

Original Article

Heterogeneous nuclear ribonucleoprotein A1 interacts with microRNA-34a to promote chondrogenic differentiation of mesenchymal stem cells

Xianglong Meng¹, Jiawen Cui², Yunsheng Wang¹, Xinuo Zhang¹, Dongyue Li¹, Yong Hai¹, Hongwu Du²

¹Department of Orthopedic, Beijing Chao-Yang Hospital Affiliated to Capital Medical University, Beijing 100020, China; ²112 Lab, School of Chemistry and Biological Engineering, University of Science and Technology Beijing, Beijing 100083, China

Received August 25, 2016; Accepted February 1, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: The mesenchymal stem cell (MSC) shows potential in degenerative disc disease (DDD) treatment. However, little is known about the function of heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) in modulating the chondrogenic differentiation of MSCs. This study aimed to investigate the role of hnRNPA1 in the chondrogenic differentiation of MSCs and potential mechanisms. Mouse MSCs C3H10 and chondrogenic ATDC5 cells were used to quantify hnRNPA1 expression. The hnRNPA1 overexpression vectors were transfected into C3H10 cells, cell viability and chondrogenic factors expressions were assessed by MTT assay, qPCR and Western blot, respectively. After microRNA-34a (miR-34a) inhibitor transfection, expressions of chondrogenic factors and the Wnt signaling were detected. RNA-binding protein immunoprecipitation (RIP) was performed to reveal the interaction between hnRNPA1 and miR-34a. Results showed that hnRNPA1 was significantly down-regulated in C3H10 compared to ATDC5. Overexpression of hnRNPA1 markedly promoted C3H10 cell viability and expressions of chondrogenic factors SRY-box 9 (SOX9), collagen II, hyaluronan synthase 2 (HAS2) and aggrecan, without significant influence on adipogenic factors. miR-34a inhibitor suppressed chondrogenic factors expressions. RIP results showed the interaction between miR-34a and hnRNPA1. Besides, hnRNPA1 promoted expressions of Wnt family member 3A (WNT3A), WNT5A and β -catenin, and these effects were abrogated by miR-34a inhibitor. We found the promotive effect of hnRNPA1 on chondrogenic factors, which might require the interaction with miR-34a and the regulation of the Wnt signaling. Thus hnRNPA1 might induce the chondrogenic differentiation of MSCs that facilitate the MSC therapy for DDD.

Keywords: Degenerative disc disease, heterogeneous nuclear ribonucleoprotein A1, miR-34a, Wnt signaling, chondrogenic differentiation, mesenchymal stem cell

Introduction

The intervertebral disc is composed of the annulus fibrosus and the nucleus pulposus, which resisting tension and compression when the body at different positions. Degenerative disc disease (DDD) may occur with age and be caused by other risk factors such as excessive activity, injury, smoking and heredity [1]. DDD is usually accompanied by the embrittlement and fracture of the disc annulus fibrosus, as well as the dehydration and atrophy of the nucleus pulposus. It is the main cause of chronic low back and neck pain [2, 3], and may develop into hasten spinal stenosis, spondylolisthesis or spine osteoarthritis [4].

Treatments of DDD including drugs, rehabilitation therapy and surgery are focused on anti-inflammatory, chondrocyte protective and decompressive effects [5, 6].

In the last decade, researches have been concentrated on stem cell therapies such as the mesenchymal stem cell (MSC) therapy for DDD. MSCs are undifferentiated pluripotency cells which can differentiate into multiple types of cells including chondrocytes under specific environment *in vivo* or *in vitro*, thus MSCs are being explored in the regeneration of damaged tissues [7]. The MSC therapy is considered to be one promising treatment for DDD, since it has been found that MSCs kept their cell viabil-

ity after transplantation and have potential to promote regeneration of the intervertebral disc [8, 9]. In order to take full advantage of MSCs in treating DDD, enhancing MSC function and chondrogenic differentiation are becoming the priorities [10, 11].

Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) is a kind of RNA-binding protein which constitutes the ribonucleoprotein (RNP) complex and regulates mRNA splicing, stability, transcription and translation [12-14]. Besides mRNA processing, hnRNPA1 can also bind to microRNAs (miRNAs), such as miR-18a, to promote miRNA processing [15]. Based on these functions, hnRNPA1 plays pivotal roles in modulation of diseases [16]. Furthermore, recent study suggested that hnRNPA1 was crucial for stem cells to differentiate into smooth muscle cells *in vitro* and *in vivo* [17], implying its application potential in the stem cell therapy. However, little is known about its role in MSCs differentiating into chondrocytes.

This study aimed to investigate the function of hnRNPA1 in the chondrogenic differentiation of MSCs. Mouse MSCs C3H10 T1/2 (C3H10) and mouse chondrogenic cells ATDC5 were used to detect hnRNPA1 expression pattern. The hnRNPA1 was overexpressed in C3H10 cells to assess its effect on chondrogenic factor expression. We also investigated miR-34a and the Wnt signaling to explore possible mechanism of hnRNPA1 in chondrogenic differentiation. The findings in this study might enrich the researches on MSC differentiation and facilitate further applications of MSC therapy in DDD.

Materials and methods

Cell culture

Mouse MSCs C3H10 (ATCC, Manassas, VA, USA) and mouse chondrogenic cells ATDC5 (RIKEN, Saitama, Japan) were used in this study. The cells were cultured in Dulbecco minimum essential medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 50 U/mL Penicillin-Streptomycin (Gibco), and incubated in humid air with 5% CO₂ at 37°C. The culture medium was changed every other day, and the cells were passaged at about 80% of cell confluence.

Cell transfection

The complete coding sequence of mouse *Hnnpa1* (GenBank Accession NM_001039129) was cloned into pcDNA3.1 vector (Thermo Scientific, Carlsbad, CA, USA) and the correction of ligation was verified by sequencing. Specific inhibitors of miR-34a, miR-146a or miR-99a, and the inhibitor negative control were synthesized by Sangon Biotech (Shanghai, China). Cell transfections were performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. One day before transfection, C3H10 cells (1×10⁵/well) were seeded in the 24-well plate and cultured in DMEM/F12 medium without antibiotics. When the cell confluence reached about 90%, cell transfection was performed: the overexpression vector of *Hnnpa1* (1 μg) or the inhibitor (200 nM) was added, and the empty vector or the inhibitor negative control was transfected as control groups. The cells were incubated at 37°C and sampled at different time points for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At 48 h post transfection, cells were collected for protein or RNA extraction.

MTT assay

The cell viability of transfected C3H10 cells was detected by MTT assay. Cells were seeded in a 96-well plate, each well containing 5×10³ cells. MTT (5 mg/mL, Sigma-Aldrich, Shanghai, China) was added to each well and the plate was incubated for another 4 h, then 150 μl dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added. The plate was slowly oscillated until all the crystal substance was dissolved. Optical density at 490 nm was detected by using a microplate reader SpectraMax i3x (Molecular Devices, Silicon Valley, CA, USA).

RNA-binding protein immunoprecipitation (RIP)

RIP of transfected C3H10 cells was performed to assess the interaction between hnRNPA1 and miR-34a using RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instruction. Cells were lysed in the RIP Lysis Buffer, and suitable proportion was stored as Input. Magnetic beads were incubated with the specific antibodies against hnRNPA1 (ab5832,

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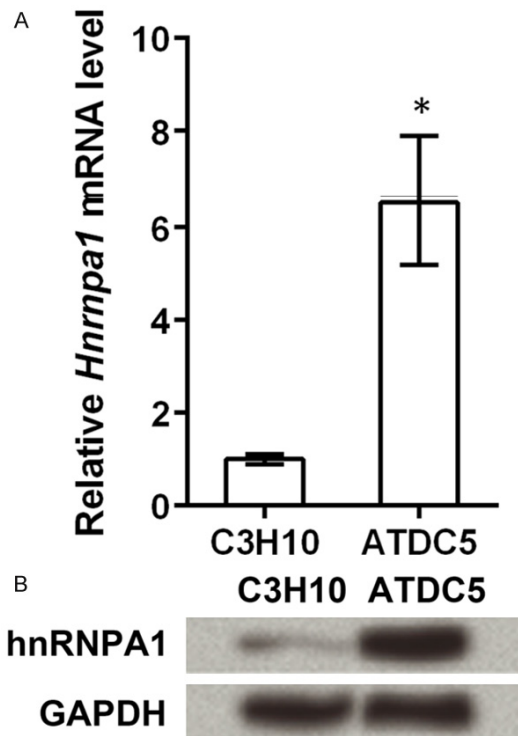


Figure 1. Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) expression in mouse mesenchymal stem cells C3H10 and mouse chondrogenic cells ATDC5. A: *Hnrnpa1* mRNA levels were quantified by qPCR. * $P < 0.05$. B: HnRNPA1 protein expressions were detected by Western blot. GAPDH was used as a reference.

Abcam, Cambridge, UK) or anti-immunoglobulin G which was provided in the kit as negative control, for 30 min at room temperature, and then been washed in the Wash Buffer twice. Cell lysates were incubated together with the magnetic beads overnight at 4°C, after that the beads were collected and washed for 6 times. During all the procedures, RNase inhibitor cocktail was added to prevent RNA degradation. RNA on the beads was extracted by the phenol-chloroform method according to the instruction. The level of miR-34a in the extracted RNAs was quantified by real-time quantitative PCR (qPCR).

qPCR

Total RNA samples of cells were extracted by using Trizol (Invitrogen) and purified by RNA Purification Kit (TIANGEN, Beijing, China). RNA was quantified by using NanoDrop 2000 (Thermo Scientific) and reverse-transcribed into complementary DNA (cDNA) by ReverAid First Strand cDNA Synthesis Kit (Thermo

Scientific). qPCR was conducted on LightCycler 480 system (Roche, Basel, Switzerland) to quantify the mRNA level of miR-34a (forward: 5'-ACACT CCAGC TGGGT GGCAG TGTCT TAGCT-3' and reverse: 5'-TGGTG TCGTG GAGTC G-3'), SRY-box 9 (*Sox9*, forward: 5'-GTACC CGCAT CTGCA CAACG-3' and reverse: 5'-GTGGC AAGTA TTGGT CAAAC TCATT-3'), collagen II (*Col2a1*, forward: 5'-AAAGA CGGTG AGACG GGAGC-3' and reverse: 5'-GACCA TCAGT ACCAG GAGTG CC-3'), hyaluronan synthase 2 (*Has2*, forward: 5'-ACAGT ATTGT GGCAC CGCAC-3' and reverse: 5'-ACCAA CCCCC ATTGA ATGTC T-3'), aggrecan (*Acan*, forward: 5'-CGGGA AGGTT GCTAT GGTG-3' and reverse: 5'-CCTGT CTGGT TGGCG TGTA-3'), peroxisome proliferator activated receptor gamma (*Pparg*, forward: 5'-ATGCT TGTGA AGGAT GCAAG-3' and reverse: 5'-TGGAG ATCTC CGCCA ACAG-3'), CCAAT/enhancer binding protein alpha (*Cebpa*, forward: 5'-GAGAC GTCTA TAGAC ATCAG-3' and reverse: 5'-GCTCT TGTTT GATCA CCAGC-3'), CCAAT/enhancer binding protein beta (*Cebpb*, forward: 5'-GACGG TGGAC AAGCT GAGCG-3' and reverse: 5'-AGCTC TCGCG ACAGC TGCTC-3') and zinc finger protein 423 (*Znf423*, forward: 5'-TGGAT GCATA AGAAG AGGGT TGA-3' and reverse: 5'-TTCTG ATCGC ACTCT GGCTC-3'). The qPCR results were analyzed by $2^{-\Delta\Delta Ct}$ method normalized to *U6* (forward: 5'-GCATG ACGTC TGCTT TGGA-3' and reverse: 5'-CCACA ATCAT TCTGC CATCA-3') or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, forward: 5'-TCAAC AGCAA CTCCC ACTCT TCCA-3' and reverse: 5'-ACCCT GTTGC TGTAG CCGTA TTCA-3').

Western blot

Protein samples of cells were extracted by ProteoPrep Total Extraction Sample Kit (Sigma-Aldrich) according to the manufacturer's instruction. Samples were separated on a sodium dodecyl sulfate-polyacrylamide gel and transferred onto a nitrocellulose membrane (Millipore). The membranes were incubated in 5% skim milk and then incubated overnight at 4°C with the specific mouse monoclonal antibodies against hnRNPA1 (ab5832, Abcam), Wnt family member 3A (WNT3A, ab81614), WNT5A (ab110073), β -catenin (ab22656), SOX9 (ab76997), collagen II (ab3092), HAS2 (ab140671), aggrecan (ab3778), PPAR γ (Sangon Biotech), CEBPA (ab15047), CEBPB (ab33481), ZNF423 (Sangon Biotech) or GAPDH (ab8245) as a reference. Then the membranes were washed in PBS for 3 times and incubated in horseradish peroxidase-conjugated goat anti-

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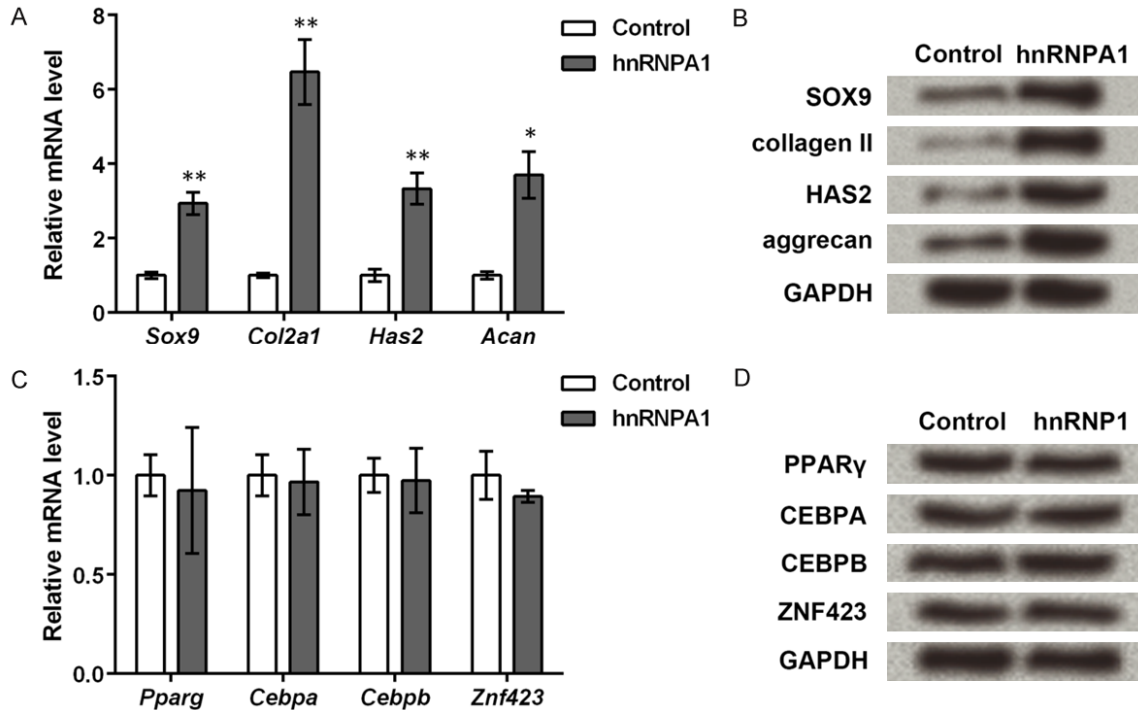


Figure 2. Expression of chondrogenic and adipogenic factors in transfected mouse mesenchymal stem cells C3H10. C3H10 cells were transfected with heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) overexpression vectors or empty vectors as Control. After 48 h of transfection, qPCR and Western blot were performed to detect the expression of chondrogenic and adipogenic factors. A: mRNA levels of chondrogenic factors SRY-box 9 (*Sox9*), collagen II (*Col2a1*), hyaluronan synthase 2 (*Has2*) and aggrecan (*Acan*). * $P < 0.05$, ** $P < 0.01$. B: Protein expressions of chondrogenic factors SOX9, collagen II, HAS2 and aggrecan normalized to GAPDH. C: mRNA levels of adipogenic factors peroxisome proliferator activated receptor gamma (*Pparg*), CCAAT/enhancer binding protein alpha (*Cebpa*), *Cebpb* and zinc finger protein 423 (*Znf423*). No significant change was detected between each two groups. D: Protein expressions of adipogenic factors PPAR γ , CEBPA, CEBPB and ZNF423 normalized to GAPDH.

mouse antibodies (ab6789) for 1 h. Signals were developed by using EasyBlot ECL Kit (Sangon Biotech) and the band density was quantified by software ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Experiments were repeated for 3 times. Results were represented as the mean \pm standard deviation. Statistical analysis was performed by SPSS 20 (IBM, New York, USA), using one-way analysis of variance or Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

hnRNPA1 promoted chondrogenic factors and cell viability of C3H10 cells

Firstly, hnRNPA1 expression level was detected in C3H10 and ATDC5 cells. Results of qPCR

showed that *hnrnpa1* mRNA was significantly lower in C3H10 compared to ATDC5 ($P < 0.01$, **Figure 1A**). Besides, Western blot results showed low expression of hnRNPA1 protein in C3H10 (**Figure 1B**), indicating that hnRNPA1 was down-regulated in C3H10 cells. It was possible that hnRNPA1 was up-regulated and played certain role in the differentiated chondrogenic cells.

Next, chondrogenic factors in C3H10 cells were quantified to analyze the role of hnRNPA1 on the chondrogenic process. With the overexpression of hnRNPA1 in C3H10 cells, mRNA levels of *Sox9*, *Col2a1*, *Has2* and *Acan* were all significantly elevated compared to the control ($P < 0.01$ or $P < 0.05$, **Figure 2A**); moreover, protein expressions of these factors were also increased (**Figure 2B**). By contrast, no significant change was found in mRNA or protein expressions of adipogenic factors PPAR γ , CEBPA, CEBPB or ZNF423 (**Figure 2C and 2D**),

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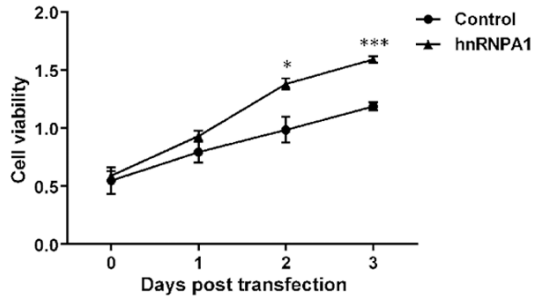


Figure 3. Cell viability changes of transfected mouse mesenchymal stem cells C3H10. C3H10 cells were transfected with heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) overexpression vectors or empty vectors as the Control. At 0, 1, 2 and 3 days post transfection, MTT assay was performed to assess cell viability, and the optical density at 490 nm was detected. Significant difference of cell viability was found at 2 d (* $P < 0.05$) and 3 d post transfection (** $P < 0.001$).

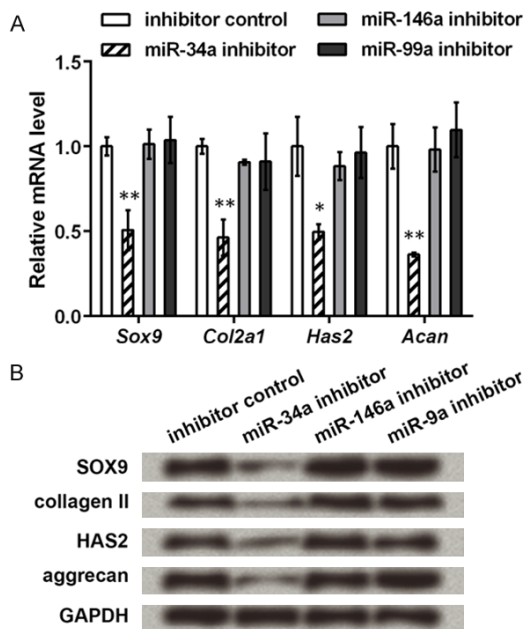


Figure 4. Expression of chondrogenic factors when miR-34a expression were inhibited in mouse mesenchymal stem cells C3H10. miR-34a inhibitor was transfected in C3H10 cells to inhibit miR-34a expression. qPCR and Western blot were performed at 48 h post transfection. Inhibitors of miR-146a or miR-99a were also respectively transfected into C3H10 cells as negative controls. A: mRNA levels of chondrogenic factors SRY-box 9 (*Sox9*), collagen II (*Col2a1*), hyaluronan synthase 2 (*Has2*) and aggrecan (*Acan*). * $P < 0.05$, ** $P < 0.01$. B: Protein expression level of chondrogenic factors SOX9, collagen II, HAS2 and aggrecan normalized to GAPDH.

further suggesting the specific promotion of chondrogenic factors by hnRNPA1.

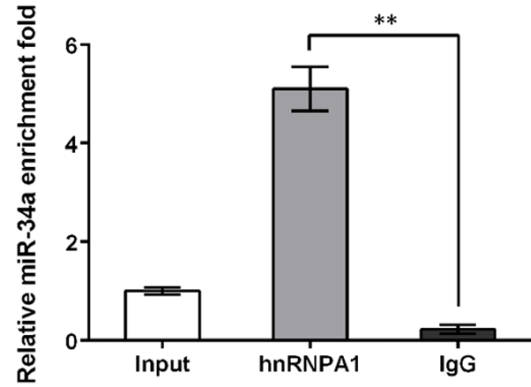


Figure 5. Interaction between heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and miR-34a. hnRNPA1 was overexpressed in mouse mesenchymal stem cells C3H10. Immunoprecipitation (IP) was performed with the antibodies against hnRNPA1 to assess hnRNPA1-miR-34a interaction at 48 h post transfection. Input contained total protein without IP. IP with anti-immunoglobulin G (IgG) was performed as a negative control. qPCR was performed to quantify the miR-34a level in IP products. Anti-hnRNPA1 antibodies immunoprecipitated significant more miR-34a compared to the negative control. ** $P < 0.01$.

MTT assay for transfected C3H10 cells was performed to investigate whether cell viability was changed by hnRNPA1 overexpression. Results indicated that hnRNPA1 overexpression significantly improved C3H10 cell viability when detected at 2 and 3 days post transfection ($P < 0.05$ or $P < 0.001$, **Figure 3**), though no obvious change was found at 0 or 1 day post transfection ($P > 0.05$). These results further supported that hnRNPA1 overexpression improved C3H10 cell viability.

miR-34a regulated chondrogenic factors

Base on existed studies reported that the miR-34a involvement in chondrogenesis [18], this study using miR-34a inhibitor transfection to assess the effect of miR-34a on chondrogenic factor expression in C3H10 cells. Results of qPCR suggested that miR-34a inhibitor significantly suppressed the mRNA level of *Sox9*, *Col2a1*, *Has2* and *Acan* ($P < 0.01$ or $P < 0.05$, **Figure 4A**), and similar expression profiles were also observed in Western blot results (**Figure 4B**). Meanwhile, inhibition of miR-146a or miR-99a did not cause obvious changes in these chondrogenic factors expressions. These results suggested that miR-34a inhibitor suppressed chondrogenic factors, implying the similar role of miR-34a and hnRNPA1 in promoting chondrogenic factors expressions.

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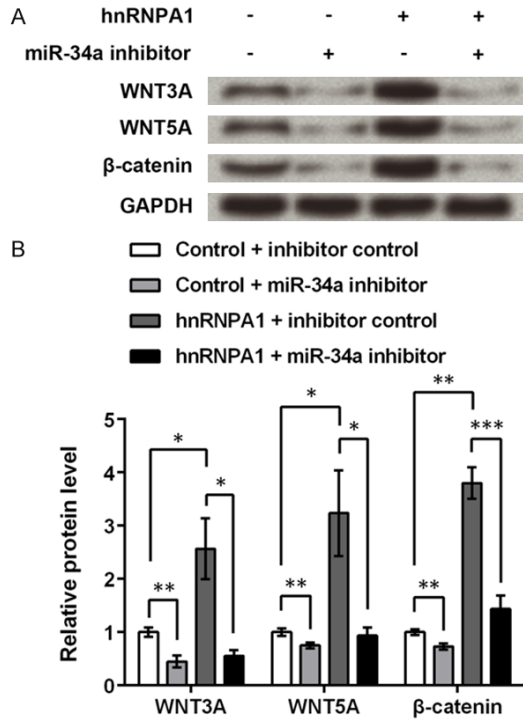


Figure 6. Regulation of the Wnt signaling pathway by heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and miR-34a. Mouse mesenchymal stem cells C3H10 were transfected with hnRNPA1 or miR-34a inhibitor, and factors in the Wnt signaling were detected by Western blot at 48 h post transfection. A: Protein levels of Wnt family member 3A (WNT3A), WNT5A and β -catenin normalized to GAPDH. B: Quantification of WNT3A, WNT5A and β -catenin protein levels based on Western blot results. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

hnRNPA1 interacted with miR-34a and regulated the Wnt signaling

We then performed RIP about C3H10 cells by overexpressing hnRNPA1 to analyze whether hnRNPA1 protein could interact with miR-34a. The qPCR results showed that miR-34a was significantly enriched by hnRNPA1 than the negative control ($P < 0.01$, **Figure 5**), and it was about 5 times of that in the Input group. This result reflected that hnRNPA1 protein was able to interact with miR-34a in C3H10 cells.

It has been reported that miR-34a targeted the Wnt signaling to affect cell capacities [19, 20], so this study further assessed factors in the Wnt signaling pathway to investigate potential mechanism of hnRNPA1 in chondrogenic differentiation. Western blot results showed that miR-34a inhibition significantly suppressed expressions of factors in the Wnt

signaling pathway, while hnRNPA1 overexpression significantly promoted it, including WNT3A, WNT5a and β -catenin ($P < 0.05$ or $P < 0.01$, **Figure 6A** and **6B**). Moreover, when miR-34a was inhibited, the promotive effects of hnRNPA1 on these factors were abrogated ($P < 0.05$ or $P < 0.001$), suggesting the pivotal position of miR-34a in hnRNPA1 regulation mechanisms.

Discussion

The chondrogenic differentiation of MSCs has great benefits for the stem cell therapy in DDD. This study revealed that hnRNPA1 was differentially expressed in mouse MSCs C3H10 and chondrogenic cells ATDC5. Overexpression of hnRNPA1 in C3H10 cells promoted cell viability and chondrogenic factors expressions. We further found that miR-34a regulated chondrogenic factors which interacted with hnRNPA1 and was involved in the regulation of the Wnt signaling pathway by hnRNPA1.

hnRNPA1 has been reported to participate in cell differentiation for its regulatory function in gene expression [17]. In this study, we found that hnRNPA1 was differentially expressed in C3H10 and ATDC5 cells, with a lower level in C3H10 that led us to explore its role in the chondrogenic differentiation of MSCs. SOX9, collagen II, HAS2 and aggrecan are markers for chondrogenic differentiation [21] and their overexpression induces the cell differentiation [22-25]. C3H10 cells that overexpressed hnRNPA1 possessed significantly higher levels of these chondrogenic factors expressions; meanwhile, the expression of four adipogenic factors PPAR γ , CEBPA, CEBPB and ZNF423 [26-28] were barely changed, suggesting that hnRNPA1 overexpression facilitates the expression of chondrogenic factors. Meanwhile, C3H10 cell viability was also elevated by hnRNPA1 overexpression. Together with the elevated chondrogenic factors, it implied that the elevation of hnRNPA1 might induce the chondrogenic differentiation of MSCs.

A previous study has shown the regulatory roles of miR-34a in chondrogenesis [29]. In this study, inhibition of miR-34a resulted in the downregulation of the four chondrogenic factors, while inhibition of miR-146a or miR-99a did not show similar effects, implying that miR-34a might be involved in the up-regulation of

these chondrogenic factors. However, miRNAs are effective gene regulators by binding to their mRNA targets, and there were studies indicated that miR-34a has inhibition effects on its target genes such as *Col2a1* [30], which seems conflicting with the results of this study. We suspected that mechanisms other than the direct modulation by miR-34a might exist in regulation of the four chondrogenic factors, and then we performed RIP to investigate the relationship between hnRNPA1 protein and miR-34a.

As shown in RIP results, hnRNPA1 protein could interact with miR-34a directly or indirectly. It was not a particular case, since hnRNPA1 has been indicated to interact with primary RNA sequence of miR-18a and accelerate the processing of miR-18a [15]. Thus it is reasonable to deduce that hnRNPA1 protein might interact with miR-34a in C3H10 cells. Though we have no valid proof to exclude the possibility that miR-34a targets *Hnrnpa1* mRNA, as predicted by TargetScan7 [31], the effect of hnRNPA1 was opposite to miR-34a inhibitor, suggesting that the interaction between hnRNPA1 protein and miR-34a might exert more forceful influence on the chondrogenic factors in C3H10 cells than miR-34a targeting hnRNPA1 mRNA. The interplay between hnRNPA1 and miR-34b needs to be confirmed in further research.

Based on the reported role of the Wnt signaling in chondrogenic differentiation [32], this study detected the expressions of WNT3A, WNT5A and β -catenin to explore the possible mechanism of the hnRNPA1-regulated chondrogenic differentiation. It has been reported that suppression of the Wnt/ β -catenin was required for the chondrogenic differentiation of human adipose-derived stem cells [33], whereas inactivating the Wnt/ β -catenin inhibited the chondrogenic differentiation of ATDC5 cells [34], both supporting the important position of Wnt in chondrogenic differentiation. This study found that hnRNPA1 overexpression promoted WNT-3A, WNT5A and β -catenin expression levels in C3H10 cells, which might be one mechanism of the hnRNPA1-induced chondrogenic differentiation. Moreover, suppression of miR-34a abrogated the up-regulation of the Wnt signaling induced by hnRNPA1, indicating the necessity of miR-34a in this mechanism. Although miR-34a targeted and inhibited the Wnt signaling in some cell types [20], based on the above dis-

ussion, the interaction of hnRNPA1 and miR-34a might generate a more powerful effect to promote the Wnt signaling, and more reliable evidences are need to be discovered.

To sum up, this study revealed the promotive effects of hnRNPA1 on chondrogenic factors expression in mouse MSCs C3H10, reflecting the potential of hnRNPA1 in inducing the chondrogenic differentiation of MSCs. The function of hnRNPA1 is possibly exerted via interaction with miR-34a and regulation of the Wnt signaling. With more depiction of integrated regulatory mechanisms, hnRNPA1 has the potential to be used in the MSC therapy for DDD.

Disclosure of conflict of interest

None.

Address correspondence to: Yong Hai, Department of Orthopedic, Beijing Chao-Yang Hospital Affiliated to Capital Medical University, No. 8, Gongtinan Road, Chaoyang District, Beijing 100020, China. E-mail: haiyong020@126.com; Hongwu Du, 112 Lab, School of Chemistry and Biological Engineering, University of Science and Technology Beijing, No. 30, Xueyuan Road, Haidian District, Beijing 100083, China. E-mail: duhongwu086@126.com

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