MicroRNA-145 Regulates Chondrogenic Differentiation of Mesenchymal Stem Cells by Targeting Sox9

Bo Yang1, Hongfeng Guo1, Yulan Zhang1,2, Lei Chen3, Dajun Ying1*, Shiwu Dong1*

1 Laboratory of Biomechanics, Department of Anatomy, The Third Military Medical University, Chongqing, People’s Republic of China, 2 Department of Anesthesiology, Chengdu Military General Hospital, Chengdu, People’s Republic of China, 3 Department of Orthopaedics, Southwest Hospital, Chongqing, People’s Republic of China

Abstract

Chondrogenic differentiation of mesenchymal stem cells (MSCs) is accurately regulated by essential transcription factors and signaling cascades. However, the precise mechanisms involved in this process still remain to be defined. MicroRNAs (miRNAs) regulate various biological processes by binding target mRNA to attenuate protein synthesis. To investigate the mechanisms for miRNAs-mediated regulation of chondrogenic differentiation, we identified that miR-145 was decreased during transforming growth factor beta 3 (TGF-β3)-induced chondrogenic differentiation of murine MSCs. Subsequently, dual-luciferase reporter gene assay data demonstrated that miR-145 targets a putative binding site in the 3′-UTR of SRY-related high mobility group-Box gene 9 (Sox9) gene, the key transcription factor for chondrogenesis. In addition, over-expression of miR-145 decreased expression of Sox9 only at protein levels and miR-145 inhibition significantly elevated Sox9 protein levels. Furthermore, over-expression of miR-145 decreased mRNA levels for three chondrogenic marker genes, type II collagen (Col2a1), aggrecan (Agc1), cartilage oligomeric matrix protein (COMP), type IX collagen (Col9a2) and type XI collagen (Col11a1) in C3H10T1/2 cells induced by TGF-β3, whereas anti-miR-145 inhibitor increased the expression of these chondrogenic marker genes. Thus, our studies demonstrated that miR-145 is a key negative regulator of chondrogenic differentiation by directly targeting Sox9 at early stage of chondrogenic differentiation.

Introduction

Bone marrow mesenchymal stem cell (MSCs) possess the potency of self-renewal[1] and multipotential differentiation, such as chondrocytes, osteoblasts, adipocytes[2]. Additionally, MSCs present themselves as a ideal candidate for the regeneration of cartilage as they possess chondrogenic differentiation potential, are easily obtained and expanded in vitro[3,4]. As chondrogenic differentiation rarely occurs spontaneously, the investigation of precise mechanisms for chondrogenic differentiation will ultimately contribute to a better understanding of skeletal development and diseases. Chondrogenic differentiation of MSCs is regulated by several transcription factors and growth factors, such as SRY-related high mobility group-Box (Sox) genes and the transforming growth factor (TGF)-β superfamily, respectively. In chondrogenesis, TGF-β stimulation is necessary for chondrogenesis derived from MSCs[2]. Preferentially, TGF-β1 or TGF-β3 is the most commonly used growth factor for chondrogenic differentiation. TGF-β3 enhances the early chondrogenesis of MSCs[3] and maintains a chondrogenic phenotype[6]. The cross-talk between TGF-β signal and transcription factors has an important role for the chondrogenesis of MSCs. For example, TGF-β receptor-regulated Smad3 and p300 cooperatively activate the Sox9-dependent transcription to promote the early chondrogenesis[7]. However, the precise mechanisms involved in this process still remain to be defined. Further studies are required to investigate the molecular mechanisms involved in the regulation of chondrogenesis of MSCs in response to the stimulation of TGF-β.

MicroRNAs (miRNAs) are short (21–24 nucleotides) noncoding RNAs, which are crucial regulators of protein-mediated gene expression[8]. After transcription, miRNA precursors are cleaved in the nucleus by Drosha[9], exported to cytoplasm by exportin[10,11], cleaved by Dicer, and then inserted into an RNA-induced silencing complex (RISC)[12]. MiRNAs repress target mRNA expression by binding the miRNAs regulatory elements (MREs) located in 3′-untranslated region (3′-UTR) of mRNAs[13]. Abundant evidence suggests that miRNAs play critical roles in controlling cellular processes such as cell proliferation, apoptosis and differentiation[14,15]. Particularly, miRNAs participate in controlling stem cells function and differentiation as evidence by the Drosha complex partners Loquacious (HIV-1 RNA binding protein 2), which is required for germ-line stem cell maintenance[16]. Additionally, Dicer-deficient mouse embryonic stem cells display severe defects in differentiation[17].

Previously, there were few studies about the mechanisms of miRNAs regulation in chondrogenic differentiation of MSCs. MiR-140 is tissue-specific for cartilage during embryonic development. It plays an important role in both cartilage development and homeostasis via regulating its downstream target genes, histone deacetylase 4 (HDAC4) and Smad3[18,19,20,21]. Lin et al found that miR-199* might affect its target gene Smad1 to regulate chondrogenic differentiation[22]. However, more
evidences of the roles of miRNAs in regulating chondrogenic differentiation is needed. In this study, to determine the roles of miRNAs in chondrogenic differentiation of MSCs, we focused on characterization of miR-145 whose expression level was gradually decreased during TGF-β3-induced chondrogenic differentiation of murine MSCs[23]. Other studies have identified the function of miR-145 involved in various oncogenic pathways [24,25,26], the differentiation of embryonic stem (ES) cells [27] and smooth muscle cells (SMCs) fate decisions [28,29,30]. Here, we show that miR-145 has a complementary role in suppressing chondrogenic differentiation of the murine embryonic mesenchymal cell line C3H10T1/2 cells. Through dual-luciferase reporter gene assay and gain- or loss-of-function experiments, we demonstrated that miR-145 can target and suppress the expression of SRY-related high mobility group-B Box gene 9 (Sox9). Sox9 is a master positive regulator of chondrogenesis and regulation of Sox9 may affect chondrogenic differentiation of MSCs [31,32,33,34]. Importantly, over-expression or suppression of miR-145 resulted in inhibiting or promoting chondrogenic differentiation, respectively. Our results suggest that miR-145 acts as a key mediator to antagonize early chondrogenic differentiation via attenuating the effect of transcription factor Sox9.

**Results**

**MiR-145 is down-regulated during TGF-β3-induced murine MSCs chondrogenic differentiation**

In our previous study, miRNA microarray technology was applied to detect miRNAs expression profiles of three different stages during chondrogenic differentiation, including murine MSCs, chondrogenic induction at 7 d, and 14 d after TGF-β3 treatment[23]. The expression of miR-145 significantly decreased during chondrogenic differentiation. Subsequently, we performed bioinformatic analyses to predict the target genes of miR-145 using Pictar [35] and Targetscan [36]. Noticeably, we found Sox9 was the potential target gene regulated by miR-145. According to the primary role of Sox9 in the process of MSCs differentiation into chondrocytes, we hypothesized that Sox9 may be inhibited by miR-145, which prevents MSCs from differentiating into chondrocytes. Furthermore, down-regulation of miR-145 may act as a positive effect on chondrogenic differentiation. Thus qRT-PCR assay was performed to validate the expression pattern of miR-145 in this study. The results confirmed that miR-145 gradually decreased in MSCs, which were induced by TGF-β3 (Figure 1).

**MiR-145 targets Sox9 by binding 3'-UTR of Sox9 mRNA**

MiRNAs inhibit mRNA expression by binding the MREs located in 3'-UTR of target mRNA. The Sox9 3'-UTR contains one putative miR-145 seed site which is bound with imperfect complementation (Figure 2A). To determine if miR-145 targets Sox9, we applied the luciferase reporter gene assay using the pMIR-REPORT Luciferase reporter. Firstly, we constructed a reporter vector containing a consensus miR-145-binding site within the 3'-UTR (pMIR-PT, Table 1) as a positive control. After we co-transfected this reporter plasmid into HEK293 cells with pre-miR-145 or its control pre-miR, we found that luciferase expression significantly decreased in the HEK293 cells transfected with pre-miR-145 (Figure 2B). These data indicate miR-145 can suppress expression of transcripts containing an exact miR-145-binding site by our luciferase reporter assay. Next, we created specific reporter vectors, which contained either two copies of the endogenous MREs found in the Sox9 mRNA 3'-UTR (pMIR-MRE, Table 1) or corresponding two copies of the MREs with a scrambled seed sequence (pMIR-MUT, Table 1). Previous studies have proven that such reporter constructed with multiple MREs is an available method for the identification of miRNAs target genes [37,38,39,40]. Co-transfection of pre-miR-145 with wild-type pMIR-MRE resulted in a suppression of luciferase gene expression, but co-transfection of pre-miR-145 and mutant pMIR-MUT did not (Figure 2B). Furthermore, the suppression effect occurred in a dose dependent manner (Figure 2C). In addition, we found that anti-miR-145 could overcome the suppression effect when it was co-transfected with pMIR-MRE and pre-miR-145. However, the effect of anti-miR-145 was abolished when pMIR-MUT was used instead of pMIR-MRE (Figure 2D). Taken together, our data suggest that miR-145 could target Sox9 by binding the MREs within the Sox9 mRNA 3'-UTR.

**MiR-145 inhibits Sox9 expression at early stage of chondrogenic differentiation**

The different degree of complementary between miRNA and its target mRNA probably determines that miRNA repress target mRNA through two distinct pathways. MiRNA suppresses mRNA translation bearing imperfect complementary target sequences and degrades mRNA bearing perfect complementary target sequences [41,42,43]. Computational algorithms predicted that miR-145 would bind to the Sox9 3'-UTR with imperfect complementation, suggesting that it may not result in Sox9 mRNA cleavage. To demonstrate whether miR-145 acts as attenuator of Sox9 protein expression, we transfected C3H10T1/2 mesenchymal stem cells with either pre-miR-145 or anti-miR-145 for 24 h, and then exposed the transfected cells to the chondrogenic differentiation medium primarily consisting of TGF-β3. We firstly performed the non-transfected control in preliminary experiment for optimizing the condition of transfection with miRNAs. At the optimal condition of transfection, there is no significant difference of Sox9 protein level compared with control. As expected for the mechanisms of miRNAs regulation, measured by western blot assay, Sox9 protein level was notably...
decreased in the cells of miR-145 over-expression and increased in the cells of miR-145 suppression at 1 d and 7 d but not at 14 d by TGF-β3 treatment (Figure 3A). However, qRT-PCR analysis showed no significant change in Sox9 mRNA level in both transfected cells (Figure 3B). Thus, these data demonstrate miR-145 inhibits Sox9 protein expression but not mRNA levels in mesenchymal stem cell line at early stage of chondrogenic differentiation.

MiR-145 inhibits early chondrogenic differentiation

To explore whether miR-145 has an effect on chondrogenic differentiation, we transfected either pre-miR-145 or anti-miR-145 into C3H10T1/2 cells. After induction of chondrogenic differentiation by medium containing TGF-β3 for 1 d and 7 d, qRT-PCR analysis of C3H10T1/2 cells transfected with pre-miR-145 showed a significant decrease in the mRNA expression levels of chondrogenesis markers including Col2a1, Agc1, COMP, Col9a2 and Col11a1 (Figure 4A). Moreover, alcian blue staining intensity were decreased following pre-miR-145 treatment for 3 d and 7 d (Figure 4B). These results reveal that miR-145 over-expression inhibits early chondrogenic differentiation. Inhibition of endogenous miR-145 expression in C3H10T1/2 cells by transfection of anti-miR-145, under the same induction conditions as above, resulted in enhancing chondrogenic differentiation as shown by a significant increase in chondrogenesis markers at mRNA level (Figure 5A) and alcian blue staining intensity (Figure 5B). The results of qRT-PCR and alcian blue staining have shown that modulation of miR-145 effected the expression of genes and GAGs related to chondrocyte after induced for 7 d. However, the same effect did not last for 14 d (Figure 4, 5). Collectively, our data demonstrate that miR-145 act as a key negative regulator of early chondrogenic differentiation.

Mir-145 has no influence on mRNA expression of C/EBPβ and C/EBPδ

To investigate whether the regulation effect of miR-145 mediated by Sox9 is specific to chondrogenesis, we measured the mRNA expression level of other Sox9 non cartilage target genes. Sox9 directly binds to the promoter regions of C/EBPβ and C/EBPδ to suppress their promoter activity, preventing adipocyte differentiation[44]. Our results showed that miR-145 has not effected the mRNA expression of C/EBPβ and C/EBPδ after induction of chondrogenic differentiation (Figure 6). It suggests that the effect which miR-145 regulate chondrogenic differentiation of MSCs mediated by Sox9 in response to TGF-β3 is a specific influence on genes associated with chondrogenesis.

Mir-145 has no influence on proliferation of C3H10T1/2 cells

Skelotogenesis is dependent upon proliferation of progenitor cells that is followed by differentiation. To investigate whether
miR-145 inhibition that leads to an increase in Sox-9 protein expression affects cell proliferation, we assessed the proliferation of C3H10T1/2 cells by direct cell counting. However, treatment of C3H10T1/2 cells with either pre-miR-145 or anti-miR-145 inhibitor did not significantly affect the cell proliferation (Figure 7).

Discussion

MiRNAs, as endogenous small molecular regulators of gene expression, play critical roles in stem cell function [45,46] and provide new insight into precisely controlling cell fate decisions. In this study, we identified that miR-145 can suppress TGF-β-mediated chondrogenesis as validated by microarray and qRT-PCR data. MiR-145 is decreased significantly in BMP-2-chondrogenic differentiation of murine MSCs. The similar expression patterns of miR-145 have been described in previous studies. MiR-145 is decreased significantly in BMP-2-induced C2C12 cells detected by microarray analysis [22]. Additionally, compared with the expression level in human cartilage chondrocytes, miR-145 is also decreased in dedifferentiation of chondrocytes as validated by microarray and qRT-PCR analysis [47]. Therefore, with the combination of our microarray and qRT-PCR data, miR-145 emerged as a candidate with significant potential to participate in the regulation of chondrogenic differentiation. Although miR-145 has been known to be involved in vascular pathogenesis by maintaining the differentiation status of smooth muscle cells (SMCs) and regulation of embryonic stem (ES) cells self-renewal program [27,48], we have identified another complementary function to inhibit Sox9 and C/EBPβ and C/EBPα at mRNA levels in the chondrogenic differentiation. However, we cannot find the certain evidence that miR-145 would affect on the terminal differentiation of chondrogenesis by a long-time effect (for 14 d). It is maybe due to the transient transfection of miRNAs. On basis of current results, we only demonstrate miR-145 can regulate chondrogenesis at early stage.

Because the condensation and proliferation of mesenchymal cells is the initial step of chondrogenesis and skeletogenesis, we used directly counting cells numbers from micromass pellets to detect the proliferation of C3H10T1/2 cells which were transfected with pre-miR-145 or anti-miR-145, respectively. Unexpectedly, there is no significant difference between pre-miR-145 or anti-miR-145 treatment pellets and their control treatment pellets. It suggest that the effect of miR-145 regulating chondrogenic differentiation depends on Sox9-mediated the promotion of its target genes associated with cartilage, such as Col2a1, Agc1 and COMP, etc..

An important feature of miRNAs is that one miRNA can regulate many target genes owing to the short seed match and imperfect base-pairing between miRNAs and their targets.
MiR-145 Regulates Chondrogenesis

A

| Condition     | Sox9 | β-actin |
|---------------|------|---------|
| pre-miR-145   | 1 d  |         |
| control       |      |         |
| anti-miR-145  | 7 d  |         |
| control       |      |         |

B

| Time  | Sox9   | β-actin |
|-------|--------|---------|
| 1 d   | 0.8    | 1.2     |
| 7 d   | 1.5    | 1.8     |
| 14 d  | 1.2    | 1.5     |
Accordingly, miR-145 may regulate the process of chondrogenic differentiation by suppressing other target genes besides Sox9. There are a few target genes correlated with chondrogenesis predicted by combination of Pictar and Targetscan, including Smad3, type-I activin receptor (ACVR1B also known as ALK4) and type-II activin receptor (ACVR2A). Smad3 has been confirmed as a positive mediator of TGF-β-induced chondrogenesis [51]. ACVR2A can be bound by activins, which belongs to the TGF-β superfamily of structurally related signaling proteins, leading to recruitment and phosphorylation of the ACVR1B. This complex, containing activins, goes on to recruit the R-Smads, Smad2 or Smad3 [52] thereby involving the regulation of chondrogenesis. Thus, miR-145 may regulate chondrogenesis by repressing not only Sox9 but also other genes.

In conclusion, our studies demonstrate that miR-145 is decreased during TGF-β3-induced chondrogenic differentiation of murine MSCs. The attenuation of miR-145 expression positively regulates its direct target gene Sox9, and results in promoting chondrogenic differentiation of mesenchymal stem cell line. Our findings indicate that miR-145 plays a key role in chondrogenesis and may provide a novel mechanism in miRNA-mediated regulation of chondrogenic differentiation of MSCs.

Materials and Methods

Cell Culture

Isolation of murine MSCs from bone marrow is previously described [23, 53]. Briefly, mouse tibia and femur marrow cavity was rinsed by the syringes containing the medium under sterile conditions, which composed of low glucose DMEM (L-DMEM), 10% fetal bovine serum (FBS, Hyclone), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma). The rinsed solution was filtered through a 70 μm filter mesh and incubated at 37 °C with 5% CO2 in a humified incubator. Culture medium was replaced after 3 h, and every 8 h repeated this step in the following 72 h. Thereafter, fresh medium was replaced every 3 d until the cells reached 90% confluence. Cells were passaged by 0.25% trypsin (Hyclone) for 2 min at room temperature. The fourth generation cells were used for the following experiments. The Balb/c mice (six to eight weeks of age) were obtained from Institute of Animal, the Third Military Medical University (Chongqing, China). The Southwest institutional Animal Care and Use Committee at the Third Military Medical University approved all animal protocols.

Transfection assay

To demonstrate the functional relevance of miR-145, pre-miR-145 (a final concentration of 50 nM), anti-miR-145 (a final concentration of 150 nM) or their negative controls were transfected, respectively, into C3H10T1/2 cells in 6-well pellets (105 cell per well) with 5 μl siPORT NeoFX transfection agent (Ambion) following the manufacturer’s instructions. After incubation for 24 h, the transfected cells were trypsinized and subjected to the chondrogenic differentiation assay. After indicated time points, the cells were harvested for mRNA and protein analysis.

Chondrogenic Differentiation Assay

Before chondrogenic differentiation, C3H10T1/2 cells ready for gain- or loss-of-function analysis, were transfected with pre-miR-145, anti-miR-145 or their negative controls. MSCs ready for miR-145 expression analysis were directly induced to chondrogenic differentiation. After transfection and incubation for 24 h, high density micromass cultures were treated as previously described [22]. The cells were trypsinized by 0.25% trypsin and modulated at a density of 105 cells/mL. 10 μl of the suspension was placed into the center of each well on a 12-well plate (Corning). After incubation for 2 h at 37 °C and 5% CO2, wells were flooded with 1 mL chondrogenic differentiation medium (Cyagen). The chondrogenic differentiation medium composed of dexamethasone, ascorbate, ITS+ Supplement, sodium pyruvate, proline and TGF-β3 was replaced every 2 d.

Quantitative RT-PCR Analysis

To validate the expression pattern of miR-145, which emerged in the microarray results, qRT-PCR was performed. U6 acted as an internal control. Total RNA was used to generate cDNA by TaqMan RT-PCR kit (Epicentre) according to manufacturer’s instructions. The RT-PCR primers are as follows: U6: 5′-CGC-CTTACGAGATTTCGTTGCTCAT-3′; miR-145: 5′-GTGCTGTACAGTGCGTGTCGGAATATTGCACTGGA-3′ for the miR-145 forward and 5′-TTGTAACCACCTGGGACGATATGG-3′ for the miR-145 reverse. qRT-PCR was performed. U6 was used as an internal control. The cycle parameters for the RT reaction were 15°C for 30 min, 42°C for 40 min, and 95°C for 5 min, in a final volume of 20 μl. Subsequently, the synthesized cDNA was used for real-time quantitative PCR with SYBR Green (Invitrogen) with the amplification parameters: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 20 s, 78°C for 20 s. The primers of real-time PCR are as follows: U6 forward: 5′-GCTTCAGGCAGCACATACTAATATT-3′; U6 reverse: 5′-CAGTGCGTGTCATGTTGCT-3′.

To determine the expression levels of Sox9, Col2a1, Agc1 and COMP, total RNA was performed RT-PCR using the Rever Tra Ace-a -First Strand cDNA Synthesis Kit (Toyobo) followed by real-time quantitative PCR with SYBR Green. β-actin acted as an internal control. The cycle parameters for the RT reaction were 42°C for 10 min, 30°C for 20 min, and 99°C for 3 min. Next, a reaction mixture (Promega) containing the SYBR Green and the appropriate primers was added to a 0.2 ml MicroAmp (ABI), together with 2 μl of cDNA template, for a final reaction volume of 20 μl. The amplification parameters were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s. The primers of real-time PCR are as follows: β-actin forward: 5′-GATCTTGTATCTTCTGATGGTGTAG-3′; Col2a1 reverse: 5′-TTGTTAACACCTTGGAAGCAGTATG-3′; Col2a1 forward: 5′-CCCGCCCTTCCCCATTATGGAC-3′; Col2a1 reverse: 5′-GGGAGGACGGTGTGGATATCA-3′; Agc1 forward: 5′-GGCCACACTTTGCATGACAGAGACG-3′; Agc1 reverse: 5′-CCCC-
Figure 4. Over-expression of miR-145 inhibits early chondrogenic differentiation of C3H10T1/2 cells. C3H10T1/2 cells were transfected with pre-miR-145 or its control respectively. (A) After 24 h, 7 d and 14 d of treatment with TGF-β3, all of cells were lysed and then the expression of chondrogenic differentiation markers, such as Col2a1, COMP, Agc1, Col9a2 and Col11a1, were measured via qRT-PCR. The relative expression level of mRNA in cells transfected with control oligonucleotide was set to one, as control. (B) After 3 d, 7 d and 14 d of treatment with TGF-β3, all of pellets were measured by alcian blue staining. Three independent experiments were done and data was represented as mean ± sd. *, p<0.05, when compared with control.

doi:10.1371/journal.pone.0021679.g004
Figure 5. Suppression of mir-145 enhances early chondrogenic differentiation of C3H10T1/2 cells. C3H10T1/2 cells were transfected with anti-miR-145 or its control respectively. (A) After 24 h, 7 d and 14 d of treatment with TGF-β3, all of cells were lysed and then the expression of chondrogenic differentiation markers, such as Col2a1, COMP, Agc1, Col9a2 and Col11a1, were measured via qRT-PCR. The relative expression level of mRNA in cells transfected with control oligonucleotide was set to one, as control. (B) After 3 d, 7 d and 14d of treatment with TGF-β3, all of pellets were measured by alcian blue staining. Three independent cell culture experiments were done and data was represented as mean±sd. *, p<0.05, when compared with control.
doi:10.1371/journal.pone.0021679.g005
Western Blot Analysis

The cell lysates from micromass cultures of C3H10T1/2 cells transfected with pre-miR-145 or anti-miR-145 were extracted with lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1% TritonX-100, 1% deoxycholate, 0.1% SDS, 1 mM PMSF and 0.2% Aprotinin (Sigma). After we measured the protein concentration, the equal protein samples were mixed with 5× sample buffer (Beyotime) and boiled. The samples were resolved by 10% SDS-PAGE gel and transferred on PVDF membrane (Millipore) by using the semi-dry transfer method. After blocking in 10% non-fat dried milk in TBST for 2 h, the blots were incubated with anti-Sox9 (Santa Cruz, diluted 1:700) or anti-β-actin antibody (Santa Cruz, diluted 1:1000) at 4°C overnight. β-actin acted as an internal control. After washing by TBST, the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz, diluted 1:2000) at room temperature for 1 h. The blots were visualized by Femto (Pierce) following the manufacturer’s instructions.

Generation of Luciferase Reporter Constructs

Target sequences for a consensus miR-145-binding site (PT), two copies of the endogenous MREs sequence in the Sox9 mRNA 3’-UTR (MRE) and corresponding two copies of the MREs with a scrambled MREs sequence (MUT) were synthesized (Invitrogen) and cloned into the SpeI/ HindIII site of the pMIR-REPORT Firefly Luciferase reporter vector (Ambion) using standard DNA techniques. The correctness of three plasmid DNA constructs, shown as pMIR-PT, pMIR-MRE and pMIR-MUT, were further identified by sequencing. All target sequences of inserts are available in Table 1.

Figure 6. Mir-145 has no influence on mRNA expression of C/EBPβ and C/EBPδ. C3H10T1/2 cells were transfected with pre-miR145, anti-miR-145 or its control, respectively. After 24 h of treatment with TGF-β3, all of cells were lysed. The expression of C/EBPβ and C/EBPδ were measured via qRT-PCR. The relative expression level of mRNA in cells transfected with control oligonucleotide was set to one, as control. Three independent cell culture experiments were done and data was represented as mean±sd. *, p<0.05, when compared with control.

doi:10.1371/journal.pone.0021679.g006
dent experiments were performed, each time in triplicate. 10 pmol of pre-miR-145 were used, respectively. To explore the efficiency of miR-145 inhibition, 2.5 pmol, 5 pmol, and 10 pmol of anti-miR-145 inhibitor were induced to chondrocytes for 24 h. Subsequently, all micromass pellets were trypsinized by 0.25% trypsin and directly counted using a hemacytometer. All experiment were done in three independent experiments and repeated counting in triplicate.

Cell proliferation assay

Directly counting cells numbers from micromass pellets was used to detect the proliferation of C3H10T1/2 cells. The micromass pellets which were treated with either pre-miR-145 or anti-miR-145 inhibitor were induced to chondrocytes for 24 h. Subsequently, all micromass pellets were trypsinized by 0.25% trypsin and directly counted using a hemacytometer. All experiment were done in three independent experiments and repeated counting in triplicate.

Statistical Analysis

Data are expressed as the mean±SD. Statistical comparisons were made between two groups with the t-test and between multiple groups with one-way ANOVA. A value of P<0.05 was considered significant unless otherwise described.

Supporting Information

Figure S1 Preliminary experiment of the non-transfect ed controls. Pre-miR-145 (a final concentration of 50 nM), anti-miR-145 (a final concentration of 50 nM), their negative controls and non-transfected controls were transfected into C3H10T1/2 cells in 6-well pellets, respectively. After transfection, cells were induced to chondrocyte by TGF-β3 for 24 h and then harvested for measurement of Sox9 protein expression using Western blot. β-actin acts as an internal control. Quantitation of the Sox9 protein level was performed using Quantity One software. The result is shown in the below panels. There is no significant difference of Sox9 protein expression on cells between transfected group and non-transfected control. Three independent experiments were done and data was represented as mean±SD. * p<0.05, when compared with control.

Acknowledgments

We wish to acknowledge Dr. Tao Wang, from the Institute of Combined Injury, Department Preventive Medical, of Third Military Medical University, for technical assistance of dual-luciferase analysis.

Author Contributions

Conceived and designed the experiments: BY SWD DJY. Performed the experiments: BY SWD HFG YLZ LC. Analyzed the data: BY SWD. Wrote the paper: BY SWD.

Figure 7. Mir-145 has no influence on proliferation of C3H10T1/2 cells. C3H10T1/2 cells were transfected with pre-miR-145, anti-miR-145 or its control, respectively. After 24 h of treatment with TGF-β3, all of pellets were trypsinized and directly counted in triplicate using a hemacytometer. Data was represented as mean±sd. doi:10.1371/journal.pone.0021679.g007

Dual-luciferase Reporter Gene Assay

For luciferase activity analysis, HEK293 cells (2×10³ cells per well) were co-transfected with 125 ng of luciferase reporter constructs, 25 ng of phRL-TK (Promega) Renilla luciferase plasmid and 10 pmol of miRNAs with 1 μl Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). The miRNAs transfected into cells were purchased from Ambion, including pre-miR-145 precursor, anti-miR-145 inhibitor and their respective negative controls. After incubation for 48 h, we carried out the luciferase assay using dual-luciferase reporter assay system (Promega) per the manufacturer’s instructions. Measurements of luminescence were performed on the luminoimeter (Glomax 20/20; Promega). In the dose-dependent experiment to explore the efficiency of miR-145 inhibition, 2.5 pmol, 5 pmol, 10 pmol of pre-miR-145 were used, respectively. Three independent experiments were performed, each time in triplicate.

References

1. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Pierzanti S, et al. (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131: 324–336.
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, et al. (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284: 143–147.
3. Osaki C, Schneider PRA, Shakhbazi M. (2000) Mesenchymal stem cells as a potential pool for cartilage tissue engineering. Annals of Anatomy-Anatomischer Anzeiger 190: 395–412.
4. Pittenger MF (2008) Mesenchymal stem cells from adult bone marrow. Methods Mol Biol 449: 27–44.
5. Fan HB, Zhang CL, Li J, Bi L, Qin L, et al. (2008) Gelatin microspheres containing TGF-beta 3 enhance the chondrogenesis of mesenchymal stem cells in modified pellet culture. Biomacromolecules 9: 927–934.
6. Melhorn AT, Schmal H, Kaiser S, Lepski G, Finkenzeller G, et al. (2006) Mesenchymal stem cells maintain TGF-beta-mediated chondrogenic phenotype in alginate bead culture. Tissue Eng 12: 1393–1403.
7. Furumatsu T, Ozaki T, Asahara H (2009) Smad3 activates the Sox9-dependent transcription on chromatin. Int J Biochem Cell Biol 41: 1198–1204.
8. Bartel DP (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281–297.
MiR-145 Regulates Chondrogenesis

9. Lee Y, Ahn G, Han JJ, Cho H, Kim J, et al. (2003) The nuclear Rnaase III Drosha initiates microRNA processing. Nature 425: 415–419.
10. Yi R, Qin Y, Macara IG, Cullen BR (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17: 3011–3016.
11. Loundes J, Gottlieb G, Coble A, Duda GE, Kutay U (2004) Nuclear export of microRNA precursors. Science 303: 95–98.
12. Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, et al. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Genes Dev 15: 2121–2131.
13. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15–20.
14. Marzluff WF, Byrom MW, Shallon J, Ford LP (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Research 33: 1290–1297.
15. Chen CZ, Li L, Lodiash HH, Bartel DP (2008) MicroRNAs modulate hematopoietic lineage differentiation. Science 320: 83–86.
16. Forstemann K, Tornari Y, Tu T, Vinog VV, Denli AM, et al. (2005) Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol 3: e236.
17. Kane Lopezou C, Muljo SA, Ganesan S, Drapkin R, et al. (2005) Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev 19: 489–501.
18. Wirthhohli E, Klossostman WP, Miska E, Alvarez-Saavedra B, Berezdov, E, et al. (2005) MicroRNA expression in zebrafish embryonic development. Science 309: 301–303.
19. Tuddenham L, Wheeler G, Nsomi-Fousara S, Waters H, HjajtJoseine MK, et al. (2006) The cartilage specific microRNA-140 targets histone deacytase 4 in mouse cells. Febs Letters 580: 4214–4217.
20. Kooyar J, Yui S, Coble A, Duda GE, Kutay U, et al. (2008) Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. Proceedings of the National Academy of Sciences of the United States of America 105: 1949–1954.
21. Fun H-N, Nicolaou PE, Soond SM, Swingler TF, Clark JM, et al. (2010) Analyzing miRNA expression identifies Smad3 as a microRNA-140 target regulated only at protein level. Rna-a Publication of the Rna Society 16: 489–494.
22. Liu FA, Kong L, Bai XH, Luan Y, Liu CJ (2009) miR-199a, a Bone Morphogenic Protein 2-responsive MicroRNA, Regulates Chondrogenesis via Smad3. Bmb Reports 44: 28–33.
23. Lewis BP, Zhang H, Yang D, Kong L, Wang Q, et al. (2009) MiR-145 suppresses cell invasion and rescues hypodactyly mice. Matrix Biology 28: 224–233.
24. Akayama H, Stadler HS, Martin JF, Edhii TM, Beachy PA, et al. (2007) Misexpression of Sox9 in mouse limbs bud mesenchyme induces polydactyly and rescues hypodactyly mice. Matrix Biology 26: 356–367.
25. Sachdeva M, Mo YY (2010) MicroRNA-145 Suppresses Cell Invasion and rescues hypodactyly mice. Matrix Biology 26: 224–233.
26. Kerek A, Grun D, Poy MN, Wolf R, Rosenberg L, et al. (2005) Combinatorial microRNA target predictions. Nature Genetics 37: 495–500.
27. Lewis BP, Shih IH, Jones-Rhozdes MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. Cell 115: 787–796.
28. Ko MH, Kim S, Hwang DW, Ko HY, Kim YH, et al. (2008) Bioimaging of the unbalanced expression of microRNA9 and microRNA145 during the neuronal differentiation of P19 cells. Febs Journal 275: 2565–2576.
29. Kong YW, Cannell RG, de Moor CH, Hill K, Garside PG, et al. (2008) The mechanism of micro-RNA-mediated translation repression is determined by the promoter of the target gene. Proceedings of the National Academy of the United States of America 105: 8866–8871.
30. Flynn AS, Li N, Thatcher EJ, Soinich-Krezel L, Patton JG (2007) Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. Nature Genetics 39: 259–263.
31. Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ (2008) MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proceedings of the National Academy of the United States of America 105: 1516–1521.
32. Doench JG, Petersen CP, Sharp PA (2003) siRNAs can function as miRNAs. Genes & Development 17: 430–442.
33. Cai X, Ambros V (2003) Role of microRNAs in plant and animal development. Science 301: 336–338.
34. Zeng Y, Yi R, Cullen BR (2003) MicroRNAs and small interfering RNAs can inhibit miRNA expression by similar mechanisms. Proceedings of the National Academy of Sciences of the United States of America 100: 9779–9784.
35. Wang Y, Sul HS (2009) Pref-1 regulates mesenchymal cell commitment and differentiation through Sox9. Cell Metab 9: 297–302.
36. Gangaraju VK, Lin HF (2009) MicroRNAs key regulators of stem cells. Nature Reviews Molecular Cell Biology 10: 116–125.
37. Lakshmipathy U, Hart RP (2009) Concise review: MicroRNA expression in multipotent mesenchymal stromal cells. Stem Cells 27: 356–363.
38. Karlén TA, Shahdadfar A, Brinchmann JE (2010) Human primary articular chondrocytes, chondroblasts-like cells and dedifferentiated chondrocytes: differences in gene, microRNA and protein expression and phenotype. Tissue Eng Part C Methods.
39. Ella L, Quintavalle M, Zhang J, Contu R, Cossa L, et al. (2009) The knockout of miR-143 and-145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. Cell Death and Differentiation 16: 1590–1598.
40. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B (1999) Sox9 is required for cartilage formation. Nat Genet 22: 85–89.
41. Liu CJ, Zhang Y, Xu K, Parsons D, Alfonso D, et al. (2007) Transcriptional activation of cartilage oligomeric matrix protein by Sox9, Sox5, and Sox6 transcription factors and CBP/p300 coactivators. Frontiers in Bioscience 12: 3809–3910.
42. Fujimura T, Tsuda M, Taniguchi N, Tajima Y, Ahsara H (2005) Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 coactivators. Journals of Biological Chemistry 280: 3835–3850.
43. Fujimura T, Nakamura JS, Nakamura JS, Nakamura JS, Nakamura JS, et al. (2005) SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. Arthritis and Rheumatism 56: 3561–3573.
44. Kanellopoulou C, Muljo SA, Ganesan S, Drapkin R, et al. (2005) MicroRNA expression in zebrafish embryonic development. Science 309: 309–311.
45. Sohmi M, Nadri S (2009) A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. Nat Protoc 4: 102–106.