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Original Article

Expression pattern and regulation of miR-19a and CCND1 in C3H10T1/2 chondrogenic differentiation

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Abstract: Mesenchymal stem cells are regulated by many different pathways even in early stage of chondrogenic differentiation that the cells' proliferation is depressed. Recent studies have supplied marker genes of chondrogenesis were potently affected by cell cycle alterations. In this study, we explored relationship between miR-19a and CCND1, a cell cycle gene, in chondrogenic differentiation and revealed the possible mechanism of miR-19a engaging in its proliferation. A cell pellet model was applied to induce chondrogenesis in C3H10T1/2 cells. In the experiment, chondrogenic differentiation was induced by TGF-β_3 in high glucose-DMEM medium containing 10% FBS and ITS. In the control group, the TGF-β_3 and ITS were omitted. Real-time PCR was performed to assess the expression of the genes associated with chondrogenic differentiation for the first, the second and the third week respectively. And toluidine blue staining was employed for the third week. Compared with the controls, the expression levels of the two functional genes Bax and Klf-4 were both down-regulated and the differences were statistically significant. In chondrogenic differentiation, the master promoter SOX-9 and the marker genes of aggrecan, collagen IIa1 and collagen Xa1 were all up-regulated significantly in the second week. CCND1 was forecasted as the possible target gene of miR-19a by means of bioinformatics. The gene expression of miR-19a was gradually up-regulated significantly compared with the controls, while the gene expression of CCND1 was gradually down-regulated and the difference was also statistically significant. The pellets subjected to toluidine blue staining appeared violet and the nuclei were dark blue and the content of extracellular matrix was increased significantly in the induced group. Together, our results showed that, in C3H10T1/2 chondrogenic differentiation, miR-19a participated in maintaining cellular undifferentiation and proliferation while the differentiation capacity was enhanced.

Keywords: microRNA, mesenchymal stem cell, chondrogenic differentiation

Introduction

MicroRNA (miRNA) is a group of endogenous non-coding RNAs with 20~24nt in length, which binds non-coding or coding region of the target messenger RNA (mRNA) according to the base-pairing rules, leading to the degradation and (or) the translation inhibition of the mRNA, and thereby resulting in the post-transcriptional inhibition on expression of target gene. By this way, mRNA can be degraded or the translation process can be suppressed rapidly and effectively, and therefore, the protein expression of the target gene will be controlled within the required level for life activities [1, 2]. In the differentiation of mesenchymal stem cells, microRNAs engaged in regulation of multiple biological processes, for instance, regulating cell cycle progression via targeting pivotal regulators (E2F, CDK, cyclin D, p21, p27, DNA polymerase, etc.) which promoted or retarded the cell cycle [3-6]. For CCND1 (cyclin D1) biological function, it regulated the cellular proliferation. Through bioinformatics prediction on the internet (*http://www.microrna.org/microrna, http://www.targetscan.org*) [7], miR-19a can specifically bind with CCND1 mRNA, regulating the cell cycle. There were also some reports on miR-19a regu-

Table 1. Nucleotide sequences for qPCR primers

Gene	Size of PCR product (bp)	Primer sequence
Bax	310	5'-GACACCTGAGCTGACCTTGG-3' (F)
		5'-GAGGAAGTCCAGTGTCCAGC-3' (R)
Klf-4	140	5'-ACTCACACAGGCGAGAAACC-3' (F)
		5'-AAGGCCCTGTCACACTTCTG-3' (R)
Sox9	108	5'-CGGCTCCAGCAAGAACAAG-3' (F)
		5'-TTGTGCAGATGCGGGTACTG-3' (R)
Aggrecan	105	5'-GCTGCAGTGATCTCAGAAGAAG-3' (F)
		5'-GATGGTGAGGGAAGACCCTA-3' (R)
Collagen IIa1	145	5'-CCAGGATGCCCGAAAATTAG-3' (F)
		5'-TTCTCCCTTGTCACCACGAT-3' (R)
Collagen Xa1	190	5'-GCAGCATTACGACCCAAGAT-3' (F)
		5'-CATGATTGCACTCCCTGAAG-3' (R)
CCND1	138	5'-ATGTTCGTGGCCTCTAAGATGA-3' (F)
		5'-CAGGTTCCACTTGAGCTTGTTC-3' (F)
18s	112	5'-CCTGGATACCGCAGCTAGGA-3' (F)
		5'-GCGGCGCAATACGAATGCCCC-3' (R)

lating cellular proliferation [8-11]. In order to investigate whether cellular proliferation was persistently suppressed in the differentiation of stem cells, the expression tendencies of miR-19a and CCND1 during the induced chondrogenic differentiation of C3H10T1/2 were verified in this study.

Materials and methods

Cell culture methods

The murine mesenchymal cell line C3H10T1/2 was purchased from ATCC (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) at 37°C in a humidified 5% CO_2 atmosphere (Thermo Fisher Scientific, USA). Cells were passaged using 0.05% trypsin (Invitrogen, Carlsbad, CA) at room temperature and collected, and then passaged to a 75 cm^2 cell culture flask in a ratio of 1:3, which was recorded as generation P1. The medium was replaced every three days, and digestion and passaging continued in the same way. All of the experiments were conducted using cells that had been passaged less than 15 times.

Pellet model formation

To induce chondrogenesis, 5×10^5 cells were grown first as a monolayer, collected using trypsin, and then pelleted by centrifugation at 1,200 rpm for 5 minutes (15 mL conical polypropylene tubes, CORNING, USA), which was described basely on literature [12, 13]. The pellets were divided into the chondrogenic induction group and the control group. The induction was then cultured in 2 mL of chondrogenic medium (high glucose DMEM containing 2% FBS, 100 nM dexamethasone [Sigma, Dorset, UK], 50 mM L-ascoribic acid 2-phosphate [Sigma, Dorset, UK], BD™ITS+Premix 1:100 [6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL bovine serum albumin, and 5.35 mg/mL linoleic acid; BD Biosciences Discovery Labware, Bedford, MA, USA] and 10 ng/mL transforming growth factor-β_3 [TGF-β_3; PeproTech EC, London, UK]). For the controls', high Glucose-DMEM containing 2% FBS was added. Then the up-right tubes were cultured in the incubator. The medium was then renewed for the first time. After that, the whole medium was refilled every three days.

Histochemical staining

The pellet samples were collected in the third week for toluidine blue staining. When the pellets harvested, it were subjected to PBS washing, fixing, embedding, deparaffinizing, and then toluidine blue staining. After deparaffinizing, the sections were subjected to heat treatment in citrate solution for 10 min. After being washed by distilled water, the fixed tissues were subjected to 0.5% toluidine blue staining for 1 min, color separation by 95% ethanol, washing by distilled water again, dehydration for 10 min in 100% ethanol, transparentizing for 10 min with xylene and sealing with neutral gum finally. Images were captured using a Leica DMI4000B microscope (Germany) fitted with an Optixcam summit series 5 MP digital camera. The photographs were assembled in Adobe Photoshop 6.0.

Total RNA extraction and reverse transcription quantitative PCR analysis

Total RNA was extracted from the pellets using TRIzol (Invitrogen, CA, USA), according to the manufacturer's instructions. cDNA was synthe-

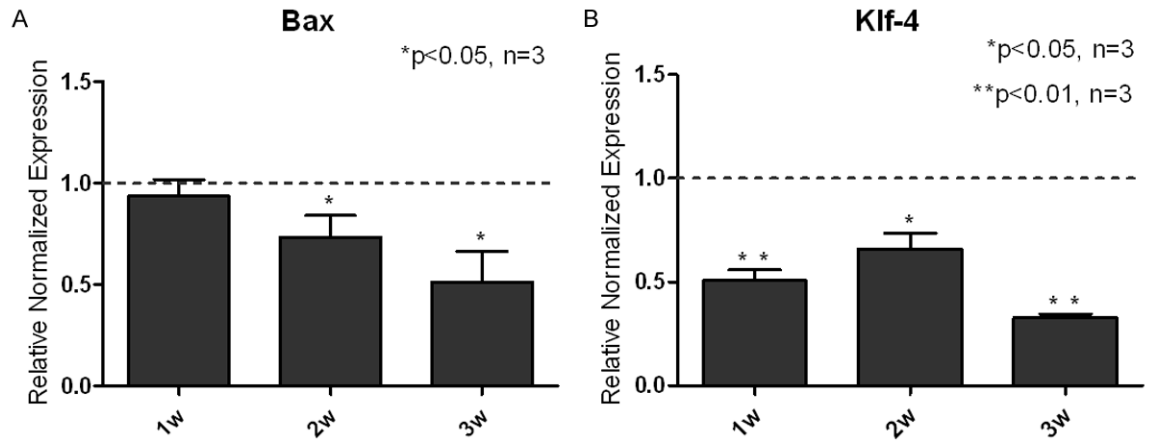

Figure 1. Chondrocyte gene expression by real-time PCR. A. Bax; B. Klf-4. Compared with the control group, Bax expression was down-regulated at the 2ed and 3rd week while the t values were 4.296 and 5.623; *P* values were 0.05 and 0.03 respectively. The Klf-4 was also down-regulated during the chondrogenic differentiation. The t values were 16.496, 7.616, 59.801 and *P* values were 0.004, 0.017, 0.000 respectively.

sized from 0.5 μg of total RNA with an oligo-dT primer using a commercially available kit according to the manufacturer's protocol (TaKaLa, Japan). The oligonucleotide primers used to amplify the target mRNA are listed in **Table 1**. Based on the instruction, the total reaction system of q-PCR was 10 μL, and the procedures for gene detection: 95°C for 10 min; 95°C for 15 s, 60°C for 30 s, 72°C for 20 s, 40 circles, with 18 s as the internal reference. And the procedures for miRNA detection: 95°C for 30 s; 95°C for 3 s, 60°C for 30 s, 40 circles, with U6 as the internal reference. The expression levels of mature miR-19a were detected using a microRNA RT-kit (TaKaLa, Japan). For the RT-reaction, 0.5 μg of total RNA was used and no-template reaction was performed, according to the manufacturer's protocol (TaKaLa, Japan). The sequence of the upstream primer: 5'-CTCGCTTCGGCAGCACA-3' and the sequence of the downstream primer: 5'-AACGCTTCACGAATTTGCGT-3'. The relative expression levels of all the genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistics

Each experiment was repeated at least three times. Data are presented as the mean ± SD. Comparisons between groups were performed using independent t-test. The statistical analysis was performed with SPSS 20.0 software and *P<0.05 and **P<0.01 indicated statistical significance.

Results

Bax and Klf-4 gene expression

During the differentiation, gene expression of Bax which regulated apoptosis of stem cells was gradually down-regulated, and steep down-regulation was observed in the second and the third week with statistical significance; the expression of Klf-4, the self-renewal function gene, was also inhibited. The differences of the first, the second and the third week were all statistically significant. These results indicated inhibition on the expression of the functional genes (Bax, Klf-4, etc.) engaging in cellular proliferation and apoptosis during the differentiation (**Figure 1**).

Marker genes expression in chondrogenic differentiation

Compared with the controls, the gene expression of the master promoter SOX-9 was up-regulated through the differentiation (**Figure 2A**), and peaked in the second week. The differences were all statistically significant. And the expression of the marker gene aggrecan peaked in the second week and then was down-regulated (**Figure 2B**). The difference of the second week was statistically significant compared with the controls. Moreover, the gene expression of col-IIa1 and col-Xa1 were gradually up-regulated and then down-regulated at last (**Figure 2C**, **2D**) as compared with the control. These results showed the transformation

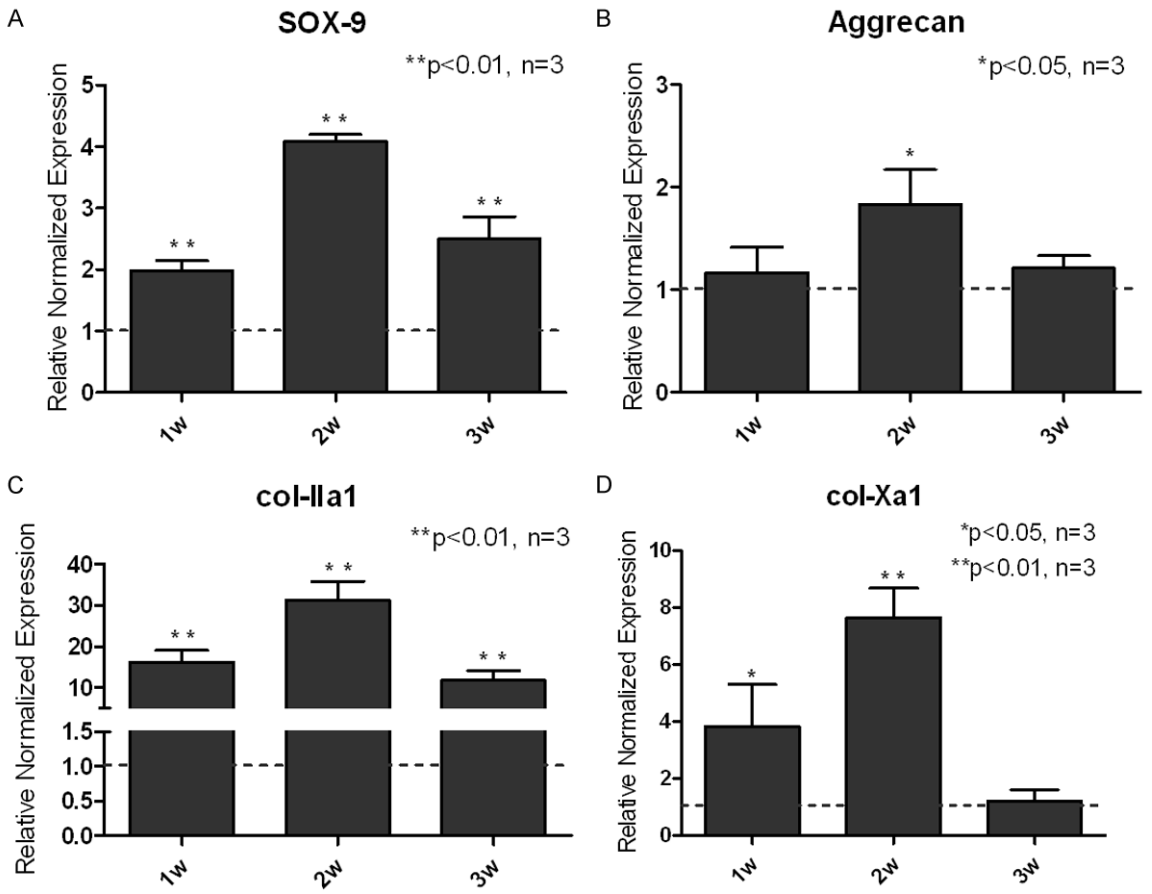

Figure 2. Chondrocyte gene expression by q PCR. A. SOX9; B. Aggrecan; C. Collagen IIa1; D. Collagen Xa1. Compared with the control group, SOX-9 expression was up-regulated at the 1st, 2ed, 3rd week while it peaked on the 2ed week and t values were 10.237, 47.515, 7.201; *P* values were 0.009, 0.000, 0.019 respectively. The marker genes of aggrecan, col-IIa1/Xa1 were all promoted, which also peaked on the 2ed week. The t values were 4.345, 11.277, 11.204 and *P* values were 0.049, 0.008, 0.008 respectively. Moreover, in the 3rd week, aggrecan and col-Xa1 were down-regulated and no significant differences were showed in compared with the control group. And the col-IIa1 was up-regulated and the t value was 7.752 and *P* value was 0.016 in the 3rd week.

of C3H10T1/2 to hypertrophic (mature) chondrocytes.

Toluidine blue staining on pellets

Toluidine blue staining was performed on the pellets after differentiation for three weeks. The matrix of the tissue appeared light blue and the nuclei were dark. Based on the sectioning result, a dense cluster of cells were observed in the induced group, especially the aggregate fibre-like tissue surround the pellets and cartilage cavity-like lacunas appeared in the central area; While in the control group, the cells were scattered loosely and the ECM was sparsely dyed and less fibre-like tissue as the similar area of the induction (**Figure 3**).

miR-19a and CCND1 gene expression

Based on the RT-PCR result, the expression of miR-19a gradually up-regulated during the differentiation, and the differences of the second and the third week were statistically significant compared with the controls; while the expression of CCND1 was inhibited during the differentiation and the differences of the second and the third week were statistically significant compared with the controls. In addition, by bioinformatics prediction on the internet (*http://www.microrna.org/microrna, http://www.targetscan.org*), miR-19a can specifically bind with CCND1 mRNA, which regulate cell cycle (**Figure 4**).

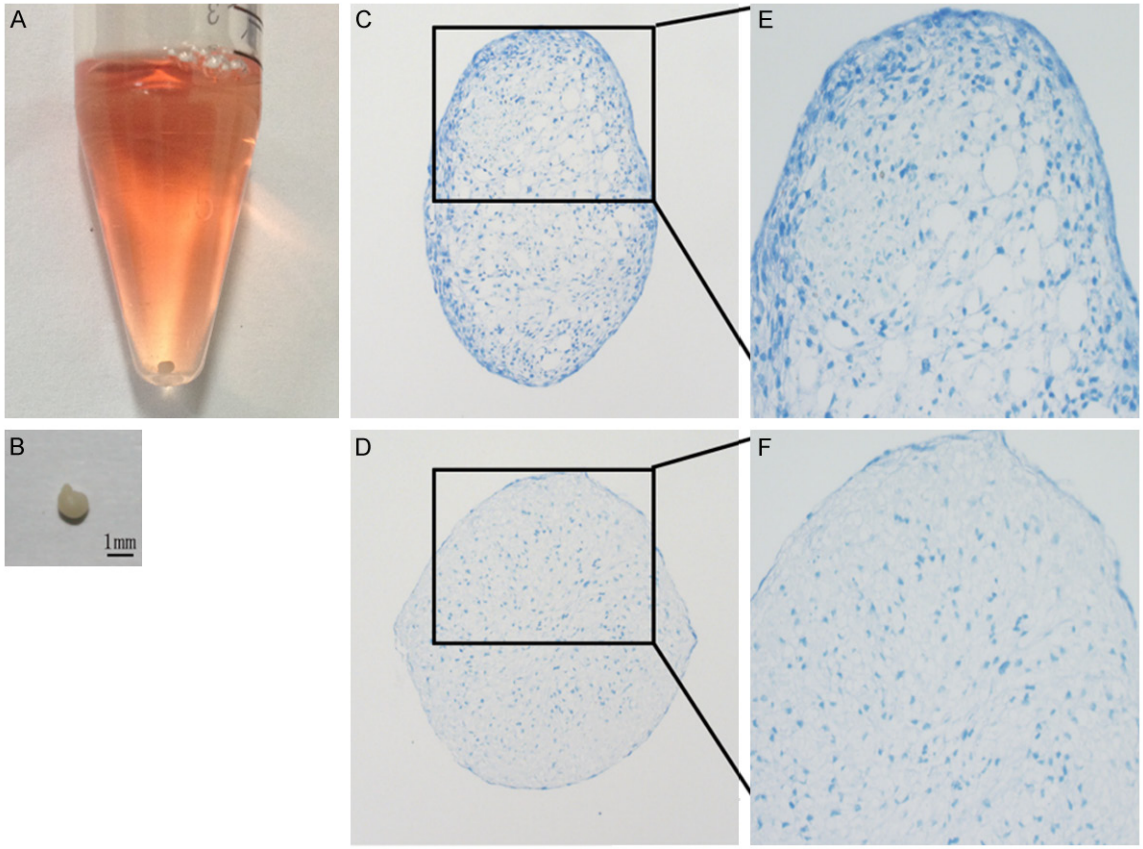

Figure 3. Histochemical staining of toluidine blue for 3 week pellet. (A, B) The pellet cultured in medium and before paraffin embedded; (C) Toluidine blue staining (induction group), ×20; (D) Toluidine blue staining (control group), ×20; (E) Toluidine blue staining of the (C) slices, ×40; (F) Toluidine blue staining of the (D) slices ×40.

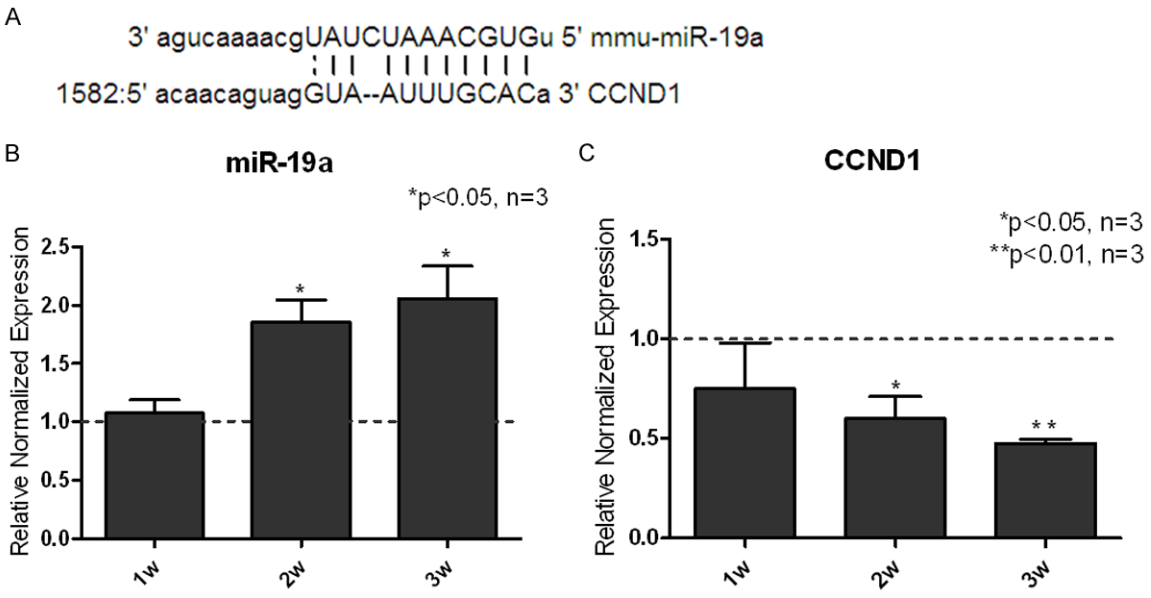

Figure 4. The gene expression of miR-19a and CCND1 in C3H10T1/2 chondrocytes differentiation. A. A diagram of miR-19a binding sites predicted from internet (http://www.microrna.org/microrna). B. In C3H10T1/2 chondrogenic differentiation, miR-19a was up-regulated in 2ed to 3rd week compared with the control group. The t values were 7.726, 6.528 and *P* values were 0.016, 0.023 respectively. C. The gene expression of CCND1 was down-regulated in the 2ed and 3rd week. The t values were 6.262, 46.783 and *P* values were 0.025, 0.000 respectively.

Discussion

Stem cell is a group of multipotent cells with self-renewal capacity as well as differentiation into various cell lineages that are sensitive to extracellular signals [14]. As it exposed in unfavourable microenvironment, embryonic stem cells (ESC) can adopt it through the microRNA pathway so as to remain in the G1/S phase [10]. Moreover, compared with tumour cells in capacity of cell division and proliferation, stem cells are under control in proliferation, division and differentiation [8, 15]. Although initial phases of oncogenesis were not fully understood, we have interest in explore regulation in cellular proliferation and differentiation that help itself to avoid excessive proliferation functional defects of tissues.

Sox9 had been known as the master transcriptional factor and promoted expression of marker genes of aggrecan, col-IIa1/Xa1 in chondrogenic differentiation [16, 17]. We found in our pellets model, the gene expression of the promoter Sox9 and the marker genes aggrecan and col-IIa1/Xa1 was all up-regulated. Histologic sectioning showed that the ECM of the pellets in the induced group was obviously intensified when compared with the controls. These observations possibly indicated there was much more ECM in the induced group and a denser fibre-like tissue compared with the controls, which constructed the pellets' formation. These results indicate the feasibility of chondrogenic differentiation of C3H10T1/2 pellets *in vitro*.

During the differentiation, the genes expression of Bax and Klf-4 was gradually down-regulated and these results indicated the cells apoptosis receded while maintained undifferentiation. The gene expression of CCND1 that engaging in the regulation on the cell cycle was also down-regulated. Based on these results, we predict the genes associated with cellular proliferation, apoptosis and undifferentiation were inhibited in the differentiation. When the master transcriptional factor of chondrogenic differentiation was triggered, the expression of the relevant marker genes gradually up-regulated; the ECM increased, thereby leading to the formation of mature cartilage.

There were special clustered microRNAs detected in pluripotent embryonic stem cells that were found in differentiated embryos and adults [18]. They display elaborate regulation in biological processes, such as renewal of stem cells and tissue differentiation and maintaining phonotypes. As the principle members of miR-17-92, a highly conserved gene cluster [19], miR-19a was commonly believed to participate in the regulation of cellular proliferation and differentiation. Some studies showed that miR-19a enhances proliferation and invasion functions of cervical cancer cells by targeting protein CUL5 [20]. Others showed that miR-19a promotes proliferation of lymphoma cells and lung cancer cells via targeting protein TNF-α [9]. In addition, miR-19a had been found to engage in the regulation of proliferation and survival of post-shock neural stem cells [21]. Furthermore, miR-19a could inhibit gene expression of PTEN, bim and CYLD1, which were inhibit factors of tumor cells and thus facilitate it growth [11]. However, miR-19a had not been reported in regulation of stem cell differentiation or proliferation by far.

It was generally considered that the proliferation of stem cells was inhibited during differentiation. In the results, the expression of miR-19a was up-regulated, indicating participation of the gene cluster miR-17-92 in the proliferation of stem cells during the differentiation and miR-19a was accompanied by the down-regulation of CCND1. Therefore, based on the Sox-9 gene expression as well as the marker genes aggrecan and col-IIa1/Xa1 associated with chondrogenic differentiation, we predict that during the differentiation, miR-19a regulated the gene expression of CCND1 and inhibit cellular proliferation. Because the intracellular targeting functions of microRNA are complex, we are incapable to specify whether miR-19a could target other genes expression. Given the nature of the miRNAs modification, this may be one mechanism that supports the chondrogenic differentiation and phonotypes maintenance.

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Disclosure of conflict of interest

None.

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