Original Research   *Highlight article*

RASL11B gene enhances hyaluronic acid-mediated chondrogenic differentiation in human amniotic mesenchymal stem cells via the activation of Sox9/ERK/smad signals

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Impact statement

RASL11B, a member of the small GTPase superfamily, has high similarity to RAS proteins, and involves some pathophysiological processes, such as inflammation, arteriosclerosis, and cancer. However, there is no available information regarding the role of *RASL11B* in chondrogenic differentiation. We show that *RASL11B* is activated in the process of HA-mediated chondrogenesis of hAMSCs, and *RASL11B* regulates the differentiation of hAMSCs into chondrocytes through the activation of Sox9 and ERK/Smad signals. The results of this study support that *RASL11B* may be used as a target in the chondroinductive differentiation of hAMSCs in the presence of HA and even in the cartilage defect repairing by HA-based stem cell therapy.

Abstract

This study aimed to elucidate the molecular mechanisms, whereby hyaluronic acid, a main extracellular matrix component of articular cartilage, promotes the chondrogenic differentiation of human amniotic mesenchymal stem cells (hAMSCs). Our previous findings indicated that hyaluronic acid combined with hAMSCs showed a marked therapeutic effect against rat osteoarthritis. In the present study, hyaluronic acid markedly enhanced the expression of chondrocyte-specific markers including *Col2α1, Acan*, and Sox9 in hAMSCs, with strong synergistic effects on chondrogenic differentiation, in combination with the commonly used inducer, transforming growth factor β3 (TGF-β3). Microarray analysis showed that Ras-like protein family member 11B (RASL11B) played a pivotal role in the process of hyaluronic acid-mediated chondrogenesis of hAMSCs. This directional differentiation was significantly inhibited by *RASL11B* knockdown, but *RASL11B* overexpression dramatically promoted the expression of Sox9, a master chondrogenesis transcriptional factor, at the levels of transcription and translation. Increased Sox9 expression subsequently resulted in high expression levels of *Col2α1* and *Acan* and the accumulation of cartilage-specific matrix components, such as type 2 collagen and glycosaminoglycans. Moreover, we observed that *RASL11B* activated the signal molecules such as ERK1/2, and Smad2/3 in the presence of hyaluronic acid during TGF-β3-induced chondrogenesis of hAMSCs. Taken together, these findings suggest that hyaluronic acid activates the *RASL11B* gene to potentiate the chondrogenic differentiation of hAMSCs via the activation of Sox9 and ERK/Smad signaling, thus providing a new strategy for cartilage defect repairing by hyaluronic acid-based stem cell therapy.

Keywords: RASL11B, hyaluronic acid, human amniotic mesenchymal stem cells, chondrogenic differentiation, Smad signaling, ERK signaling

Introduction

Articular cartilage, a type of hyaline cartilage, has no blood vessels or nerves. Thus, the self-repair or regeneration of articular cartilage damage is quite limited.[1,2] Articular cartilage damage most commonly occurs in the knee due to trauma, osteoporosis, or degenerative osteoarthritis. As the disease advances, the patient's activity is gradually limited and due to the loss of joint function leads to disability. At present, the most commonly recommended treatment for cartilage damage is pharmacotherapy with non-steroidal anti-inflammatory agents, which can delay the progression of disease, and improve the symptoms of patients, but they

do not reverse the pathological process and most of these drugs have obvious side effects. Therefore, an alternative therapeutic strategy is urgently required. Cartilage transplantation technique such as autologous chondrocyte implantation, and osteochondral allograft is a very promising therapeutic approach. However, some disadvantages including an insufficient supply of cells, adverse effects of donor site, and immunological rejection have deeply impacted the efficacy. Mesenchymal stem cells (MSCs) may offer an improved starting material for generating the large numbers of functional chondrocytes required.[3]

In the past few decades, as multifunctional cells, MSCs have become well known for their ability to maintain an undifferentiated status and be induced to differentiate into desired cell types for the purpose of cell replacement under the corresponding culture conditions.[4] Among these cells, human amniotic MSCs (hAMSCs) derived from placental tissue have significant advantages, including the absence of ethical issues, low immunogenicity, potent paracrine activity, and non-tumorigenic properties. Thus, hAMSCs are recognized as an ideal seed cell source with broad clinical application prospects for regenerative medicine,[5,6] especially for cartilage damage.[7,8] However, the optimal technique to directly and efficiently regulate their differentiation into chondrocyte requires further investigation. Numerous previous studies have shown that some growth factors including insulin-like growth factor (IGF), fibroblast growth factors (FGF), transforming growth factor β (TGF-β), and bone morphogenetic proteins (BMP) have the potential to induce cartilage formation.[9–11] These factors can regulate the expression of chondrogenic-associated genes by activating intracellular protein kinase A (PKA), TGF-β, protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and other signals, thus inducing the differentiation of MSCs into chondrocytes.[12] Nevertheless, these induction approaches are not sufficient to enable MSCs to efficiently generate functional chondrocytes.

The extracellular matrix plays a vital role in maintaining the function of chondrocytes and signal transmission between chondrocytes and the organism.[13–15] Hyaluronic acid (HA) is one of the main extracellular matrix components,[16] and it is recognized as a functional ligand influencing cell migration, proliferation, and differentiation.[17,18] Furthermore, HA is commonly used as a scaffold material in tissue engineering of cartilage, where it plays a key role in cartilage regeneration and in attenuating cartilage degeneration in early osteoarthritis.[19,20] In our previous study, a cocktail of HA and hAMSCs transplantation showed a significant therapeutic effect against rat knee osteoarthritis.[21] Also, HA displayed a similar function of routine chondroindutive chemicals in hAMSCs and a synergistic effect on the chondrogenic differentiation of hAMSCs in combination with chondroinductive chemicals.[21] However, how does HA modulate the chondrogenic differentiation of hAMSCs, and its underlying molecular mechanism remains to be elucidated.

The sex-determining region Y-box (Sox9), a transcription factor with a high mobility group DNA-binding domain, is required for chondrocyte differentiation of MSCs and cartilage formation, in which Sox9 binds to chondrocyte-specific enhancer elements in the collagen type 2 gene (*Col2α1*) and activates *Col2α1* expression after chondrogenic mesenchymal condensations.[22] Thereby, Sox 9 is expressed in all prechondrocytic and chondrocytic cells, while its expression is dramatically downregulated in hypertrophic chondrocytes. Furthermore, the cartilage formation is a multi-step cell differentiation process that is modulated by a complex signaling network. Here members of the TGF-β superfamily play an indispensable role in almost every aspect of cartilage formation and maintenance, including coagulation, proliferation, terminal differentiation, and the maintenance of chondrocytes.[23–25] Many previous studies showed that TGF-β stimulates chondrogenesis through the downstream small mothers against decapentaplegic (Smad) signaling.[26,27] Smad3 is involved in enhanced transcriptional activity of Sox9 in human MSCs.[27] In addition, an increasing evidence suggests that the MAPK signaling pathway plays an essential role in MSCs-differentiated intracellular signaling, in which extracellular signal-regulated kinase (ERK) mainly participate in chondrogenesis.[28,29] ERK showed either inhibitory or promotive effects on chondrocytes differentiation.[30] Furthermore, our recent study has shown that Smad and ERK signals co-regulate the expression of type II collagen and aggrecan in CD44-induced chondrogenic differentiation.[31] In addition, the ERK signal can regulate the Sox9 expression in MSCs and chondrocytes.[30] Therefore, Sox9/ERK/Smad signals may involve chondroinductive differentiation in hAMSCs by the combination of HA and TGF-β3.

In the present study, the gene targeted by HA to promote the chondrogenic differentiation of hAMSCs was identified by microarray analysis and the downstream signaling pathway was also investigated for the first time.

Materials and methods

Reagents

HA (300 kD) was obtained from the Seebio Biotech Inc. (Shanghai, China). Dulbecco's modified Eagle medium-low/high glucose (LG/HG-DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAAs), GlutaMAX, trypsin, and β-mercaptoethanol were purchased from GIBCO Industries Inc. (Los Angeles, CA, USA). Dexamethasone (Dex), β-glycerol phosphate disodium (β-Gly), *L*-ascorbic acid (Asc), and collagenase II, DNase I were all purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (D-PBS), penicillin, and streptomycin were purchased from Sino-American Biotech Inc. (Shanghai, China).

The isolation and culture of hAMSCs

After gaining informed consent, we conducted 15 term placentas obtained from healthy individuals undergoing caesarean delivery in accordance with the guidelines of the Ethical Committee of Affiliated Hospital of Zunyi Medical University (Zunyi, China). In brief, the amniotic membrane was stripped from the placental tissue using medical forceps, repeatedly washed with D-PBS containing

100 U/mL of penicillin and 100 μg/mL of streptomycin to remove residual blood, and the amniotic tissue was shredded. To the amniotic membrane pieces, the mixture (0.05% trypsin containing 0.02% EDTA-2Na) was added twice to its volume. Samples were shaken and digested at 190 r/min for 45 min at 37°C in a shaking incubator, and then were filtered using a 300-mesh sieve. Subsequently, the supernatant was discarded and the above procedure mentioned was repeated once for the digestion of amniotic membrane pieces. Next, the above amniotic membrane pieces were then washed once with D-PBS containing antibiotics. An equal volume to amniotic membrane pieces of type II collagenase solution (0.5 mg/mL) containing 0.05 mg/mL DNase I was added and the mixture was digested at 37°C, 190 r/min for 1 h in a shaking incubator. Finally, after the amniotic membrane pieces were completely digested until flocculent, samples were filtered through a 300-mesh sieve. The filtrates were collected and centrifuged at 1500 r/min for 11 min to obtain hAMSCs. The harvested cells were plated in 25-cm^2 flasks at a density of 2×10^5 cells/flask in the LG-DMEM supplemented with 10% (v/v) FBS, 1% (v/v) NEAA, 1% (v/v) L-GlutaMAX, and 10 ng/mL human bFGF. The cells were then maintained at 37°C in a humid atmosphere consisting of 5% CO_2 and 95% air. When the degree of cell confluence reaches 80–90%, subculture is carried out after digestion. Cells at passage 2 (P2) were used for further experiments.

Phenotypic analysis of hAMSCs by flow cytometry

hAMSC-specific surface markers were identified by flow cytometry. For the flow cytometry analysis, all primary antibodies, including PE-conjugated anti human monoclonal CD29, CD11b, CD34, CD44, CD45, and HLA-DR antibodies; FITC-conjugated anti human CD90 and CD45 antibodies; APC-conjugated anti human CD 73 antibody; Cy5.5-conjugated anti human CD 105 antibody; or their corresponding isotype controls were purchased from BD Pharmingen (San Diego, CA, USA). In brief, the third passage of cells in the logarithmic phase was collected and suspended at a density of 2×10^6 cells/mL in D-PBS containing 0.1% (m/v) bovine serum albumin (BSA). After adding the appropriate antibody, approximately 4×10^5 cells were co-incubated for 25 min. The labeled cells were then washed with D-PBS and analyzed on a BD FACSCalibur system using the CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Screen for target genes involved in hyaluronic acid-induced chondrogenic differentiation of hAMSCs

The microarray technique was employed to screen possible target genes. Briefly, total RNA was extracted from the following groups tested, including a negative control group (NC), a positive drug TGF-β3 control group (PG), and a combined group with PG+HA (CG). Referring to the RNAiso Plus extraction kit (Takara Bio, Dalian, China) instructions, RNA was extracted from hAMSCs at day 21 after induction. Subsequently, A NanoDrop ND-1000 instrument (Thermo Fisher Scientific, Waltham, MA, USA) was employed to assess the quantity and quality of RNA. The collection and analysis of microarray data were performed by Shanghai Kangcheng Bioengineering Co., Ltd (Shanghai, China). Microarray data were analyzed to identify differentially expressed mRNAs and were screened for genes involved in the chondrogenic differentiation of hAMSCs using an Arraystar Human array. A comparative analysis of whole genome expression profiles was performed for the NC, PG, and CG groups (Figures S1 and 2).

Analysis of the effects of RASL11B overexpression and knockdown

Recombinant adenovirus expression vectors, including HBAD-GFP, HBAD-h-RASL11B-1–3*flag-GFP, HBAD-h-RASL11B-shRNA1-GFP, HBAD-RA SL11B-shRNA2-GFP, and HBAD-RASL11B-shRNA3-GFP were constructed by Shanghai Hanheng Biotechnology Co., Ltd (Shanghai, China). The map of the selected interference vector, pHBAd-U6-MCS-CMV-GFP, is shown in Figure S4.

To determine the optimal multiplicity of infection (MOI), P2 hAMSCs were seeded at a density of 2×10^5 per well in 6-well plates (Corning, NY, USA) in basal medium. Different concentrations of adenovirus were then added in the logarithmic growth phase, at MOI values of 10, 30, 50, 100, and 200 and the medium was replaced with the fresh medium after 6 h of incubation. Finally, green fluorescent protein expression was measured after 72 h. The optimal MOI was chosen based on the fluorescence reaching 80% and a minimum amount of virus required (Figure S5).

To analyze the effects of overexpression and knockdown of the *RASL11B* gene, P2 hAMSCs were seeded at a density of 2×10^5 per well in 6-well plates and were then transfected with the appropriate vector in the logarithmic growth phase. A normal control group (NC, no virus solution) and a null-vector negative control group (vector-NC; transfected with no-load virus, HBAD-GFP) were included along with an RASL11B-overexpression group (h-RASL11B, transfected with HBAD-h-RASL11B -1–3*flag-GFP) and an RASL11B-knockdown group (sh-RASL11B, transfected with HBAD-RASL11B shRNA3-GFP). After 4–8 h, virus-containing media were changed to normal media and total RNA was extracted on days 7, 14, and 21 after induction. RT-qPCR was then used to analyze the overexpression and knockdown of the *RASL11B* gene.

Directional differentiation of hAMSCs into chondrocytes

For chondrocyte differentiation, P2 hAMSCs were plated at a cell density of 2×10^5 per well in 6-well plates and incubated at 37°C until reaching 80% confluence, the medium was then replaced with the fresh medium, with or without the adenovirus vector, for 4–8 h. Chondrogenic differentiation experiments not involving adenoviral vectors included a negative control group (NC), an HA treatment group (HG), a positive drug control group (PG), and a PG + HG combined group (CG). For the NC group, hAMSCs were grown only in the basal media that consisted of HG-DMEM supplemented with 10% (v/v) FBS, 1% (v/v) L-GlutaMAX, and 1% (v/v) NEAA. The HG group was added with

0.05 mg/mL HA, the PG group was added with a commonly used inducer, TGF-β3, at 10 ng/L, and the CG group was added with 0.05 mg/mL HA and 10 ng/L TGF-β3. Chondrogenic differentiation experiments involving adenovirus vectors included a null-vector negative control group (vector), an RASL11B-overexpression group (h-RASL11B), an RASL11B-knockdown group (sh-RASL11B). Upon successful transfection, each of the above groups included PG and CG samples. For long-term chondrogenic differentiation, the above active ingredients were added to each group when the media was changed, which occurred every three days.

Toluidine blue staining

After culturing in different chondrogenic induction media for 21 days, hAMSCs were washed with PBS for three times. They were then fixed at 24–28°C for 30 min with 4% paraformaldehyde. After washing with PBS for three times, cells were stained with 1% toluidine blue in the dark for 30 min at 24–28°C. Finally, the cells were washed three times with PBS again, dried, and observed under a microscope.

Immunohistochemical staining

P3 hAMSCs or chondrogenically differentiated cells were fixed at 24–28°C for 30 min with 4% paraformaldehyde and then washed three times with PBS. For immunostaining, the cells were permeabilized at 24–28°C for 15 min with 0.3% (v/v) Triton X-100 and then washed with PBS. Subsequently, they were blocked at 24–28°C for 1 h with 5% BSA containing 0.1% Triton X-100, and were then incubated with the following primary antibodies, anti-CK19 (Sigma, St Louis, MO, USA), anti-vimentin (Sigma), or anti- type II collagen (ab34712; Abcam, Cambridge, UK) overnight at 4°C. After washing off unbound primary antibody with PBS, the cells were re-incubated at 37°C for 30 min, and were then treated in the dark for 1 h at 37°C with horseradish peroxidase (HRP)-conjugated secondary antibodies. Finally, the cells were washed with PBS again, and were then counterstained with hematoxylin to visualize the nuclei. The images were taken under a microscope.

Quantitative reverse transcription polymerase chain reaction assay

Following the manufacturer's protocols, total RNA was extracted in hAMSCs at days 7, 14, and 21 after induction using an RNAiso Plus Kit and cDNA was synthesized using a PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). qRT-PCR was performed using a standard SYBR Green PCR Kit (Qiagen, Hilden, Germany) and was processed on a CFX96 Touch real-time PCR detection system with CFX Manager Software (Bio-Rad, Hercules, CA, USA). List of the primers for all genes tested in the study is shown in Table 1. The comparative cycle threshold (Ct) method was used to calculate the relative expression level of the genes tested. β-actin was used as an internal control for normalization.

Western blotting analysis

The protein expression of Sox9, Col2α1, and Acan on the 7th, 14th and 21th day of differentiation, and ERK1/2, p-ERK1/2, Smad2/3, p-Smad2/3 on the 7th day of differentiation were analyzed by Western blotting assay. Total protein was extracted from cultured hAMSCs using a total protein extraction kit (Applygen, Beijing, China). Protein concentration was quantified using a BCA kit, according to the manufacturer's recommendations. After denaturing, the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transfer, membranes were incubated overnight at 4°C with the following primary antibodies including mouse anti-human Sox9, mouse anti-human Col2α1, mouse anti-human Acan, mouse anti-human β-actin, mouse anti-human ERK1/2, rabbit anti-human p-ERK1/2, mouse anti-human smad2/3, and mouse anti-human p-smad2/3. After washing for three times with tris-buffered saline/Tween 20 (TBST), HRP-labeled rabbit anti-mouse IgG or goat anti-rabbit IgG was added to the membranes and they were incubated at 24–28°C for 1 h. The membranes were then washed with TBST again and visualized using an enhanced chemiluminescence (ECL) luminescent solution (Beyotime, Shanghai, China). Finally, the membranes were exposed in the Bio-Rad ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA) and the resulting images were preserved. ImageJ 1.46a software (NIH, Bethesda, MD, USA) was employed to analyze the protein bands. β-Actin was used as an internal reference in the study.

Statistics

All experimental data are presented as mean ± SD and were obtained from at least three individual experiments. The experimental data were analyzed statistically by SPSS v. 13.0 software (IBM, Armonk, NY, USA) and significance of difference between two groups was determined by one-way ANOVA and/or Student's t test. A value of $P < 0.05$ was considered statistically significant.

Results

Characterization of hAMSCs

To ensure the quality of cultured cells, we identified their phenotypic characteristics. As shown in Figure 1(a), hAMSCs were stained strongly for the mesenchymal cell marker vimentin, but were negative for the epithelial cell marker cytokeratin 19 (CK19), by immunohistochemical staining. Furthermore, flow cytometric analysis showed that hAMSCs highly expressed CD90 (91.30% ± 5.31%), CD73 (91.22% ± 5.19%), CD105 (96.88% ± 3.28%), and CD44 (98.12% ± 3.49%), but did not express CD34, CD45, CD11b, CD19 and the MHC class II antigen molecule, HLA-DR. In addition, these hAMSCs can differentiate into osteoblasts, adipocytes, and chondrocytes as described in our previous report.[5] Therefore, these results suggested that the isolated cells of mesenchymal origin met the definition and minimum criteria of mesenchymal stromal cells, as

Table 1. Primer sequences of target genes.

Gene	Sequence (5′→3′)	GenBank ID	Length of product (bp)
RASL11B	For: AAGCGAAGAACCTCCCTCATT	NM_023940.2	77
	Rev: AGGGCTTGCTTAAACCTCCTC		
Sox9	For: GCGGAGGAAGTCGGTGAAGA	NM_000346.3	82
	Rev: GAAGATGGCGTTGGGGGAGA		
Col2α1	For: CAACACTGCCAACGTCCAGAT	NM_001844.4	121
	Rev: TCTTGCAGTGGGCTGCCTTAT	NM_033150.2	
Acan	For: GTGCCTATCAGGACAAGGTCT	NM_001135.3	167
	Rev: GATGCCTTTCACCACGACTTC	NM_013227.3	
ERK1	For: CTACACGCAGTTGCAGTACAT	NM_002746.2	157
	Rev: CAGCAGGATCTGGATCTCCC		
ERK2	For: TCTGGAGCAGTATTACGACCC	NM_138957.3	134
	Rev: CTGGCTGGAATCTAGCAGTCT		
Smad1	For: CAGCCTCTTAGCTCAGTTCCG	NM_005900.2	144
	Rev: CTCCCAGGAGAGTTTGGGTAA		
Smad2	For: CCGACACACCGAGATCCTAAC	NM_005901.5	125
	Rev: GAGGTGGCGTTTCTGGAATATAA		
Smad3	For: TGGACGCAGGTTCTCCAAAC	NM_001145102.1	90
	Rev: CCGGCTCGCAGTAGGTAAC		
Smad5	For: TCTCCAAACAGCCCTTATCCC	NM_001001420.2	113
	Rev: GCAGGAGGAGGCGTATCAG		
Smad8	For: ATGTGATTTACTGTCGCGTGT	NM_005905.5	132
	Rev: GGCGGTAGTGGTAAGGGTTAAT		
β-actin	For: TGGCACCCAGCACAATGAA	NM_001101.3	186
	Rev: CTAAGTCATAGTCCGCCTAGAAG		
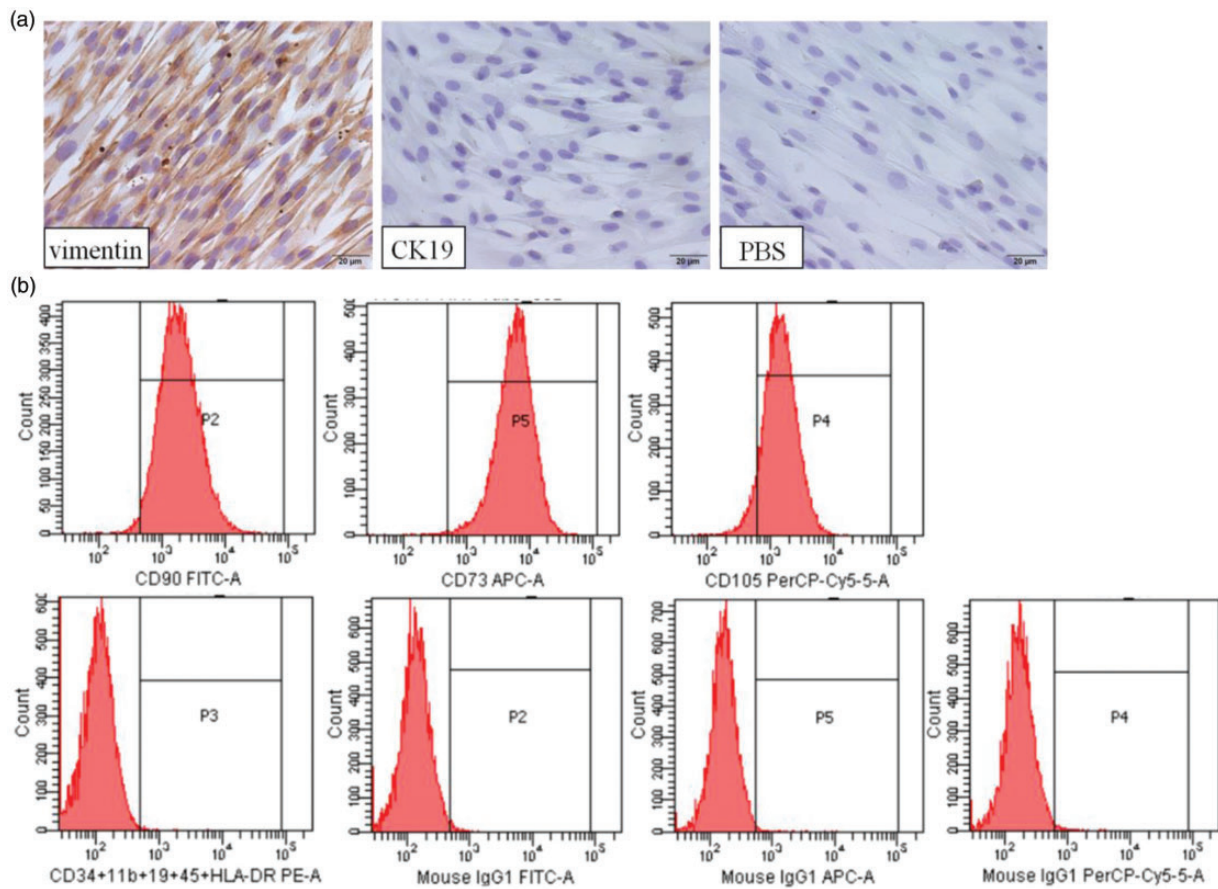
Figure 1. Characterization of hAMSCs. (a) The expression of vimentin and cytokeratin (CK19) was measured in hAMSCs. Brown staining denotes the positive expression for vimentin and/or CK19. PBS: negative control. The nuclei were counterstained with DAPI (blue). Scale bar = 100 μm. (b) Flow cytometry analysis of hAMSCs, including samples and homotypic controls. (A color version of this figure is available in the online journal.)

proposed by the International Society for Cellular Therapy.[31]

HA promoted chondrogenic differentiation of hAMSCs

Differentiated chondrocytes can secrete structural extracellular matrix components, including type II collagen and glycosaminoglycan, to form cartilage tissue. hAMSCs were cultured under different chondroinductive conditions for 21 days and the deposition of cartilage-specific matrix components, such as glycosaminoglycan and type II collagen was observed to determine the effect of HA on the chondrogenic differentiation of hAMSCs. Glycosaminoglycan synthesis in the extracellular matrix was examined by toluidine blue staining and type II collagen synthesis was analyzed by immunohistochemical staining. As shown in Figure 2(a) and (b), HA alone had no significant effect on chondrogenic differentiation at a dose of 0.05 mg/mL, but when combined with TGF-β3, could significantly increase the accumulation of the cartilage-specific matrix components, glycosaminoglycan, and type II collagen, compared with PG cells. Similar to the TGF-β3, further study showed that HA could give rise to a dramatic upregulation of *Sox 9*, a master chondrogenesis transcription factor, on day 14 after induction of chondrogenic differentiation, as well as

chondrocyte-specific gene *Col2α1* on day 7 at transcriptional level (Figure 2(c)). Furthermore, CG group exerted a very strong synergistic effect on up-regulation of chondrogenic genes, such as *Col2α1* and *Acan*, on day 14 as shown in Figure 2(c).

RASL11B regulated the chondrogenic differentiation of hAMSCs during synergistic induction with HA and TGF-β3

To clarify the underlying mechanism of HA synergism with TGF-β3 in the promotion of the chondrogenesis of hAMSCs, we screened for genes involved in the chondrogenic differentiation of hAMSCs using an Arraystar Human mRNA array. A comparative analysis of whole genome expression profiles was performed between groups. A total of 113 differentially expressed mRNAs were identified by whole-genome expression profiling, of which 8 showed large differences, and thus were possibly related to chondrogenic differentiation (Tables S1 and 2). Here these genes were screened using the NCBI Gene database. However, the validation of eight candidate genes by qRT-PCR did not show complete consensus with the results of whole-genome microarray, only both *RASL11B* and *DLX5* indicated a slightly better consistent trend (Figure S2). Further study presented that overexpression of *DLX5*
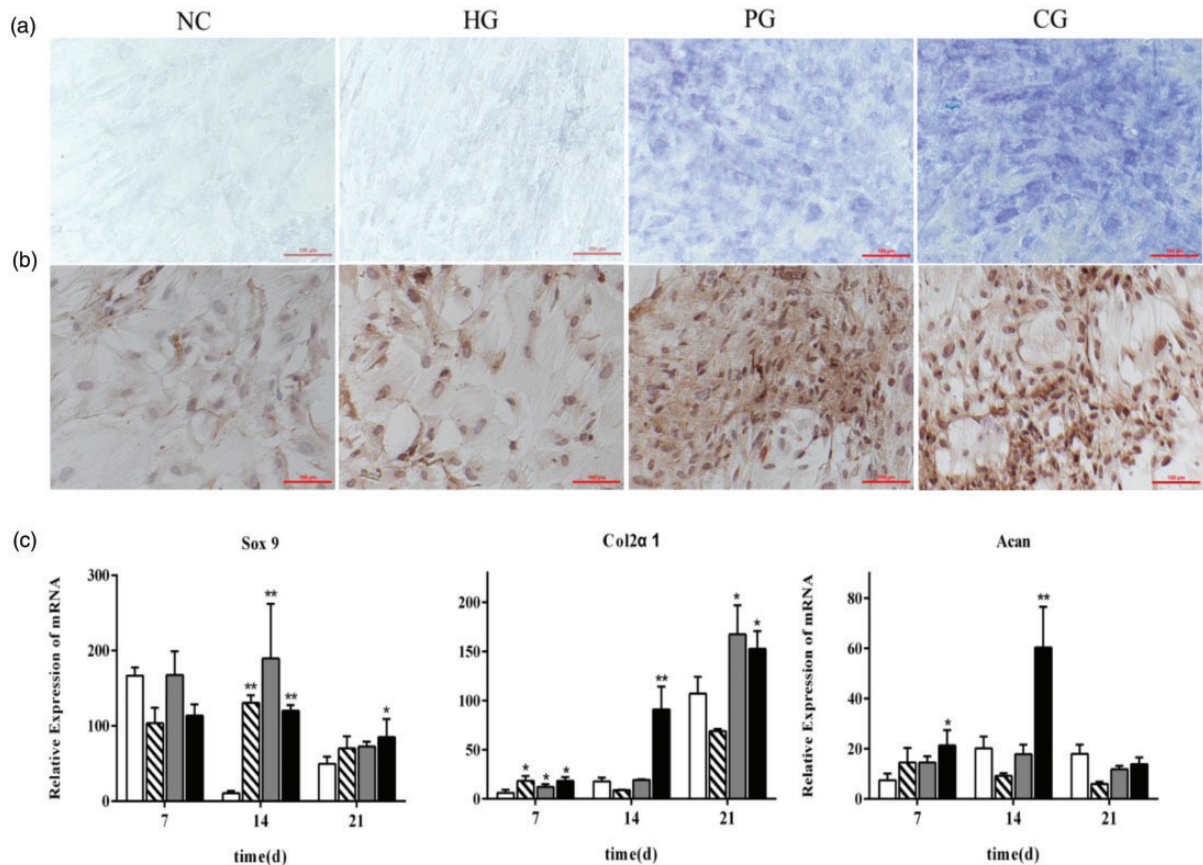
Figure 2. Effect of hyaluronic acid on the chondrogenic differentiation of hAMSCs. (a) Glycosaminoglycan detected by toluidine blue staining. (b) Type II collagen assessed by immunohistochemical staining. (c) Expression of chondrocyte-specific genes, such as Sox9, Col2α1, and Acan was detected by qRT-PCR on days 7, 14, and 21 after the induction of chondrogenic differentiation. NG: negative control group (white bar); HG: hyaluronic acid treatment group (hatched bar); PG: positive drug control group (gray bar); CG: PG + HG combined group (dark bar). *P < 0.05, **P < 0.01, vs. NC. (A color version of this figure is available in the online journal.)

did not give rise to a marked change of cartilage-specific markers, including glycosaminoglycan, and collagen type II in hAMSCs (Figure S3). By contrast, overexpression or knockdown of *RASL11B* could significantly alter the expression of chondrogenic markers as shown in Figure 3 (c) and (d). Therefore, *RASL11B* was possibly associated with HA-mediated chondrogenic differentiation. Based on this finding, this study mainly focused on the effects and mechanisms of the target gene, *RASL11B*, on the chondrogenic differentiation of hAMSCs.

The adenovirus vectors, HBAD-h-RASL11B-1–3*flag-GFP and HBAD-RASL11B shRNA3-GFP, were transfected into hAMSCs to enhance or inhibit RASL11B expression, respectively. Before performing the chondrogenic differentiation experiments, we determined that the optimal multiplicity of infection (MOI) was 100, when the fluorescence intensity was 80% and virus usage was at its lowest (Figure S5). Upon successful transfection, *RASL11B* mRNA was measured in hAMSCs at days 7, 14, and 21 after induction. As shown in Figure 3(a) and (b), the *RASL11B* mRNA expression was significantly up-regulated in HBAD-h-RASL11B-1–3*flag-GFP-transfected hAMSCs and knocked down in HBAD-RASL11B shRNA3-GFP-transfected hAMSCs. Moreover, these effects persisted for more than

21 days. The hAMSCs transfected with adenoviruses at different experimental groups were then induced to undergo chondrocyte differentiation, as shown in Figure 3(c) and (d). Knockdown of the *RASL11B* gene in the CG group (sh-RASL11B-CG) resulted in a significant decrease in the secretion of glycosaminoglycan and the expression of type II collagen, compared with the vector-CG group on day 21 after the induction of chondrogenic differentiation. Meanwhile, overexpression of the *RASL11B* gene in the CG group (h-RASL11B-CG group) led to a significant increase in the accumulation of cartilage-specific extracellular matrix components in hAMSCs, including glycosaminoglycan and collagen type II.

Effect of RASL11B on the expression of chondrocyte-specific genes and proteins in hAMSCs during the synergistic induction of differentiation by HA and TGF-β3

After a preliminary confirmation of the modulatory effect of *RASL11B* on chondrogenic differentiation, we further investigated the effect of *RASL11B* on chondrogenic differentiation-related genes and proteins on days 7, 14, and 21 after induction with HA. During the chondroinductive differentiation of hAMSCs, the expression levels of all
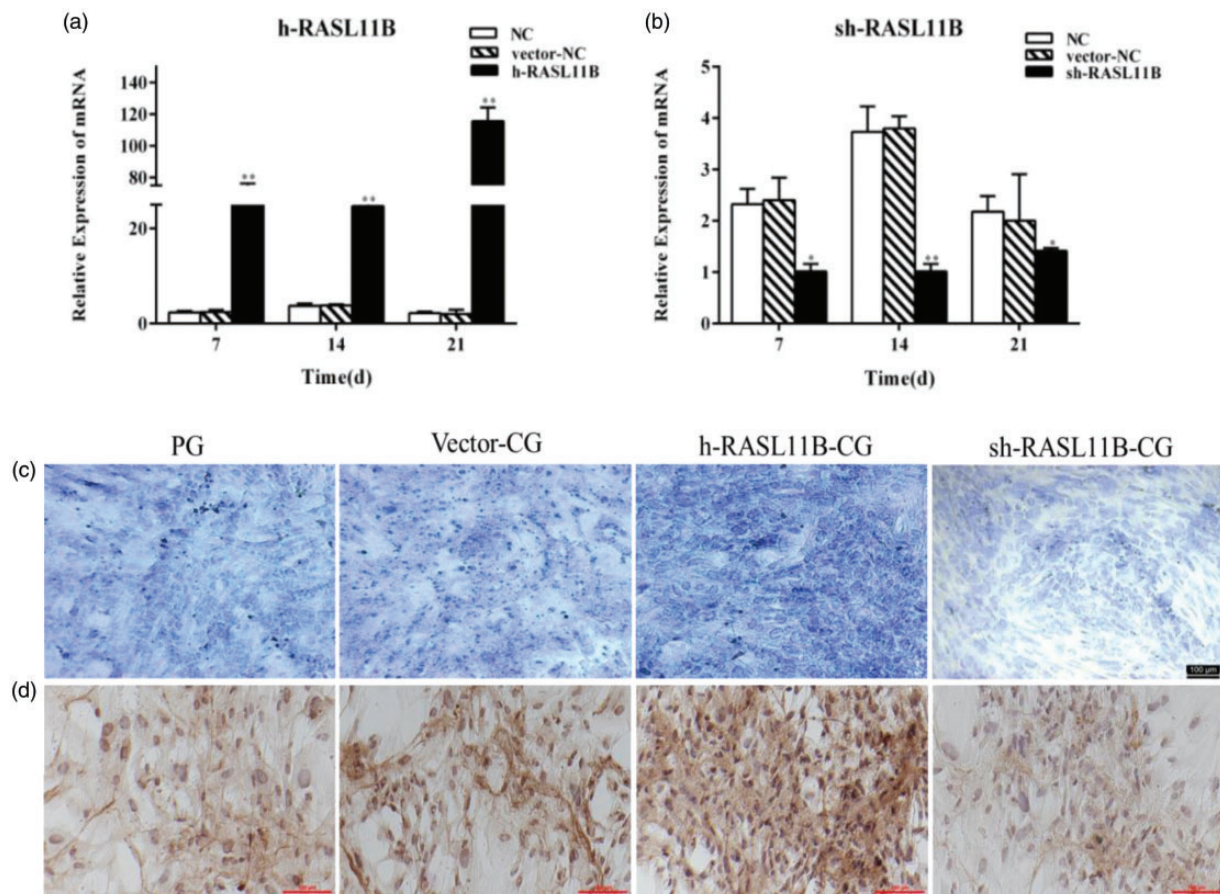
Figure 3. Effect of RASL11B on the chondrogenic differentiation of hAMSCs on day 21 after induction. (a and b) Analysis of the effects of *RASL11B* gene overexpression and knockdown. (c) Glycosaminoglycan detected by toluidine blue staining. (d) Type II collagen detected by immunohistochemical staining. NC: normal control group; vector-NC: null-vector negative control group; h-RASL11B: overexpression of *RASL11B* group; sh-RASL11B: knockdown of *RASL11B* group; PG: positive drug control group; vector-CG: null-vector CG group; h-RASL11B-CG: CG group with overexpression of *RASL11B*; sh-RASL11B-CG: CG group with knockdown of *RASL11B*. *$P < 0.05$, **$P < 0.01$, vs. NC. (A color version of this figure is available in the online journal.)
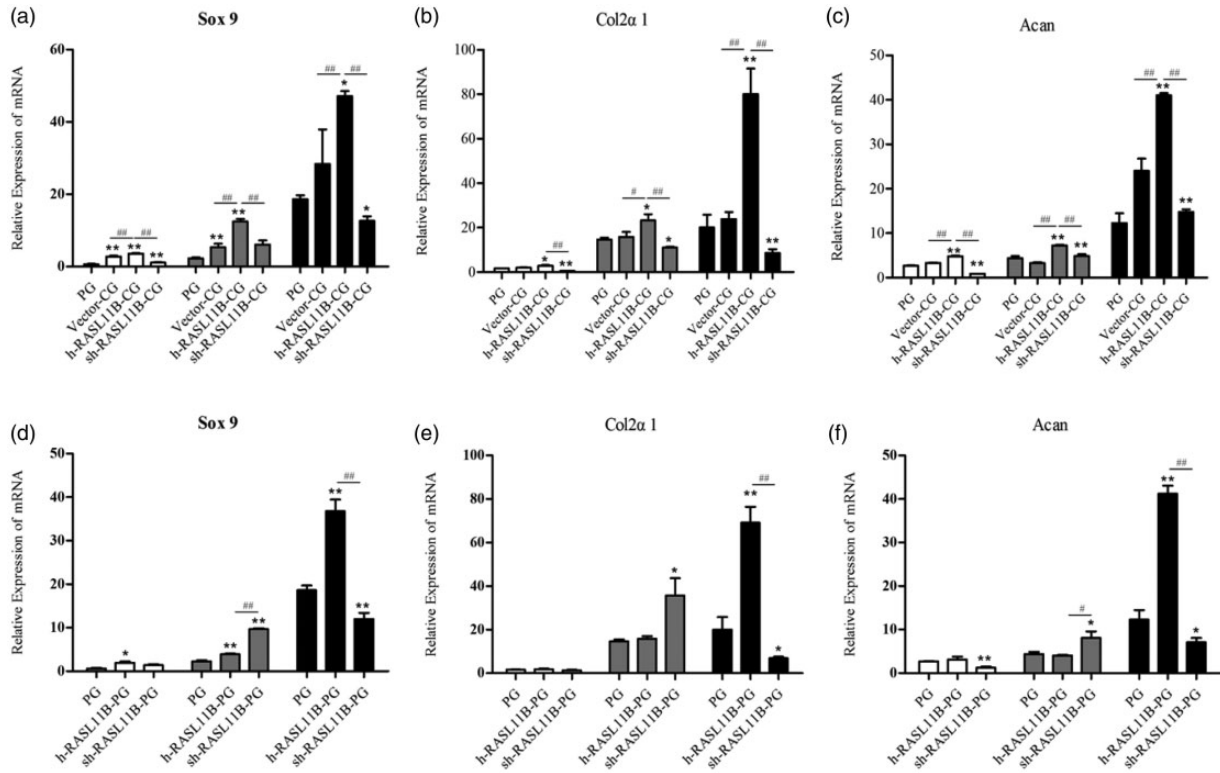
Figure 4. Effect of *RASL11B* on the transcriptional level of chondrogenesis-related genes in hAMSCs. Gene expression was evaluated by qRT-PCR on days 7 (white bar), 14 (gray bar), and 21 (dark bar) after the induction of chondrogenic differentiation. (a and d) Relative mRNA expression level of *Sox9*; (b and e) relative mRNA expression level of *Col2α1*; (c and f) relative mRNA expression level of *Acan*. PG: positive drug control group; vector-CG: null-vector CG group; h-RASL11B-CG: CG group with overexpression of *RASL11B*; sh-RASL11B-CG: CG group with knockdown of *RASL11B*; h-RASL11B-PG: PG group with overexpression of *RASL11B*; sh-RASL11B-PG: PG group with knockdown of *RASL11B*. Data are presented as mean $\pm$ SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, vs. PG; #$P < 0.05$, ##$P < 0.01$, vs. vector-CG or sh-RASL11B-CG.

three of the chondrogenesis-related genes, *Sox9*, *Col2α1*, and *Acan* were increased in a time-dependent manner in each different CG group, as shown in Figure 4(a) to (c). Furthermore, overexpression of RASL11B in the CG group (h-RASL11B-CG group) remarkably up-regulated all chondrogenesis-related genes tested, i.e. *Sox9* (Figure 4 (a))*, Col2α1* (Figure 4(b)), and *Acan* (Figure 4(c)), in a time-dependent manner, when compared with the PG and vector-CG groups ($P < 0.05$ or $P < 0.01$). The relative expression of these genes reached their highest levels on day 21. In comparison with the PG and vector-CG groups, knock-down of *RASL11B* in the CG group (sh-RASL11B-CG group) led to a significant decrease in the expression levels of all genes tested (Figure 4(a) to (c); $P < 0.05$ or $P < 0.01$), in which only *Sox9* expression on day 14 had no difference compared with the vector-CG group (Figure 4 (a)). In addition, a significant change for all genes tested at the transcriptional level was shown between the h-RASL11B-CG group and the sh-RASL11B-CG group (Figure 4(a) to (c), $P < 0.01$).

Similar to the CG group, the PG also gave rise to a time-dependent up-regulation for the expression level of three chondrogenic-related genes (Figure 4(d) to (f)). In contrast to the aforementioned CG groups, however, overexpression or knockdown of RASL11B did not result in a time-dependent expression in the PG group. For example, the transcriptional levels of *Col2α1* and *Acan* on day 14 were

higher than those on day 21 in the sh-RASL11B-PG group (Figure 4(e) and (f)). Interestingly, overexpression of RASL11B did not result in a significant increase in the transcriptional levels of *Col2α1* or *Acan* on days 7 or 14, compared with the PG group. By contrast, *RASL11B* knockdown significantly increased the relative expression levels of all three genes, *Sox9*, *Col2α1*, and *Acan*, on day 14, when compared with both other groups (Figure 4(d) to (f)). However, the changes in expression observed for all three genes on day 21 were similar to those in the corresponding CG groups.

At the translational level, all three chondrogenesis-related genes, *Sox9*, *Col2α1*, and *Acan* showed similar responses to their transcriptional level responses for all CG groups, in that overexpression of *RASL11B* in the CG group (h-RASL11B-CG group) resulted in a significant increase in the levels of Sox9 (Figure 5(b)), Col2α1 (Figure 5(c)), and Acan (Figure 5(d)) proteins in a time-dependent manner on day 7, 14, and 21 after synergistic induction with HA, compared with the PG and vector-CG groups ($P < 0.05$ or $P < 0.01$). Moreover, the relative expression of these proteins reached their highest levels on day 21. Meanwhile, knockdown of *RASL11B* resulted in a significant decrease in Sox9 (Figure 5(b)) and Acan (Figure 5(d)) protein levels in comparison with the PG group during chondrogenic differentiation of hAMSCs, with the exception of Acan levels on day 7. However, *RASL11B*
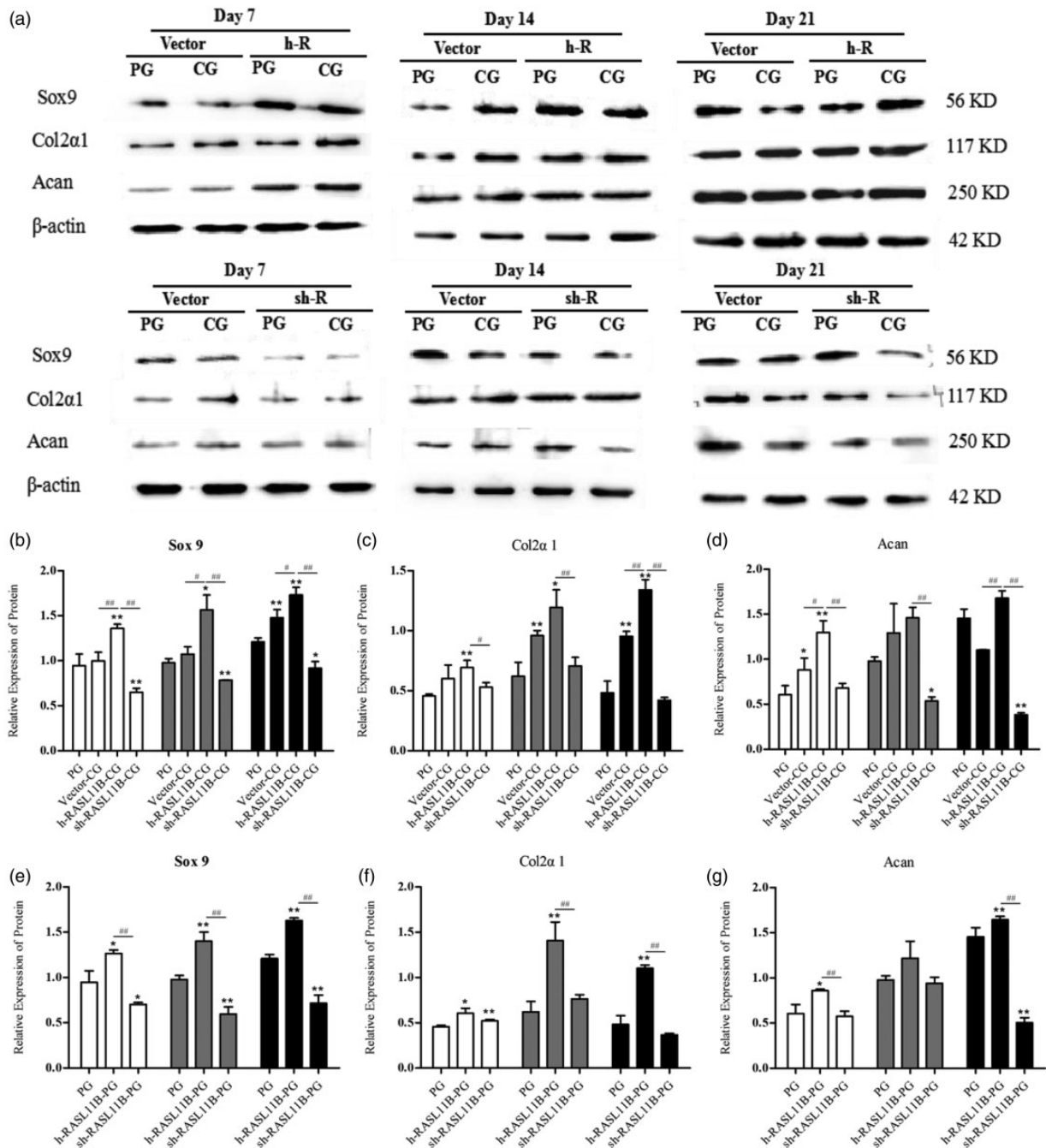
Figure 5. Effect of *RASL11B* on the protein levels of chondrogenesis-related factors in hAMSCs. These factors were detected by Western blotting on days 7 (white bar), 14 (gray bar), and 21 (dark bar) after induction of chondrogenic differentiation. (a) Western blotting results; (b and e) relative expression level of Sox9 protein; (c and f) relative expression level of Col2α1 protein; (d and g) relative expression level of Acan protein. PG: positive drug control group; vector-CG: null-vector CG group; h-RASL11B-CG: CG group with overexpression of *RASL11B*; sh-RASL11B-CG: CG group with knockdown of *RASL11B*; h-RASL11B-PG: PG group with overexpression of *RASL11B*; sh-RASL11B-PG: PG group with knockdown of *RASL11B*. Data are presented as mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, vs. PG; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. vector-CG or sh-RASL11B-CG.

knockdown had no effect on Col2α1 protein levels, as shown in Figure 5(c). Similar to the results of their transcriptional levels (Figure 4(a) to (c)), a significant change in the levels of all proteins tested was shown between the h-RASL11B-CG group and the sh-RASL11B-CG group (Figure 5(b) to (d)), $P < 0.05$ or $P < 0.01$. As shown in Figure 5, we also examined the effect of *RASL11B* on the chondrogenesis-related markers, Sox9, Col2α1, and Acan after induction by TGF-β3 (PG group, Figure 5(e) to (g)).

Unlike the transcriptional level results (Figure 4(d) to (f)), all three proteins in each different PG group (Figure 5(e) to (g)) showed a similar trend to their corresponding CG group (Figure 5(b) to (d)) during chondrogenic differentiation, in which h-RASL11B promoted the expression of all three proteins tested and sh-RASL11B suppressed the expression of Sox9 on days 7, 14, and 21 and Acan on day 21 in comparison with the PG group alone. Moreover, sh-RASL11B did not affect the relative expression of Col2α1 on
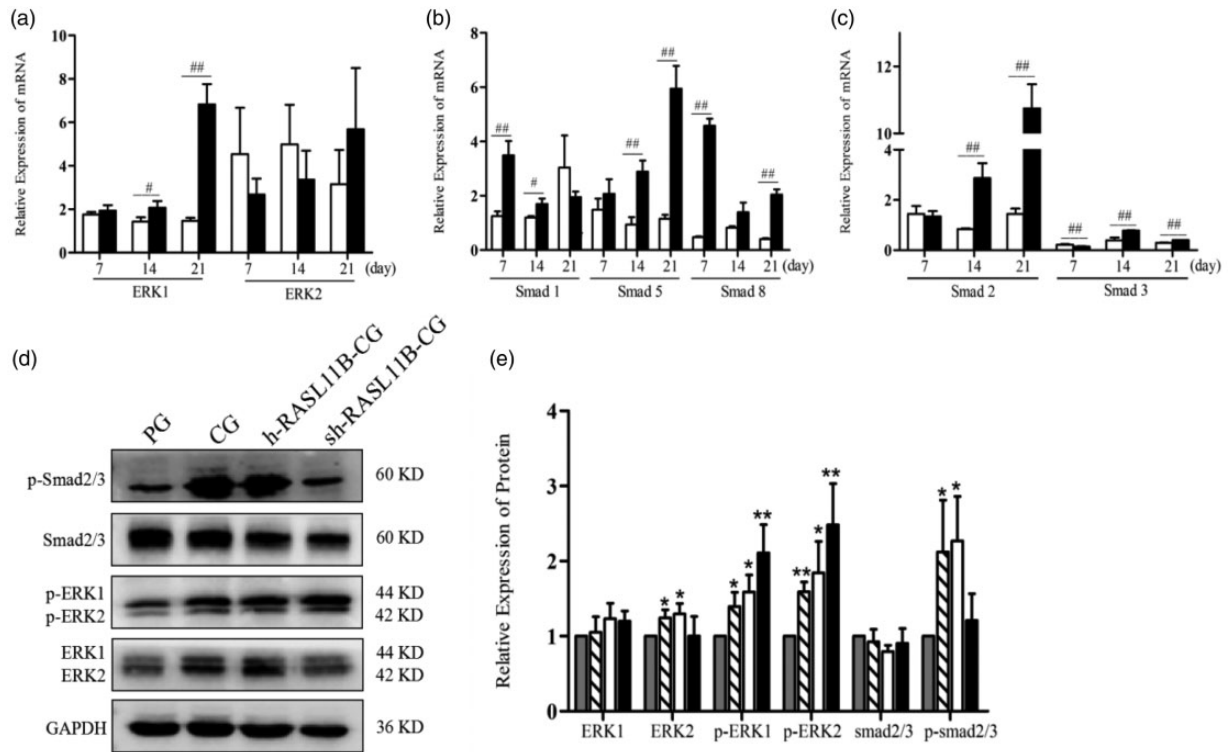
Figure 6. Relative expression of ERK/Smad pathway-related genes and proteins. (a) Relative mRNA expression levels of *ERK1* and *ERK2*; (b) relative mRNA expression levels of *Smad1*, *Smad5*, and *Smad8*; (c) relative mRNA expression levels of *Smad2* and *Smad3*. (d) Western blotting bands for Smad2/3, ERK1/2, p-Smad 2/3, and p-ERK1/2 at day 7 after differentiation. (e) Quantitative analysis of Western blots at day 7 after differentiation. PG (grey bar); CG (hatched bar); h-RASL11B-CG (white bar); sh-RASL11B-CG (dark bar). Data are presented as mean $\pm$ SD ($n = 3$). PG: positive drug control group; CG: PG + hyaluronic acid (concentration: 0.05 mg/mL). $^{*}P < 0.05$, $^{**}P < 0.01$, vs. PG; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. h-RASL11B-CG.

days 7, 14, or 21 (Figure 5(f)) or Acan on days 7 or 14 (Figure 5(g)), after differentiation induction by TGF-β3.

Smad and ERK signals are involved in the synergistic induction of chondrocyte differentiation by HA and TGF-β3 in hAMSCs

To further determine the possible mechanism underlying the synergistic effect of chondroinductive differentiation in hAMSCs by HA and TGF-β3, we first investigated key genes involved in the TGF-β/BMP and ERK1/2 signaling pathways by RT-qPCR on days 7, 14, and 21 after the overexpression or knockdown of *RASL11B* (Figure 6(a) to (c)). There was no change in *ERK1* expression at different time points when *RASL11B* was overexpressed and the expression level of *ERK1* was higher in the sh-RASL11B-CG group than in the h-RASL11B-CG group, as shown in Figure 6(a). Furthermore, *RASL11B* knockdown led to the up-regulation of *ERK1* expression in a time-dependent manner, reaching a highly significant difference on day 21 compared with the h-RASL11B-CG group. *RASL11B* overexpression resulted in a trend toward the up-regulation of *ERK2*, but without statistical significance, on days 7 and 14 compared with *RASL11B* knockdown. However, on day 21, the expression level of *ERK2* was lower in the h-RASL11B-CG group than in the sh-RASL11B-CG group (Figure 6(a)).

Major Smad family members were also investigated, as shown in Figure 6(b) and (c). sh-RASL11B up-regulated the expression of all Smads tested, compared with h-RASL11B,

during the chondrogenic differentiation of hAMSCs, with the exception of *Smad1* on day 21 and *Smad2* and *Smad3* on day 7. *Smad1* and *Smad8* showed the highest transcriptional expression levels on day 7 in the sh-RASL11B-CG group, while *Smad5* showed the highest level of on day 21. The sh-RASL11B gave rise to a highly significant change in the expression level of *Smad2*, in a time-dependent manner, compared with h-RASL11B in the CG group (Figure 6(c)). *Smad2* expression reached its highest level on day 21, whereas the highest level of *Smad3* expression was seen on day 14. Similar to the ERK expression results, overexpression of *RASL11B* could not significantly alter the expression level of any of the Smads tested at different time points.

Subsequently, we focused further on the changes in expression of ERK1/2 and Smad2/3 at the translational level and their phosphorylation levels on day 7 after the chondrogenic differentiation of hAMSCs. As shown in Figure 6(d) and (e), the expression of ERK1/2 and Smad2/3 proteins was consistent with the result at the mRNA level, with no evident difference in expression levels between h-RASL11B-CG and sh-RASL11B-CG groups. However, the phosphorylation levels of ERK1/2 were higher in the groups with abnormal RASL11B expression than in the PG and CG groups. The order of ERK1/2 phosphorylation levels was found to be PG < CG < h-RASL11B-CG < sh-RASL11B-CG. For Smad2/3 phosphorylation, the CG group had higher levels than the PG group

and the sh-RASL11B-CG showed significantly lower levels than the CG and h-RASL11B-CG groups However, Smad2/3 phosphorylation levels were only slightly higher in the h-RASL11B-CG group than in the CG group. Accordingly, these results showed that RASL11B activated the ERK/Smad signaling pathway during HA-induced chondrogenic differentiation.

Discussion

HA is one of the main extracellular matrix components in hyaline cartilage as well as in mesenchyme during the early stage of chondrogenesis. Thus, HA and HA-based biomaterials have been used to modulate stem cell chondrogenesis and stem-cell based cartilage tissue engineering.[32] Our previous study showed that HA alone or in combination with routine inducer exerted a positive effect on the chondroinductive differentiation of hAMSCs, and in the hAMSCs transplantation against rat knee osteoarthritis as well.[21] In the study, we found that HA facilitated the expression of chondrocyte-specific genes including $Sox9$, $Col2\alpha1$, and $Acan$ to some extent. In particular, the combination of HA and TGF-β3 showed a potent synergistic effect for the expression of $Col2\alpha1$ and $Acan$ on day 14 after chondrogenic induction (Figure 2(c)). Here the expression of $Col2\alpha1$ reached the highest value on day 21 for all groups tested, while the expression of $Sox9$ and $Acan$ was decreased. As we know, the transition of chondrocytes to hypertrophy and terminal differentiation is a common phenomenon during the late stage of chondrogenic differentiation. It is unclear whether these cells still retained a chondrocyte phenotype but not a hypertrophic chondrocyte one in this study. As described previously, the duration of common protocols of $in\ vitro$ chondroinductive differentiation was 21–28 days.[5,32–34] Furthermore, the hypertrophy of chondrocytes was inhibited in the presence of TGF-β.[35] In the study, a common protocol of TGF-β3-based $in\ vitro$ chondroinductive differentiation was employed in hAMSCs. In addition, the addition of HA was conducive to the maintenance of stemness during the proliferation of differentiation of hAMSCs.[5,6] To achieve a more stringent control of hAMSCs differentiation to chondrocyte, however, the effect of HA on hypertrophic chondrocyte during induction of chondrogenesis in hAMSCs and possible mechanism should be investigated in further study.

Induction of the chondrogenic differentiation in stem/progenitor cells is accurately regulated by essential transcription factors and complex signals, such as TGF-β, BMPs, Wnt/β-catenin, and MAPK.[12,15,22,23,27,32] However, the molecular mechanisms underlying this process remain largely unclear. Here we used whole-genome microarray analysis and qRT-PCR validation to investigate the mechanisms of HA-mediated regulation of chondrogenic differentiation induced by TGF-β3. The expression of differential gene $RASL11B$ was significantly changed after addition of HA during the process of chondrogenic differentiation of hAMSCs. Furthermore, using gene ontology information, the $RASL11B$ gene was predicted to be involved in HA-mediated chondrogenesis.

The microarray is an ideal research tool for comparative analysis of whole-genome gene expression changes due to its high-throughput. However, the simultaneous measurement of thousands of gene transcripts coupled with each step in the experimental process can introduce experimental errors that easily introduce a certain level of false-positive result. Thus, false-positive results for gene microarray are always present. In the study, eight differential expressed genes with high fold changes did not get a desired result in the qRT-PCR validation test (Figure S2). Also, the candidate $DLX5$ that indicated a relatively concordance between qRT-PCR and microarray data could not significantly alter the HA-mediated chondrogenesis in hAMSCs (Figure S3). Only $RASL11B$ was well correlated with the HA-mediated chondrogenic differentiation of hAMSCs.

$RASL11B$, a member of the small GTPase protein superfamily, has high similarity to RAS proteins. It is expressed in many tissues, but especially in the placenta and primary macrophages.[36] The protein encoded by the $RASL11B$ gene does not anchor to the cell membrane, as it lacks the characteristics of other RAS proteins, such as the presence of prenylation sites.[36] It has demonstrated that RASL11B is involved in TGF-β1-mediated pathophysiological processes including arteriosclerosis inflammation and cancer.[36,37] However, nothing is known about the physiological function of $RASL11B$ in developmental processes or in pathophysiologies.[36,37] A recent study showed that $RASL11B$ negatively regulates the proliferation of retinal precursor cells in the developing zebrafish eye.[38] Another research group found that the long non-coding RNA, maternally expressed gene 3, up-regulates $RASL11B$ to inhibit cell proliferation, invasion, and migration, induce cell cycle arrest, and promote apoptosis, by suppressing $miR-7$ in clear cell renal cell carcinoma.[39] However, there is no information available on $RASL11B$ only and no experimental data regarding its role in chondrogenic differentiation.

Subsequent functional studies showed that the overexpression of $RASL11B$ increased the expression level of chondrogenic markers, including Col2α1 and Acan, at the mRNA and protein levels and the accumulation of cartilage-specific matrix components, such as type 2 collagen and glycosaminoglycan. Thus, $RASL11B$ overexpression promoted the chondrogenic differentiation of hAMSCs. By contrast, knockdown of $RASL11B$ decreased the expression of chondrogenic markers and the accumulation of cartilage-specific matrix components. Therefore, we next aimed to determine the mechanism whereby $RASL11B$ regulated the HA-mediated chondrogenic differentiation of hAMSCs induced by TGF-β3.

Growth factors, such as TGF-βs, BMPs, and IGF-1, play crucial roles in the $in\ vitro$ chondrogenic differentiation of MSCs. Among these growth factors, TGF-βs (TGF-β3 or TGF-β1) are widely known to efficiently induce the chondrogenic differentiation of MSCs.[40–42] The underlying mechanism of TGF-β-mediated chondrogenesis generally involves TGF-β first binding to the TGF-β type 2 receptor (TGFβR2) on the plasma membrane. A heteromeric complex of TGFβR1, also known as anaplastic lymphoma kinase 5, is subsequently formed and phosphorylated

following the activation of TGFβR2.[40,41] Activated TGFβR1 initiates the phosphorylation of Smad2/3 and phosphorylated Smad2/3 then combines with co-Smad (Smad4). The resulting Smad heteromeric complex translocates into the nucleus where it regulates the transcription of downstream target genes through directly binding to Smad-binding elements.[40,41] Among these target genes, *Sox9* is a master transcriptional factor. Smad signaling activates the Sox9 transcription factor, which increases the expression of chondrocyte-specific markers, such as *Col2α1* and *Acan*.[40,43] In the study, Sox9 expression was significantly up-regulated, in a time-dependent manner, at the mRNA and protein level, after continuous overexpression of *RASL11B* in the CG group (HA plus TGF-β3 induction). Consistent with the changes of Sox9 expression, chondrocyte-specific markers, such as Col2α1 and Acan, also exhibited a time-dependent increase in expression (Figures 4 and 5). Finally, the accumulation of cartilage-specific matrix components, such as glycosaminoglycans and type 2 collagen, was markedly elevated on day 21 after the induction of differentiation, in comparison to the null-vector CG group (Figure 3). By contrast, knockdown of *RASL11B* in hAMSCs resulted in significantly lower expression levels of Sox9, Col2α1, and Acan. Interestingly, continuous overexpression of *RASL11B* in the PG group (TGF-β3 induction) did not lead to high *Sox9* mRNA expression levels at the early stage of chondrogenic differentiation (days 7 and 14) or affect the expression of *Col2α1* or *Acan* mRNA. Therefore, high levels of *RASL11B* expression were closely associated with the addition of HA during the TGF-β3-induced chondrogenic differentiation of hAMSCs. As previously mentioned, Smad signaling activates the transcription of the downstream target gene, *Sox9*, which eventually triggers the expression of *Col2α1* and *Acan* during the TGF-β3-induced chondrogenic differentiation of MSCs.[40,43] Therefore, we further assessed the effect of the *RASL11B* gene on Smad signaling in hAMSCs induced to differentiate by the combined treatment of HA and TGF-β3. Our results showed that Smad signaling was significantly different between CG groups with overexpression or knockdown of *RASL11B*, at all differentiation stages (Figure 6). In comparison to overexpression of *RASL11B*, knockdown of *RASL11B* was shown to markedly increase the expression of *Smad1/5/8* at the transcriptional level during the chondroinductive differentiation. Furthermore, *RASL11B* knockdown also gave rise to a significant increase of *Smad2* and *Smad3* at the mRNA level on days 14 and 21 after induction differentiation. However, the transcriptional level of *Smad3* on day 7 was evidently higher in the group overexpressing *RASL11B* than in the group with *RASL11B* knockdown, while the transcriptional level of *Smad2* has no difference between both the groups. Consistent with the transcriptional level of *Smad 3*, *RASL11B* overexpression significantly increased the phosphorylation levels of Smad2/3 on day 7 after chondrogenic differentiation of hAMSCs. As described previously, Smad2/3 phosphorylation is known to block terminal differentiation and promote the continual deposition of type 2 collagen and generation of stable hyaline-like cartilage, whereas Smad1/5/8 phosphorylation is associated with terminal differentiation,

hypertrophic differentiation, and mineralization during the chondrogenic differentiation of bone marrow MSCs (BMSCs).[26,42] Therefore, Smad2/3 phosphorylation is the dominant signaling pathway for the chondrogenic differentiation of BMSCs. This suggests that blockage of Smad1/5/8 activation not only prevents the hypertrophic differentiation of BMSCs, but also results in a more stable cartilage construct without mineralization. Therefore, in the study, *RASL11B* overexpression was found to inhibit the expression of Smad1/5/8 during all stages of chondrogenic differentiation (Figure 6(b)). This indicated that the addition of HA was conducive to the eventual formation of stable hyaline-like cartilage during the TGF-β3-induced chondrogenic differentiation of hAMSCs, instead of inducing the terminal differentiation of the chondrocytes. In addition, Smad2 and Smad3 play differential roles in the TGF-β-induced chondrogenic differentiation in BMSCs. Recent studies have indicated that both knockdown and overexpression of Smad2 have a minor inhibitory effect on the TGF-β1-induced chondrogenic differentiation in human BMSCs, while knockdown and overexpression of Smad3 strongly inhibit chondrogenic differentiation.[27] Thus, Smad3 has a more dominant role than Smad 2 in the TGF-β1-induced chondrogenic differentiation in human BMSCs and chondrogenic differentiation may rely on a sufficient quantity of Smad3. However, regardless of the overexpression or knockdown of *RASL11B*, *Smad3* mRNA levels were markedly lower than *Smad2* mRNA levels during the entire differentiation process in the present study (Figure 6(c)). Therefore, the pro-chondrogenic differentiation effects of *RASL11B* overexpression in the CG group may not be completely attributable to the activation of Smad2/3. Thus, HA-mediated chondrogenic differentiation via *RASL11B* is not limited to TGF-β signaling.

The small GTPase protein, RAS, is an upstream protein of the RAS/ERK signaling pathway,[44] while RASL11B has also high similarity to RAS. Thus, RASL11B may trigger the RAS/ERK signaling pathway during the induction of the chondrogenic differentiation of hAMSCs by the combined treatment with HA plus TGF-β3. Our results showed that *ERK1* mRNA levels were markedly different between CG groups with the overexpression or knockdown of *RASL11B*, in the mid-to-late stage of chondrogenic differentiation. At the early stage of differentiation (day 7), ERK1/2 phosphorylation levels were increased in all CG groups tested, compared with the PG group, most notably after *RASL11B* knockdown. Recent studies have demonstrated that ERK signaling has either stimulatory or inhibitory effect on chondrogenic differentiation.[32,45] For example, Wu *et al.*[32] reported that ERK signaling promotes Sox9 expression and chondrogenic differentiation in the 2000 kDa HA-induced chondrogenesis in adipose-derived MSCs, while Han *et al.*[45] found that the inhibition of ERK signaling enhances the simvastatin-induced differentiation of chondrocytes. However, further investigation is required to understand whether ERK signaling regulates Smad signaling or directly activates downstream target genes, such as *Sox9*, *Col2α1*, and *Acan* during the chondrogenic differentiation of hAMSCs.

In summary, our study demonstrated that HA enhanced the TGF-β3-induced chondrogenic differentiation in hAMSCs by targeting *RASL11B*. The overexpression of *RASL11B* triggered the expression of chondrocyte-specific markers, such as *Col2α1* and *Acan*, through the activation of Sox9 and ERK/Smad signaling. This eventually resulted in chondrogenic differentiation of hAMSCs, with the accumulation of cartilage-specific matrix components, such as glycosaminoglycans and type 2 collagen. Collectively, our findings suggest that *RASL11B* plays a key role in HA-mediated chondrogenic differentiation and that this occurs via ERK and Smad signaling pathways during early chondrogenesis.

Authors' contributions: YL performed partial experiments and drafted the manuscript. A-TW and Q-F Zhang collected partial experimental data. R-ML participated in the experimental design. J-H Xiao made substantial intellectual contributions to conception and design, analysis and interpretation of data, and edited the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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