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The microRNA expression profiles of mouse mesenchymal stem cell during chondrogenic differentiation

Bo Yang[1], *Hongfeng Guo*[1], *Yulan Zhang*[1,2], *Shiwu Dong*[1,*] *& Dajun Ying*[1,*]
[1]Laboratory of Biomechanics, Department of Anatomy, the Third Military Medical University, Chongqing 400038, [2]Department of Anesthesiology, Chengdu Military General Hospital, Chengdu 610083, P.R. China

MicroRNAs are potential key regulators in mesenchymal stem cells chondrogenic differentiation. However, there were few reports about the accurate effects of miRNAs on chondrogenic differentiation. To investigate the mechanisms of miRNAs-mediated regulation during the process, we performed miRNAs microarray in MSCs at four different stages of TGF-β3-induced chondrogenic differentiation. We observed that eight miRNAs were significantly up-regulated and five miRNAs were down-regulated. Interestingly, we found two miRNAs clusters, miR-143/145 and miR-132/212, kept on down-regulation in the process. Using bioinformatics approaches, we analyzed the target genes of these differentially expressed miRNAs and found a series of them correlated with the process of chondrogenesis. Furthermore, the qPCR results showed that the up-regulated (or down-regulated) expression of miRNAs were inversely associated with the expression of predicted target genes. Our results first revealed the expression profiles of miRNAs in chondrogenic differentiation of MSCs and provided a new insight on complicated regulation mechanisms of chondrogenesis. [BMB reports 2011; 44(1): 28-33]

INTRODUCTION

Bone marrow mesenchymal stem cells (MSCs) possess the potency of self-renewal and multipotential differentiation. In vitro, MSCs can be induced to differentiate into several cell types, including chondrocytes (1), osteoblasts (2), adipocytes (3), discus intervertebralis cells (4), ligament cells (5), etc. In vivo, MSCs participate in permanent and temporary chondrogenesis during embryo development process (6, 7). Articular cartilage formation is a process of permanent chondrogenesis, which is formed from primary MSCs condensed, differentiation into stable chondrocytes and secretion of extracellular matrix. Additionally, the process of entochondrostosis is a type of temporary chondrogenesis. The characters of MSCs that they can be obtained with less injury and manipulated in vitro more conveniently make them a promising cell substitute for chondrocytes in cartilage regeneration. At present, autologous MSCs have been transplanted in human injured or osteoarthritis (OA) knees for repair of articular cartilage defects (8, 9). However, unexpected results from ectopic transplatation of MSCs also have been reported, such as hypertrophy, mineralization and vascularisation (10, 11). Therefore, for safe clinical application of MSCs for repair of cartilage defects, it is crucial to better comprehend the mechanisms of controlling MSCs differentiation. However, the precise regulation mechanisms are still poorly understood.

MicroRNAs (miRNAs) are a class of approximately 22nt single-stranded non-protein coding RNAs, which identify target mRNA through 5'-seed sequences interactions with miRNAs regulatory elements (MREs) located in 3'-untranslated region of target mRNA (12). Abundant evidence suggest that miRNAs play critical roles in controlling cell process such as cell proliferation, apoptosis and differentiation (13, 14). Furthermore, miRNAs expression profiling has been applied to investigate the function of miRNAs in regulating self-renewal and differentiation of stem cells. Comparing miRNAs expression profiling of undifferentiated and differentiated human embryonic stem cells (hECs) suggests that miRNAs may involve in maintenance of pluripotency and differentiation (15). Combining the expression pattern of miRNAs and mRNAs during murine development indicates that miRNAs may regulate the maintenance or differentiation of murine ES cells via their target genes (16). Furthermore, a few studies also have identified miRNAs expression profiling in differentiating MSCs, suggesting that miRNAs act as important regulator in controlling MSCs fate(17-19). Taken together, investigation of miRNAs expression pattern would provide new insights into regulation of MSCs fate decisions.

Previously, there were few studies about the mechanisms of miRNAs regulating chondrogenesis. MiR-140 is tissue-specific expressed in cartilage during embryonic development and play important role in chondrocytes proliferation and differentia-

*Corresponding author. Shiwu Dong, Tel: 86-23-68752225; Fax: 86-23-68752226, 400038; E-mail: dongshiwu@163.com, Dajun Ying, Tel: 86-23-68752226; Fax: 86-23-68752226; E-mail: ydj96@yahoo.com.cn
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tion. It has been validated that histone deacetylase 4 (HDAC4) is its downstream target gene (20-22). Another research showed that miR-199* might effect its target gene SMAD1 to regulate chondrogenic differentiation of MSCs (23).

In this study, we investigation expression profiles of miRNAs in MSCs at the different stages of chondrogenic differentiation. Furthermore, several potential target genes were found that they were correlated with the process of chondrogenesis through bioinformatics software. In addition, the mRNA levels of four predicted target genes expressed inversely correlated to the expression level of corresponding miRNAs. These results reveal that these miRNAs might participate in the regulation of the process via a series of target genes. Our study may provide a new clue for exploration the precise roles of miRNAs in chondrogenic differentiation of MSCs in future.

RESULTS

Identification the phenotype of MSCs and chondrogenic-differentiated MSCs

Mouse bone marrow derived MSCs was isolated by different plastic adhesiveness from other cell types of bone marrow. MSCs were passaged and the fourth generation cells maintained theirs spindle shape (Fig. 1a). After a few days (about 10 d) there were in the monolayer chondrocytes cultures some small areas where the cells began to form multilayered nodules (Fig. 1b). The phenotype of MSCs was performed immunophenotype analysis by using flow cytometry. The expression of CD34 and CD45 (hemopoietic stem cell surface antigen markers) of isolated MSCs were negative, while CD44 and CD90.2 were positive (Fig. 2). After induction of chondrogenic differentiation by TGF-β_3, histology and immunohistochemistry staining were performed to detect the expression of proteoglycans and type II collagen. The results revealed that proteoglycans (Fig. 1c) and type II collagen (Fig. 1d) obviously expressed in experiment groups treated with TGF-β3.

Expression profiles of miRNAs

MiRNAs are important regulators of the differentiation process of stem cells. To understand the precise molecular mechanisms by which TGF-β3 induced MSCs chondrogenesis, MSCs were treated with TGF-β3 and their total RNA was extracted at four different stages of chondrogenesis (induced at 0 d, 7 d, 14 d and chondrocytes). Afterwards, microarrays were performed to detect the relative expression levels of miRNAs. By comparison analysis, we observed eight significantly up-regulated (Fig. 3a) and five down-regulated miRNAs (Fig. 3b) in the process of chondrogenic differentiation of MSCs ($P < 0.05$). Mir-140* was the most significantly up-regulated and miR-132 was the most significantly down-regulated. Interestingly, as the miRNA cluster, miR-143/145 and miR-132/212 expressed as similar down-regulated tendency. MiRNAs expression data were subjected to hierarchical clustering (Fig. supple data 1), revealing the relative expression of differentially expressed miRNAs. Taken together, the expression levels of many miRNAs were modulated in the process of chondrogenic differentiation of MSCs, suggesting that miRNAs may involve in regulating the process.

Validation of miRNA and mRNA expression by real-time quantitative PCR

To identify microarray data, two of the significantly up-regu-

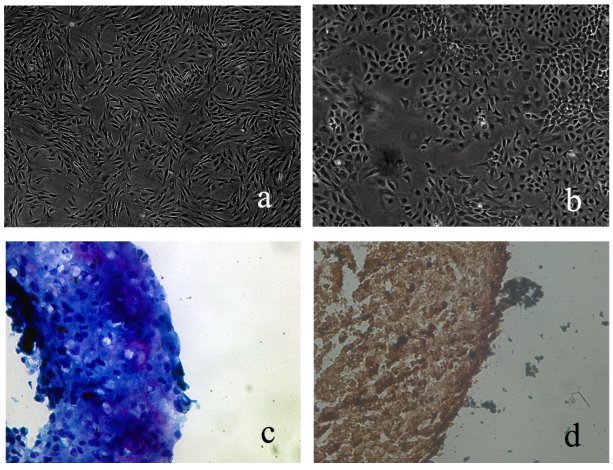

Fig. 1. Morphological features and identification of cultured cells. (a) The fourth passaged MSCs remained spindle-shaped (×100). (b) More confluent chondrocytes at 10 d (×100). (c) Toluidine blue staining of MSCs chondrogenic differentiated at 14 d (×400). (d) Immunohistochemistry stained for type II collagen of MSCs chondrogenic differentiated at 14 d (×400).

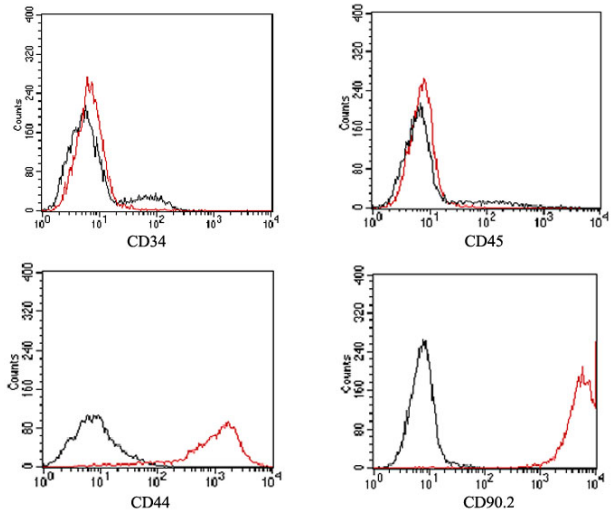

Fig. 2. Immunophenotype analysis of MSCs by using flow cytometry. CD34 and CD 45 negatively expressed, and CD44 and CD90.2 positively expressed (black curve represented isotype control).

lated (miR-140*, miR-30a) and two of down-regulated (miR-132, miR-212) were selected to perform qPCR. As shown in Fig. 3c, the qPCR results exhibited a high coincidence with our microarray results. Although the mechanisms by which miRNAs regulates their targets are not completely understood, miRNAs can fine-tune the expression of target gene under transcriptional control in mammalian (24, 25). To further characterize the miRNAs expression profile in relation with the expression of target genes, the expression of four predicted target genes, namely Sox6, ACVR1B, Runx2 and ADAMTS5, were chosen to analyze by qPCR. The functions of four predicted target genes were well-known to involve the process of chondrogenesis. As expected, the expression levels of four target genes were inversely correlated to the levels of corresponding miRNAs (Fig. 3d). These results suggest that these differential expressed miRNAs may be modulating the expression of genes required during chondrogenic differentiation of MSCs.

Target gene prediction

By the combination of targetScan, PicTar and miRanda online target gene prediction software, we predicted the target genes of differential expressed miRNAs. On the basis of previous studies on genes functions for chondrogenesis, we screened the miRNAs whose target genes were related to chondrogenesis. Among these miRNAs, miR-140*, miR-140, miR-30a, miR-132/212 and miR143/145 were found that the functions of their potential target genes were correlated with chondrogenic differentiation (Table Supple data 1). In these candidate miRNAs, miR-140 has been previously described as chondrogenesis-related miRNA, while the others not.

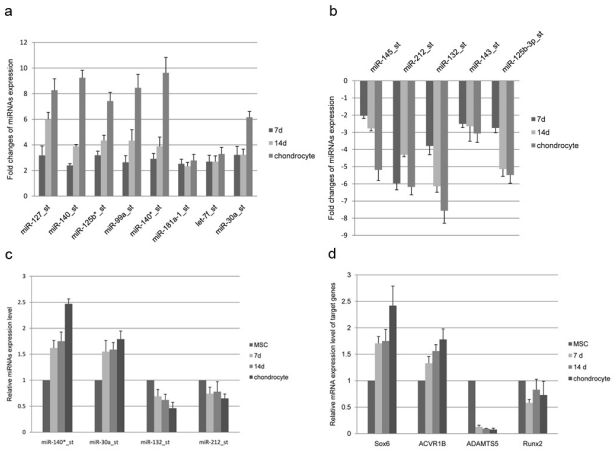

Fig. 3. Specific miRNAs and mRNAs expression during chondrogenic differentiation analyzed by microarray and qPCR. (a) Eight up-regulation miRNAs in microarray. (b) Five down-regulation miRNAs in microarray. (c) Validation of four differential expressed miRNAs by qPCR. (d) Validation of four mRNAs of predicted target genes by qPCR.

DISCUSSION

Chondrogenic differentiation of MSCs is a process which needed organism to precisely regulate the cells interactions with extracellular matrix and multiple differentiation factors. Various adhesion molecules, signaling pathways and transcription factors play important roles in the process. Considering notable effect of miRNAs in regulating cell differentiation, we performed microarray to obtain miRNAs expression profiles of mouse MSCs at four different stages of chondrogenic differentiation. Our study first demonstrated a more refined miRNAs expression profile during the complicated differentiation process, which should be responsive to TGF-β3. We observed the expression of thirteen miRNAs were significantly changed, including eight up-regulated and five down-regulated. Among these miRNAs, four remarkably differential expressed miRNAs were subsequently identified by qPCR, revealing the reliability of our microarray data. The similar expression patterns of miR-140, miR-145 and miR-143 in chondrogenic differentiation also have been described in previous studies (23, 26).

Furthermore, we predicted the target genes of these differential expressed miRNAs by bioinformatics. As shown in Table 1, there were some target genes' functions which were associated with chondrogenesis. Importantly, the expression levels of miRNAs and target mRNAs, which were identified by qPCR, showed that two up-regulate miRNAs (miR-140*, miR-30a) were associated with decreased expression of their corresponding predicted target mRNA (ADAMTS5, Runx2), while two down-regulated miRNAs clusters (miR-132/212, miR-143/145) were associated with increased expression of their corresponding predicted target mRNA (Sox6, ACVR1B). The expression changes of predicted target genes, which were inverse to the change of miRNAs, suggest that miRNAs may regulate the chondrogenic differentiation by inducing degradation of mRNAs of target genes.

Besides four genes identified by qPCR, several predicted target genes probably involved in the miRNAs-mediated regulation network. Among these potential target genes, we noticed that transcription factor Sox family member, Sox9 (miR-145) and Sox6 (miR-143, miR-132/212) play their respective roles in different stages of chondrogenic differentiation (27). As Sox9 downstream effectors, Sox6 and L-Sox5 (miR-132/212) co-expressed with Sox9 in MSCs and chondrocytes precursors, and interacted with each other to regulate chondrogenesis (28). Additionally, a recent study demonstrated that transcription factor RelA (miR-140*) might enhance Sox9 promoter to impact chondrogenic differentiation (29). Opposite to the effect of Sox family, another important transcription factor, Runx2 (miR-30a and miR-140*) interacted with multiple factors to suppress chondrogenic differentiation in the earlier period (30). Therefore, the process of chondrogenic differentiation would be regulated by miRNAs via these key transcription factors.

Among the predicted target genes, a series of SMADs family

members which include SMAD1 (miR-30a), SMAD2 (miR-132/212), SMAD3 and SMAD5 (miR-145) are involved in signaling pathways in regulation of chondrogenesis (31, 32). The molecules of signaling pathway are important factors in the process of chondrogenesis. Previously, SMAD1 has been confirmed to be a target gene of miR-199* during chondrogenesis (23).

Interestingly, we observed two miRNAs function clusters (miR-143/145 and miR-132/212) among five significantly down-regulated miRNAs. These miRNAs clusters might interact with each other to enhance the synergistic regulation effects. The function of miR-143/145 in smooth muscle cell (SMC), tumors, and stem cells have been investigated (33-35), demonstrating that they play a considerable role in regulating cells fate decision of multiple type cells. Sox9 and Sox6 were their potential target genes respectively. It might result in attenuating the suppression effect of Sox9 and Sox6 if expression of miR-143/145 was remarkably down-regulated, thereby enhancing the synergistic effect of Sox9 and Sox6 on promoting chondrogenesis. Considering down-regulation of miR-143/145 cluster might be critical factors in regulating chondrogenesis, we were performing to investigate the functions of miR-143/145.

Previous study demonstrated that miR-132/212 cluster was highly over-expressed in neurons and might participate in neurons differentiation (36). In our study, miR-132 significantly down-regulated in the process, suggesting that it would has second complementary function to regulate chondrogenesis. Among the predicted target genes of miR-132/212, Shh, Sox5 and Sox6 were well-known to be positive regulators of chondrogenesis. Other target genes, ERK2 and SMAD2 were signaling molecules in BMP-MAPK and TGF-β-SMAD signaling pathway, respectively. However, their effects related to chondrogenic differentiation might be differential in diverse cell types, induction conditions and cell differentiation stages. For example, in TGF-β1-induced hMSCs chondrogenic differentiation, suppressed activity of ERK1/2 resulted in significantly decreased secretion of type II collagen, suggesting ERK1/2 might positively regulate chondrogenesis (37). Contrarily, ERK1/2 acted as a negative regulator in mesenchymal chondrogenic differentiation of chick embryo limb bud (38). Therefore, further studies need to carry on for identification of the target genes and their precise functions in chondrogenic differentiation.

In conclusion, we applied microarray technology and exhibited miRNAs expression profiles in different stages of chondrogenic differentiation of mouse MSCs. By bioinformatics prediction, we obtained several target genes tightly correlated with the process of chondrogenesis of the candidate miRNAs. Our results suggest that miRNAs should be the important regulators in the course of chondrogenic differentiation and might provide a guide for further investigation about the mechanisms of miRNAs regulating chondrogenesis.

MATERIALS AND METHODS

Cell culture

The Balb/c mice (six to eight weeks of age) were obtained from Institute of Animal, the Third Military Medical University (Chongqing, China). The local institutional Animal Care and Use Committee approved all animal protocols. The protocol of MSCs isolation referred to it previously described (39). The fourth generation cells were used for the following experiments.

Chondrocytes was isolated from Balb/c mouse femoral head and tibial plateau cartilage surface. After scraped by operating knife blade, cells were dissociated by 0.25% trypsin for 1 h and incubated in 0.01% type II collagenase (Invitrogen) for 10 h at 37°C. After centrifuged at 1,500 rpm for 5 min, the cells were re-suspended with DMEM-F12 (Hyclone) containing 10% FBS and incubated at 37°C. The first generation cells were used for the following experiments.

Flow cytometer (FCM) analysis

MSCs was harvested and modulated at a cell density of 5×10^5/ml. PE/Cy7-conjugated anti-CD34 (Santa cruz), PE-conjugated anti-CD45, PE-conjugated anti-CD44 and PE-conjugated anti-CD90.2 (Biolegend) were added into 100 μl cell suspension respectively, and then cell suspension was incubated for 90 min at 37°C. After washed by PBS twice, FCM analysis was performed using 200 μl re-suspension.

Induction of Chondrogenic differentiation of MSCs

After trypsinization, MSCs were induced by chondrogenic differentiation induced medium (Cyagen), which contained dexamethasone, ascorbate, ITS+ supplement, sodium pyruvate, proline and TGF-β3. Briefly, cell density was modulated to 5×10^5/ml and 500 μl cell suspension was centrifuged at 1,000 rpm for 5 min with 15 ml polypropylene. The induced medium was replaced every 3 d. The cell pellets were then performed staining and total RNA extraction. Three biological replicates were performed for all cultured cell during differentiation (induced at 0 d, 7 d, 14 d and chondrocytes).

Histology and immunohistochemistry staining

The pellets to be processed for staining were routinely fixed by 4% paraformaldehyde, dehydrated and paraffin imbedded. 5 μm sections were stained by 0.1% toluidine blue for 2 min. Sections were immunohistochemistry stained for type II collagen using rabbit polyclonal antibody (Santa cruz). Following deparafinization, rehydration, antigen retrieval under boil, nonspecific antibody binding blocked. Sections were incubated with primary antibody (1 : 150 diluted) overnight at 4°C and followed to be rinsed with PBS. Sections were then incubated with biotin-conjugated anti-rabbit IgG secondary antibody (1 : 200 diluted, Jackson) for 60 min. The antibody-biotin conjugates were detected with streptavidin-biotin-horseradish peroxidase complex applied for 30 min using diaminobenzidine (Sigma) as substrate.

Microarray and data analysis

MiRNAs microarray technology was applied to detect miRNAs expression profiles of four different experiment groups, including mouse MSCs, chondrogenic induction at 7 d, 14 d and terminal chondrocyte. All samples were dissolved in Trizol (Invitrogen) according to the manufacturer's instructions. The experiments of miRNAs expression profile microarray were performed in Capital Bio Corporation (Beijing, China). The array contains 46,228 probes comprising 7,815 probe sets, including controls. Content is derived from the Sanger miRBase miRNA database v11 (April 15, 2008, http://microrna.sanger.ac.uk). Briefly, the procedures included total RNA extraction, samples quality control, miRNAs isolation using MirVana® miRNA isolation Kit (Ambion), FlashTag™ Biotin Labeling miRNAs (Genisphere), hybridization to Affymetrix® GeneChip® miRNAs microarray (Affymetrix), microarray washing, staining and scanning. Every detection parameter of miRNAs microarray was in accordance with quality control standards. T-test was performed for comparison analysis between two experiment groups and P value were calculated. When the miRNA expression level changed at least $\pm$2-fold and P value was less than 0.05, miRNA was considered significantly differential expression and selected to qPCR validation.

Real-time quantitative PCR

To validate the microarray results of different experiment groups, four significantly differential expressed miRNAs were selected to perform qPCR. U6 was acted as a internal control. Furthermore, four predicted target genes were performed qPCR to identify their mRNA expression pattern at the different stages of chondrogenic differentiation. GAPDH was acted as a internal control. Total RNA were used to generate cDNA using RT-PCR detection kit according to manufacturer's instructions (Epicentre). Subsequently, the synthesized cDNA were performed qPCR using qPCR System (Promega) with SYBR Green (Invitrogen). Data was analyzed using $2^{-\Delta\Delta Ct}$ method. All experiments were done in triplicate.

Target gene prediction

To predict miRNAs target genes, the combination of TargetScan (http://www.targetscan.org), Pictar (http://pictar.mdc-berlin.de) and miRanda (http://microrna.sanger.ac.uk/targets) three online software was used. All differentially expressed miRNAs were selected for target gene prediction.

Data analysis

Data were represented as mean $\pm$ SD from three independent experiments. T-test was performed for comparison analysis using SPSS 13.0. A value of $P < 0.05$ was considered as statistically significant. Hierarchical clustering of miRNA expression was performed using CLUSTER 3.0 and TreeView Software.

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