Abstract. The aim of the present study was to investigate the presence and biological function of microRNA-92a (miR-92a) in chondrogenesis and cartilage degeneration. Human adipose-derived mesenchymal stem cells (hADSCs) in micromass and chondrocyte-like ATDC5 cells were induced to chondrogenesis, and primary human/mouse chondrocytes (PHCs/PMCs) and chondrogenic ATDC5 cells were stimulated with interleukin-1β (IL-1β). An miR-92a mimic/inhibitor was transfected into the ATDC5 cells using lipofectamine 2000. Gene expression was analyzed using reverse transcription-quantitative polymerase chain reaction. Alcian blue was used to stain the cartilage nodules and chondrogenic micromass. The potential target genes, signaling pathways and functions of miR-92a were examined using miRanda, miRDB, CLIP-Seq, TargetScan and Kyoto Encyclopedia of Genes and Genomes. The expression of miR-92a was elevated in the chondrogenic ATDC5 cells and hADSCs, and also in the IL-1β-induced ATDC5 cells, PMCs and PHCs. Forced expression of miR-92a enhanced the expression levels of col9a2 and aggrecan. A total of 279 genes were predicted as potential target genes of miR-92a. The phosphoinositide 3-kinase/Akt, ErbB and focal adhesion kinase pathways, extracellular matrix (ECM)–receptor interaction and the mammalian target of rapamycin (mTOR) signaling pathway were suggested to mediate the effects of miR-92a on chondrogenesis and cartilage degeneration. These results demonstrated that miR-92a was involved in chondrogenesis and the chondrocyte response induced by IL-1β. miR-92a positively contributed to the expression of col9a2 and of aggrecan.

Introduction

Cartilage tissues are degenerated and are destroyed in osteoarthritic joints, which are more prevalent in elderly individuals (1). Although arthroplasty can efficiently relieve the symptoms of osteoarthritis, implant loosening is inevitable in the years following arthroplasty (1). Tissue engineered cartilage has been suggested as an improved substitution for conventional arthroplasty. Therefore, it is necessary to understand the molecular mechanisms underlying cartilage generation and degeneration.

Subsequent to mesenchymal condensation, mesenchymal stem cells sense cell-cell and cell-extracellular matrix (ECM) contact, which is termed focal adhesion (2), followed by differentiation into chondrocytes and expression of ECM. There are other exogenous stimuli and intracellular signaling pathways regulating chondrogenesis and cartilage degeneration, including the phosphoinositide 3-kinase(Akt), ErbB and focal adhesion kinase pathways, extracellular matrix (ECM)-receptor interaction and the mammalian target of rapamycin (mTOR) signaling pathway were suggested to mediate the effects of miR-92a on chondrogenesis and cartilage degeneration. These results demonstrated that miR-92a was involved in chondrogenesis and the chondrocyte response induced by IL-1β. miR-92a positively contributed to the expression of col9a2 and of aggrecan.

Key words: chondrogenesis, cartilage, osteoarthritis, microRNA-92a, col9a2
Materials and methods

The Ethics Committee of Sun Yat-Sen University (Guangzhou, China) approved the experiments performed in the present study. Procedures involving human subjects were performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from the patients prior to inclusion in the study. The experiments involving mice were performed in accordance with the Laboratory Animal Center of Sun Yat-Sen University and the Guide for the Care and Use of Laboratory Animals.

Primary chondrocyte isolation. Subsequent to obtaining informed consent, primary human chondrocytes (PHCs) were isolated from the cartilage of patients undergoing hip surgery. The patients included two females, aged 31 and 24 years, who were undergoing surgery for a femoral neck fracture at the First Affiliated Hospital of Sun Yat-Sen University. Patients with degraded cartilages, local or systemic immunological disorders or tumors were excluded from the investigation. The cartilage was carefully cut into sections and digested sequentially in pronase (cat. no. 10165921001; Roche Diagnostics, Basel, Switzerland) for 90 min and collagenase P (cat. no. 11213865001; Roche Diagnostics) for ~7 h on a 37˚C stirring-plate. The chondrocytes were then collected by centrifugation (1,000 x g for 3 min) of the digestion solution and then were rinsed with Ca/Mg-free phosphate-buffered saline (cat. no. 14190-094; Gibco Life Technologies, Paisley, UK) three times. The chondrocytes were seeded into flasks containing Dulbecco’s modified Eagle’s medium (DMEM/F12; cat. no. SH30023.01B; GE Healthcare Life Sciences, Logan, UT, USA) with 5% fetal bovine serum (FBS; cat. no. 10165-092; Gibco Life Technologies, Paisley, UK) three times. The chondrocytes were then subcultured when cells reached 90–100% confluence during the subcultures. The culture medium was replaced every 2 days and the cells were subcultured when cells reached 90-100% confluence during the expansion culture. All the experiments were completed within 20 passages. The chondrogenic differentiation was induced using ITS+ Premix (15-17). The chondrogenic culture medium was then replaced daily.

PHCs were cultured in DMEM/F12, 5% FBS, 1% penicillin and streptomycin and ITS+ at 37˚C in a 5% CO₂ humidified atmosphere.

The PMCs were cultured for expansion in M199 (cat. no. 11150-059; Gibco Life Technologies), 10% FBS, 1% penicillin and streptomycin, basic fibroblast growth factor (cat. no. 450-33; PeproTech, Oakland Park, CA, USA) and epidermal growth factor (cat. no. 315-09; PeproTech), at 37°C in a 5% CO₂ humidified atmosphere.

Interleukin-1β (IL-1β)-treated chondrocytes. ATDC5 cells were maintained in chondrogenic medium with 1% ITS+ for 14 days at 37°C to form chondrogenic ATDC5 cells. Chondrogenic ATDC5 cells, PHCs and PMCs, at the fourth passage, were treated with recombinant IL-1β (cat. no. 200-01B; PeproTech) at 1 ng/ml for 4 h (18-20).

Morphological analysis. The stained ATDC5 cells were fixed in formalin for 4 h at room temperature, and were stained with 1 mg/ml alcan blue 8GX for 20 min at room temperature, followed by examination using microscopy (Axio Imager Z1; Carl Zeiss AG, Oberkochen, Germany). The micromass was harvested at 0, 7 and 14 days. The micromass was examined by imaging with the M205 FA microscope [Leica Microsystems AG, Heerbrugg, Switzerland]. The micromass was fixed in formalin, embedded in paraffin, and stained with alcan blue (14). Images were then captured under microscopy.

Reverse transcription-quantitative polymerase chain reaction (RT–qPCR) assays. Total RNA was extracted from the cells using an miRNeasy Mini kit (cat. no. 271004; Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration and purity of the extracted RNA was analyzed using an Epoch Multi-Volume Spectrophotometer System (BioTek Instruments, Inc., Winooski, VT, USA). The cDNA of...
was obtained from mRNA and miRNAs using a PrimeScript® miRNA cDNA Synthesis kit (cat. no. DRR350; Takara Bio, Inc., Otsu, Japan), according to the manufacturer’s instructions.

Semi-qPCR was performed using SYBR® Premix Ex Taq™ II (cat. no. DRR081; Takara Bio, Inc.) and a Bio-Rad IQ5 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The concentration of reagents and cycling conditions were according to the manufacturer’s instructions. The cycles began at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Ten nanograms of cDNA was added into the 25 µl reaction volume. The primer sequences are presented in Table I.

Transfection assays. The condition and efficiency of transfection assays were verified using a CY3-labelled siR‑Ribo™ Transfection Control (cat. no. siN05815122149‑1‑1; Guangzhou RiboBio Co., Ltd., Guangzhou, China). The ATDC5 cells (4x10^4) were seeded into a 6-well plate with DMEM/F12 with 10% FBS, and were allowed to grow at 37˚C until they had reached 50-70% confluence. Lipofectamine® 2000 transfection reagent (cat. no. 11668; Invitrogen Life Technologies, Carlsbad, CA, USA) was then used to transfect the micrON™ mmu-miR-92a-3p mimic/inhibitor (cat. nos. miR10000539-1-2 and miR20000539-1-2; Guangzhou RiboBio Co., Ltd.) and micrON™ mimic/inhibitor negative control (cat. no. miR01101‑1‑2 and miR02101‑1‑2; Guangzhou RiboBio Co., Ltd.) into the cells, according to the manufacturer's instructions. Subsequent to 6 h transfection, chondrogenic differentiation was induced by replacing the medium with chondrogenic medium containing 1% ITS+ Premix.

Target prediction. The potential target genes of miRNAs were predicted using the following online algorithms: miRanda (August 2010 release; http://www.microrna.org/), miRDB (MirTarget2_v4.0; http://www.mirdb.org/miRDB/), CLIP-Seq (2012-03-28; http://mirtarclip.mbc.nctu.edu.tw/) and TargetScan (version 6.2; http://targetscan.org/). Genes predicted by three or four separate algorithms were considered as potential target genes.

Based on these predicted target genes, the signaling pathways potentially regulated by miR-92a were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG, kobas2.0-20120208; http://www.genome.jp/kegg/) database and the possible function of miR-92a was predicted.

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

| Gene               | Primer sequence (5’-3’)                     |
|--------------------|---------------------------------------------|
| Mmu/hsa-U6         | Forward: CTCGCTTCGGCAGCACA  
                      | Reverse: AACGCTTACGAATTTGCGT        |
| Mmu-GAPDH          | Forward: TGTGTCGGTCTGCTGAGATCTGA  
                      | Reverse: TTGCTGGTTGAGATTCTGAGGAGGG |
| Mmu/hsa-mir-92a    | TATTCGACTTGGCCCGCCGCTG  
                      | Forward: CCCGCCTTCCCAATTAGGAC  
                      | Reverse: GGGATTAAGAGCTCAAGGTGTGTTT |
| Mmu-col2a1         | Forward: TTCTGCTGATAATTCTGAGGGACAGAGAGG  
                      | Reverse: GCACTCACTGACTCGGTGGG       |
| Mmu-Sox9           | Forward: ATGCTTTACGCTTGCTACCAA  
                      | Reverse: GCCATGTTTGTGAGTGTGAG       |
| Mmu-Col10a1        | Forward: ATGCATTCAGCTATCCTGAGCGCAGAGAG  
                      | Reverse: AAGATGGAAGATTTGCTGAGCTGTG |
| Mmu-run2           | Forward: GCCATCGTGATGTTCTGCTGCTGCTGCTGCTG |
| Hsa-GAPDH          | Forward: GGAGCGAGATCCCTCCAAAT  
                      | Reverse: GGCTGTTTGTGAGTGTGAG       |
| Hsa-mmp13          | Forward: TCCCTATGTTGGGATAAACATATG  
                      | Reverse: GCCATCGTGAAAGATCTGTGAAAAT |
| Hsa-col2a1         | Forward: GAGGGCAATAGCAGGTTCAGTA  
                      | Reverse: TGGGGTCAATGTCATAGTGG      |
| Hsa-col10a1        | Forward: CACCAAGCATTCCAGAGGATCC  
                      | Reverse: AGGTTTGTGCTGATAGCTC       |

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Results

Expression of miR-92a is elevated in chondrogenic differentiation. The micromass produced from the hADSCs in chondrogenic medium was embedded in paraffin, cut into sections and stained with alcian blue (Fig. 1A). The expression levels of miR-92a, col2a1 and col10a1 increased in the chondrogenic hADSCs (Fig. 1B). The expression levels of mir-92a and the chondrogenic marker of col2a1 peaked at day 7 of chondrogenic induction, and the hypertrophic marker of col10a1 peaked at day 14.

Statistical analysis. All experiments were performed in triplicate. The quantitative data was expressed as the mean ± confidence interval (mean ± 1/2 CI). Differences between the groups were analyzed using Student’s t-test or analysis of variance with SPSS, version 13.0 (SPSS, Inc., Chicago, IL, USA). The least significant difference test and Tamhane’s T2 test were used in conditions with, and without, equal variances, respectively. The qualitative data was analyzed using Fisher’s Exact test. P<0.05 was considered to indicate a statistically significant difference.
Following 14 days of chondrogenic differentiation with ITS+ Premix, ATDC5 cells exhibited marked staining with alcian blue, compared with the cells without ITS+ Premix (Fig. 2A and B). The expression of col2a1 peaked at day 14, coll0a1 peaked at day 28 and miR-92a peaked at day 21 (Fig. 2C).

Expression of miR-92a is increased in IL-1β-treated chondrocytes. Expression levels of miR-92a and mmp13 were upregulated in the PHCs and PMCs treated with 1 ng/ml IL-1β for 4 h, compared with the control (Fig. 3A and B). The expression levels of miR-92a and mmp13 were elevated, and that of col2a1 was suppressed in a time-dependent manner.

Table II. Relative mRNA expression levels in ATDC5 cells transfected with miR-92a mimic or inhibitor.

| Gene | Mimic (50 nM) | Mimic (100 nM) | Inhibitor (50 nM) | Inhibitor (100 nM) |
|------|---------------|----------------|------------------|-------------------|
|      | Fold change P-value | Fold change P-value | Fold change P-value | Fold change P-value |
| Col2a1 | 0.89 0.24 0.14 | 0.74 0.02 0.10 | 1.11 0.14 0.10 | 0.40 <0.001 0.10 |
| Coll0a1 | 1.10 0.17 0.06 | 1.11 0.12 0.01 | 1.36 <0.001 0.12 | 0.75 0.001 0.05 |
| Comp | 0.61 <0.001 0.07 | 1.24 <0.001 0.07 | 0.91 0.014 0.06 | 0.24 <0.001 0.04 |
| Agc | 3.52 0.001 0.47 | 6.89 <0.001 0.76 | 0.56 <0.001 0.01 | 0.26 <0.001 0.01 |
| Mmp-13 | 1.94 0.004 0.47 | 3.03 <0.001 0.14 | 1.00 0.994 0.03 | 0.67 <0.001 0.02 |
| Co9a2 | 3.80 0.01 0.60 | 14.97 <0.001 1.95 | 0.37 <0.001 0.09 | 0.10 <0.001 0.02 |
| Sox9 | 2.45 <0.001 0.26 | 4.15 <0.001 0.50 | 1.18 0.15 0.16 | 1.10 0.40 0.23 |
| Runx2 | 1.59 <0.001 0.16 | 2.16 <0.001 0.12 | 0.91 0.19 0.02 | 1.00 0.98 0.13 |

Expression of chondrogenic markers in ATDC5 cells transfected with miR-92a mimic or inhibitor at the indicated doses. Subsequent to transfection, ATDC5 cells were cultured in chondrogenic medium with ITS+ Premix for 4 days. Chondrogenic markers were measured using reverse transcription-quantitative polymerase chain reaction. Co9a2 and aggrecan were markedly upregulated in the miR-92a mimic groups, but were downregulated in the inhibitor groups, in a dose-dependent manner. No clear trends were observed for the other markers. miR-92a, microRNA-92a; SD, standard deviation.
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in the chondrogenic ATDC5 cells treated with 1 ng/ml IL-1β (Fig. 3C).

Col9a2 and aggrecan may be regulated by miR‑92a. In order to investigate the effect of miR-92a on chondrogenesis, the expression levels of miR-92a were manipulated via transfection with a mimic or inhibitor. The altered expression of miR-92a affected chondrogenic markers, increasing the expression levels of col9a2 and aggrecan in a dose-dependent manner, compared with the untransfected control. In addition, the miR-193b-3p inhibitor reduced the expression levels of col9a2 and aggrecan in a dose-dependent manner, compared with the control (Table II).

Predicted target genes, signaling pathways and functions of miR‑92a.
The four algorithms, miRanda, miRDB, CLIP-Seq and TargetScan, were used for prediction of the miR-92a target genes. The general distribution of the predicted potential target genes is shown in Fig. 4. A total of 279 genes were predicted as potential target genes of miR-92a. In order to minimize possible false positive predictions, only genes predicted by three or four algorithms were identified as potential target genes of miR-92a. miR-92a, microRNA-92a. Numbers refer to genes predicted by one of the four software programs.

Table III. Predicted signaling pathways, based on the potential target genes of miR-92a.

| Signaling pathway             | P-value | Predicted target gene                                                                 | Function in chondrogenesis                                                                 |
|------------------------------|---------|---------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|
| PI3K-Akt                     | 0.064   | Sgk3, Phlp2, Pten, Pik3r3, Tsc1, Itga5, Itga6, Colla2, Akt1, Bcl2111 and Itgav         | Synergizing with runx2 to enhance normal hypertrophic differentiation and endochondral bone growth; promoting matrix synthesis and chondrocyte survival in adult articular chondrocytes (22). |
| ErbB                         | 0.076   | Akt1, Pik3r3, Map2k4 and Braf                                                          | Contributing to expression of aggrecanases and matrix metalloproteinases, delayed chondrogenesis and inhibition of the PI3K-Akt signaling pathway via downstream MAPK activation (23). |
| Focal adhesion                | 0.014   | Rap1b, Pten, Pik3r3, Itga5, Itga6, Braf, Colla2, Akt1 and Itgav                        | Inhibiting chondrogenesis via expression of actin and activation of the RhoA/ROCK pathway (24). |
| ECM-receptor interaction      | 0.024   | Colla2, Sdc2, Itga5, Itga6 and Itgav                                                   | Inhibiting chondrogenesis via Itga5-mediated cellular-ECM interaction (25). |
| mTOR                         | 0.007   | Pten, Tsc1, Pik3r3, Braf and Akt1                                                     | Reducing bone growth and hypertrophy; enhancing insulin-like growth factor I mediated proteoglycan synthesis in adult articular chondrocytes (5). |

PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; ROCK, Rho-associated protein kinase; ECM, extracellular matrix; mTOR, mammalian target of rapamycin.

PBK, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; ROCK, Rho-associated protein kinase; ECM, extracellular matrix; mTOR, mammalian target of rapamycin.
Figure 5. Predicted signaling pathways mediating the effects of miR-92a on chondrogenesis and cartilage degeneration. Predicted potential target genes of miR-92a are indicated by the red boxes. (A) PI3K-Akt, (B) ErbB and (C) focal adhesion signaling pathways were predicted based on the potential target genes of miR-92a. miR-92a, microRNA-92a; PI3K, phosphoinositide-3 kinase; mTOR, mammalian target of rapamycin.
Previous studies have suggested a role for miR-92a in renal tumorigenesis via the gene expression of VHL (26), and in human acute promyelocytic leukemia via the expression of p63 (27). An additional study identified the positive effects of miR-92a on the proliferation, differentiation and survival of chondrogenic progenitors via the targeting of nog3, an inhibitor of the bone morphogenetic protein (BMP) signaling pathway (28). Although miR-92a was observed to contribute to chondrogenesis by enhancing the expression of col2a1 in the study by Ning et al (28), no significant trend in the expression of col2a1 was not observed in the present study following transfection of either the miR-92a mimic or inhibitor. This discrepancy may be due to differences in experimental subjects and signaling pathways. In the study by Ning et al (28), the BMP signaling pathway (smad2/3) was observed to mediate the effects of miR-92a on in vivo pharyngeal chondrogenesis. In the present study, cultured ATDC5 cells were used for the investigation of miR-92a and chondrogenesis, which are associated with the autocrine transforming growth factor-β (smad2/3) signaling pathway (29).

In the study by Ning et al (28), the morphological defects resulting from the inhibition of nog3, one of the target genes of miR-92a’s, were partially reversed by p53 co-inhibition, suggesting a contribution of miR-92a-nog3-apoptosis/proliferation to in vivo morphological regulation of pharyngeal cartilage formation. During chondrogenesis, high levels of type 9 collagen and aggrecan are expressed, along with additional matrix proteins to form the cartilage matrix, with col9a2 and aggrecan considered as chondrogenic markers (30). Col9a2 and aggrecan were previously demonstrated to be associated with a number of diseases, including osteoarthritis (31,32), degeneration of intervertebral discs (33,34) and multiple epiphyseal dysplasia, characterized as the deformed deposition

Figure 5. Continued. Predicted signaling pathways mediating the effects of miR-92a on chondrogenesis and cartilage degeneration. Predicted potential target genes of miR-92a are indicated by the red boxes. (D) ECM-receptor interaction and (E) mTOR signaling pathways were predicted based on the potential target genes of miR-92a. miR-92a, microRNA-92a; PI3K, phosphoinositide-3 kinase; mTOR, mammalian target of rapamycin.
of cartilage at the ends of the bones (32,35,36). The present study hypothesized that col9a2 may be another mediator of the degeneration of cartilage, followed by miR-92a knockdown. For the upstream regulation of col9a2 and aggrecan, Sox9 has been previously suggested as to be critical in initiating the expression of col9a2 and aggrecan (37), although multiple enhancers have been observed to initiate expression of aggrecan (38). However, more detailed information is required on the regulation of the expression levels of col9a2 and aggrecan in order to identify the cure for these diseases.

In the present study, the results indicated that miR-92a may contribute to the upregulation of col9a2 and aggrecan, without enhancing the expression of sox9. These results provided novel insight into the upstream regulation of col9a2 and aggrecan, beyond what is already known about sox9 in relation to col9a2 and aggrecan. In addition, the results suggested another possible mechanism of a miR-92a-col9a2-cartilage deformity axis contributing to cartilage deformity following miR-92a knockdown. Further investigations are required in order to verify the effect of miR-92a on the in vivo expression levels of col9a2, aggrecan, and cartilage degeneration, and to determine the underlying mechanisms.

Several previous studies investigating miRNAs used one or two algorithms to predict the target genes, with subsequent mechanistic experiments, based on the predicted genes (39,40). However, each of these widely used algorithms has an intrinsic false positive rate. The false positive rate is 22-31% for TargetScan, 24-39% for miRanda and ~30% for PicTar (41). In the present study, four algorithms were used, and an intersection set of predicted genes from at least three algorithms was identified as a potential target gene. Based on the potential target genes, KEGG analysis was then used to predict several signaling pathways that possibly contribute to the effect of miR-92a on chondrogenesis. KEGG is a database, which is usually used for the prediction of function and signaling pathways from large scale molecular information of high-throughput experiments, including sequencing. This prediction method enables the minimization of false positive rates and assist in understanding the possible function of miR-92a in a wider context (42). Investigations of underlying mechanisms can be performed using these predictions, including luciferase reporter assays of miR-92a and 3'-UTR of Akt1.

In conclusion, the present study demonstrated the presence of miR-92a in chondrogenesis and the chondrocyte response induced by IL-1β. The positive contribution of miR-92a in the expression of col9a2 and aggrecan was observed and the PI3K-Akt, ErbB and focal adhesion kinase pathways, ECM-receptor interaction, and mTOR signaling pathway were indicated as potential mediators of the effects of miR-92a on chondrogenesis and cartilage degeneration.

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