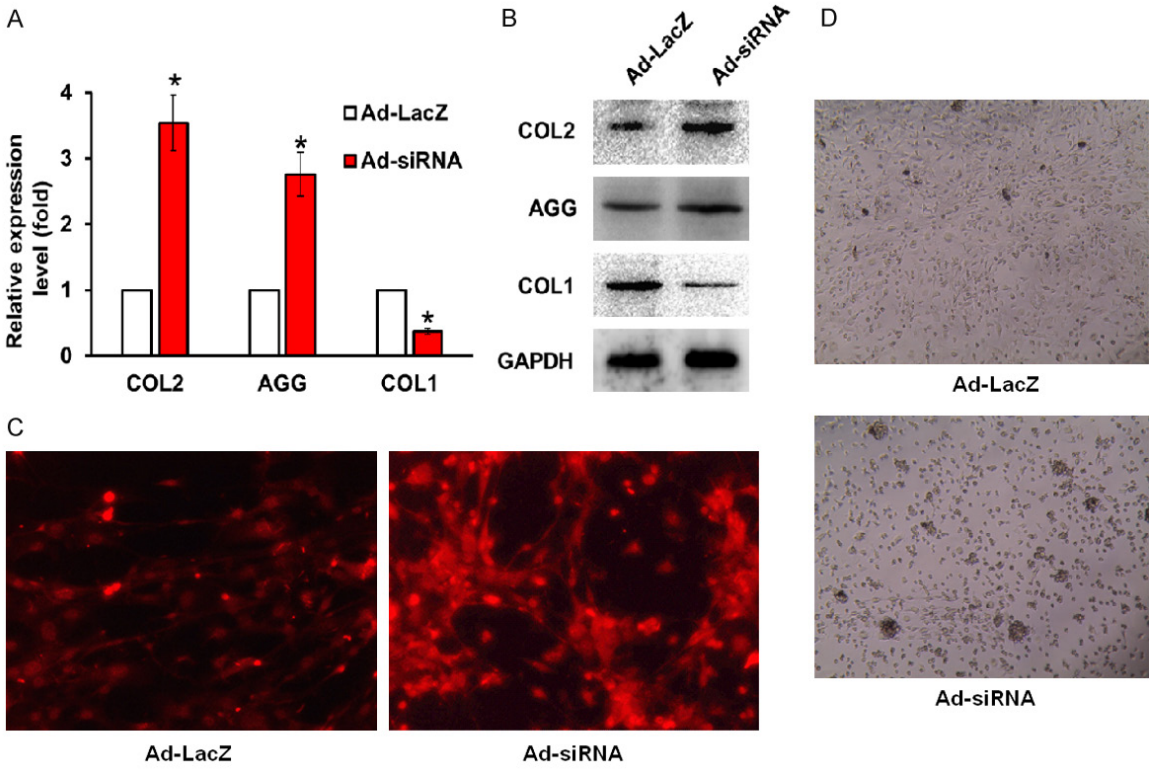

Figure 4. Gene expression with SREBP-2 knockdown. A. Relative expression of COL2, COL1, and AGG in RNA from chondrocytes infected with Ad-LacZ and Ad-siRNA, as determined using real-time PCR. The mRNA level from Ad-LacZ was used as the control, *, $P<0.05$ compared with control. B. Relative expression of COL2, COL1, and AGG in protein from chondrocytes infected with Ad-LacZ and Ad-siRNA, as determined using western blot, with GAPDH as the control. C. Expression of COL2 in chondrocytes infected with Ad-LacZ and Ad-siRNA, as determined using immunofluorescence staining. The protein level from Ad-LacZ was used as the control. D. Chondrocytes cells infected with Ad-LacZ or Ad-siRNA exhibit different morphology (Bars = 20 μm).

many passages of chondrocyte culture, we chose passages 1 as the cell for deep investigation to maintain the phenotype of chondrocytes.

Disadvantages of primary cells include the difficulty to obtain a sufficient number of cells, the diversity of cells within the culture as compared to other cell lines [16], and the fact that the cells are not easy to transfect, thereby limiting the possibility to alter the expression of target genes (via loss- or gain-of-function mutation) and elucidate their role in OA. When coming up to use chondrocytes for proteomic analysis, we had to take the stage of dedifferentiation into account as the fibroblast-like cell may interfere with actual in vivo physiological expression patterns.

Conclusions

In the present study, we have developed practical and reliable methods to isolate and culture chondrocytes from articular of adult mouse and to implement acute gene knock-down in these chondrocytes via adenoviral infection. The culture and adenoviral infection of adult mice articular chondrocyte described here should prove a powerful tool for further research for understanding the mechanism and developing therapeutic strategy for OA at the cellular or subcellular level.

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Disclosure of conflict of interest

None.

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