Cellular and Molecular Biology

Wang *et al. Cell. Mol. Biol.*2016, 62 (12): 97-101
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.12.17

Original Research

MiRNA-378 controls cell proliferation in rabbit umbilical cord mesenymal stem cells

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Abstract: Mesenchymal stem cells (MSC) are known to have the ability to differentiate into various lineages of mesenchymal tissue. They are widely distributed in a variety of tissues in the body and are also present in the foetal environment. MicroRNAs (miRNAs) are a fundamental class of biological molecules that play a crucial role in development. In this study, microRNA-378 and its predicted target (Sufu, suppressor of fused homolog) were used to study proliferation of rabbit umbilical cord mesenymal stem cells, luciferase reporter assay was used to assess and confirm the binding sequence of 3′untranslated region between microRNA-378 and Sufu. The results showed that miRNA-378 overexpression reduced Sufu gene, and promoted cell proliferation of rabbit umbilical cord mesenymal stem cells via cell viability and BrdU testing, which molecular mechanisms were down-regulation of apoptosis genes after miRNA-378 overexpression. In conclusion, our research shows that the microRNA-378 promoted the cell proliferation of rabbit umbilical cord mesenymal stem cells, and this mechanism was miRNA-378 reduced the expression of Sufu, increased cell proliferation.

Key words: microRNA, cell proliferation, Mesenymal Stem Cells, Umbilical Cord

Introduction

Mesenchymal stem cells (MSCs) was bone marrow stromal cells in the initial research of MSCs, but the MSCs were isolated from the fetal liver, lung, heart ,other parenchymal organs and umbilical cord blood following with the recent research and they have the semblable character with bone marrow MSCs. So, they can all be called mesenchymal stem cells which to have similar shape, antigens and differentiation potential with MSCs (1, 2). Because of its rapid amplification *in vitro*, low immunogenicity and easy transfection of foreign genes, MSCs are the ideal cell source for tissue engineering. Umbilical cord mesenchymal stem cells (UCMSCs) have been focused on in recent years(3, 4), It has been confirmed that the MSCs can be isolated from mesenchymal of the umbilical cord and can different into cartilage cells and nerve cells(5, 6). It is indicated that UCMSCs may be promising source cell for tissue engineering therapeutics.

MicroRNAs (miRNAs) are a fundamental class of biological molecules with a crucial role in development(7), the dysfunction of which has been linked to cancer(8), among other biological processes. Genes encoding miRNAs, which are found in most eukaryotes, produce short (18~25 nt) RNAs that bind to mRNA transcripts and down-regulate their expression either through mRNA destabilization or translational repression (8, 9). miRNAs regulate the expression of genes by guiding the RNA induced silencing complex (RISC) to a target sequence, which is usually located at the 3'untranslated region (UTR) of mRNAs (10). Thus, miRNAs are potentially key post-transcriptional regulators in stem cell self-renewal and differentiation. Distinct sets of miRNAs have been shown to be specifically expressed in embryonic stem cells (11, 12). Knock-out or knock-down of Dicer causes embryonic lethality and loss of stem cell populations (13, 14). Argonaute family members, key components of the RNA-induced silencing complex (RISC), are required for maintaining germline stem cells in various species (15, 16).These observations together support a role for miRNAs in stem cell biology.

microRNA-378 (miRNA-378) is a specific miRNA that can induce angiogenesis in tumors. Experimental studies show that miRNA-378 transfection significantly enhances cell viability and inhibits cell apoptosis. And then miRNA-378 is closely associated with stem cell survival and vascular differentiation. In this study, rabbit UCMSCs were transfected with miRNA-378 and detected regulation of miRNA-378 and Sufu to demonstrate the role of miRNA-378 in cell proliferation of rabbit UCMSCs.

Materials and Methods

Isolation and culture of rabbit UCMSCs

All animal experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at College of Wildlife Resources, Northeast Forestry UnIVersity. The Wharton's jelly was obtained from three-weeks-old rabbit embryos under the sterile conditions, washed 3 times with PBS, removed amniotic membrane and vascular, cut in to about 1mm3 small pieces, and then digested for 15 minute under 37°C using the 0.1% collagenase type

Received July 8, 2016; **Accepted** October 25, 2016; **Published** October 31, 2016

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IV (sigma, US). Enzymatic digestion was then neutralized with L-DMEM (Gibco, USA) supplemented with 10% (v/v) FBS (Biochrom, Germany). The suspension was filtered with 74-µm-mesh sieve, and centrifuged at 200g for 5 min at room temperature. The cell pellet was resuspended with complete medium containing L-DMEM, 10% (v/v) FBS, 2 mM L-glutamine, and 104 IU/mL penicillin/streptomycin. The cell suspension was plated and incubated at 37°C with 5% CO2. At 24 hr after initial plating, the cells were washed twice with PBS to remove non-adherent cells. When the cells reached 80% confluence, trypsinization juice containing 0.25% trypsin and 0.01% EDTA (Gibco) was added to dissociate the cells from plates, then trypsinization was terminated with complete medium. Cells were subcultured into new plates, and after 3-4 passages, the cells were purified.

Assay of luciferase actIVity

The 3′UTR fragments for Sufu were generated by PCR using the following primers: 5′-CTTGGAGTGTGGTTTGGTTCA-3′ and 5′-TCAGGCTCAGAGAGGGCAT-3′and cloned into the psiCHECK-2 vector (Promega) downstream from the Renilla luciferase cassette. The predicted miRNA-378 binding site was mutated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). HEK293T cells were grown in a 96-well plate and co-transfected with the luciferase reporter vector together with a miRNA precursor or a negatIVe control (20 nM; Ambion) using Attractene (Qiagen) according to the manufacturer's instructions. ActIVities of firefly and Renilla luciferase were analyzed using the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection.

Expression of Pre-miRNA-378 by recombinant adenoviruses

Recombinant adenoviruses were generated by using AdEasy technology (17). The sequence of pre-miRNA-378 was synthesized by Sangon Biotech (Shanghai, China), cloned into an adenoviral vector and then used to recombine adenoviruses in HEK293 cells. The adenoviruses designated as AdBMP9 also express GFP as a marker for monitoring infection efficiency. Analogous adenovirus only expressing monomeric GFP (Ad-GFP) was used as a control. Real time PCR was used to evaluated the expression level of miRNA-378 after virus infection. miRNAs were isolated from cells using microRNA isolation kit (Applied Biosystems, USA) according to the manufacturer's instructions. cDNA synthesis was carried out with the High Capacity cDNA synthesis kit (Applied Biosystems, USA) using 2 ng of RNA as template. The miRNA sequence-specific reverse transcription PCR primers for miR-124 and endogenous control U6 were purchased from Ambion (USA). Real-time PCR analysis was carried out using Applied Biosystems 7500 real-time PCR system. The gene expression threshold cycle (CT) values of miRNAs from each sample were calculated by normalizing with internal control U6 and relatIVe quantitation values were plotted. The target genes (Sufu) were detected by western blotting.

Western blotting

Sufu, the target gene of miRNA-378, was detected by western blot analysis following overexpression of miRNA-378. Cells were lysed using M-PER Protein Extraction Reagent (Pierce, USA) supplemented with a protease inhibitor cocktail (Abcam,USA). Protein concentrations of the extracts were measured using the BCA assays (Pierce, USA) and equalized with extraction reagent. Equal amounts of extracts were loaded and subjected to SDS-PAGE, followed by transfer onto nitrocellulose membranes. Sufu antibody (Abcam,USA, 1:200) and horseradish peroxidase-coupled secondary antibodies were purchased from Santa Cruz, USA (1:100). Membranes were probed using ultra-enhanced chemiluminescence western blotting detection reagents. Actin (Santa Cruz, USA; 1:500) was used as an internal control.

Cell viability and BrdU analysis of rabbit UCMSCs

To test viability using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) (18), rabbit UCMSCs were reseeded in six-well plates, detected role of miRNA-378 in cell proliferation, Briefly, culture medium in each well was replaced with 500 µl of fresh medium containing 10 µl of 5 mg/ml MTT stock solution (Sigma-Aldrich, St. Louis, MO). Medium was removed after 5 h and replaced with 500 µl dimethyl sulfoxide (DMSO, Sigma-Aldrich) per well and held for 10 min at 37 °C, after which samples were mixed and absorbance was read at 540 nm. Bromodeoxyuridine (BrdU) analyses were used to measure cell proliferation (BrdU incorporation into DNA of proliferating cells). Cells were treated with 10 µM BrdU (Sigma-Aldrich) for 24 h and then fixed for immunofluorescent antibody assays.

Statistical Analyses

All experiments were done at least three times in trip triplicate and statistical significance of the results was assessed using Student's t-test.

Results

miRNA-378 expression after transfection in rabbit UCMSCs

To determine the functions of miRNA-378 in rabbit UCMSCs, we transfected the pre- miRNA-378 into rabbit UCMSCs for over-expression. The expression of miRNA-378 was quantified by real time-PCR and northern blotting at 72th hr after transfection. As shown in Figure 1, miRNA-378 levels were significantly elevated by the pre-microRNA transfection.

Sufu is a direct target of miRNA-378 in rabbit UCMSCs

miRNA target genes are likely to have relatIVely long and conserved 3′UTR (19). We noticed that Sufu has a long evolutionarily conserved 3′UTR, so we used the miRDB algorithm (20) to search for miRNAs that could potentially regulate Sufu. The miRNA-378 has two putatIVe target binding sites in Sufu in the rabbit genome (Figure 2). To directly test whether miRNA-378 targets Sufu, we cloned the 3′UTRs of Sufu downstream of a luciferase reporter, and co-transfected these reporter

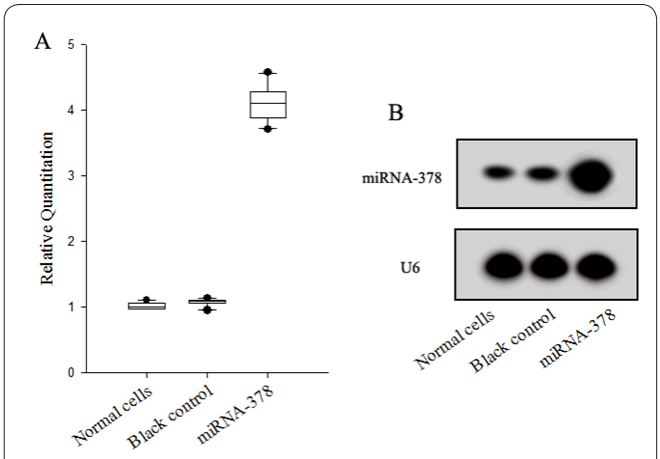

Figure 1. Rabbit UCMSCs were transfected with adenoviruses as described in the Methods, and the expression of miRNA-378 was quantified by real time PCR (A) and Northern blotting (B).

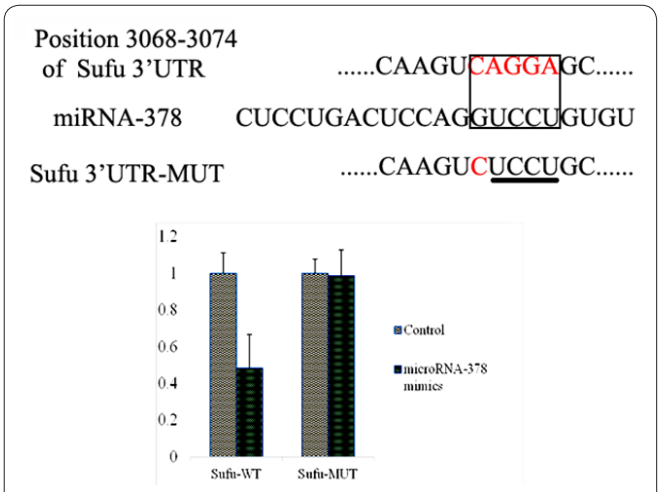

Figure 2. Sufu is target of miRNA-378. miRNA-378 complementary sites with 3′UTR of Sufu. The mutant sequence (Sufu-MUT) is identical to Sufu-WT construct except for four point mutations disrupting base-pairing at the 5′end of miRNA-378. Mutating the miRNA-378 target site in the 3′UTR of Sufu abolishes inhibition of luciferase activity by endogenous miRNA-378 in 293 cells.

constructs along with miRNA precursors into the human cell line HEK293T. Co-expression of miRNA-378 was found to effectIVely downregulate luciferase expression in constructs with these 3′UTRs (Figure 2). Mutations in the seed sequence of the predicted miRNA-378 binding sites within Sufu abolished the inhibitory effects of miRNA-378 on luciferase expression. The pre-miRNA-378 sequence were synthesized and over-expressed in rabbit UCMSCs, expression of miRNA-378 was quantified by real-time PCR at 72 h after transfection. As shown in Figure 1, miRNA-378 levels were significantly elevated after transfection. Protein expression of Sufu, the putatIVe target gene, was performed on miRNA-378-transfected cells using the Image J tools comparatIVe method (Figure 3), and the results showed that the protein level of Sufu was down-regulation.

miRNA-378 promotes cell proliferation

Cell proliferation after miRNA-378 over-expression, evaluated by MTT and BrdU incorporation assays, indicated that 1-5 days after transfected miRNA-378, that transfected miRNA-378 increased cell viability in rabbit UCMSCs ($p<0.01$; Figure 4). Formazan crystal absorbance of formazan in transfected miRNA-378 groups were higher than other groups, and BrdU data agreed with MTT assays.

Apoptosis gene expression after transfected miRNA-378

The p53-p21 pathway is crucial for preventing pro-

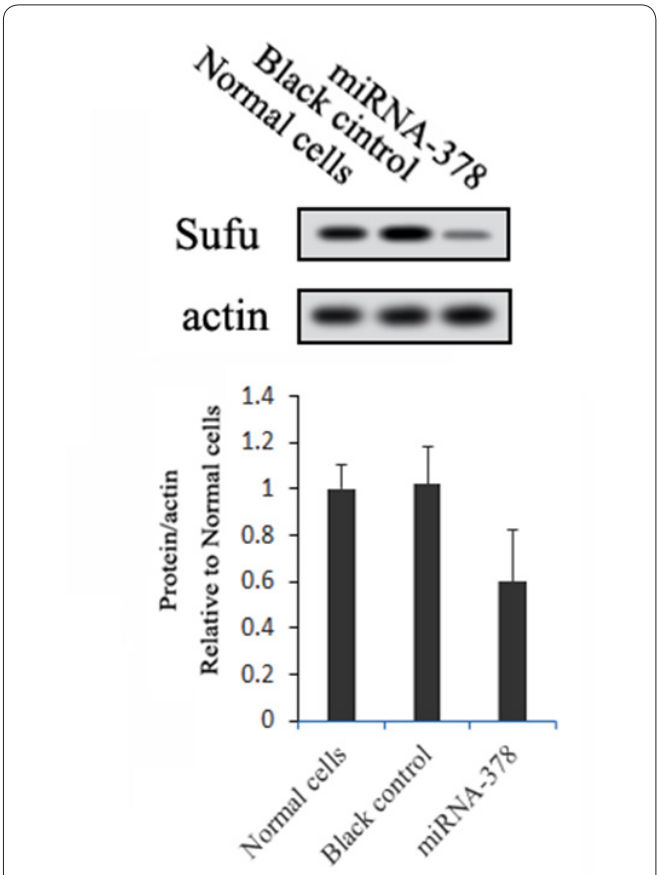

Figure 3. Effect of miRNA-378 on protein level of Sufu in rabbit UCMSCs, Quantification of Sufu in UCMSCs transfected with miRNA-378, Normal cells or Black control for 72 h.

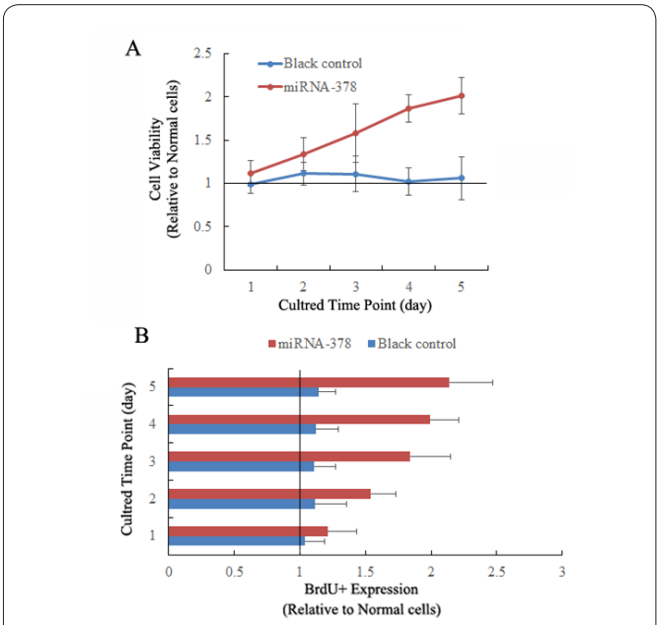

Figure 4. Rabbit UCMSCs proliferation after miRNA-378 transfection. (A) Cell viability in 1- to 5-day-cultured rabbit UCMSCs after miRNA-378 transfection via MTT assay. (B) Cell proliferation in 1- to 5-day-cultured rabbit UCMSCs after miRNA-378 transfection via BrdU labeling indicating more proliferation with miRNA-378 transfection.

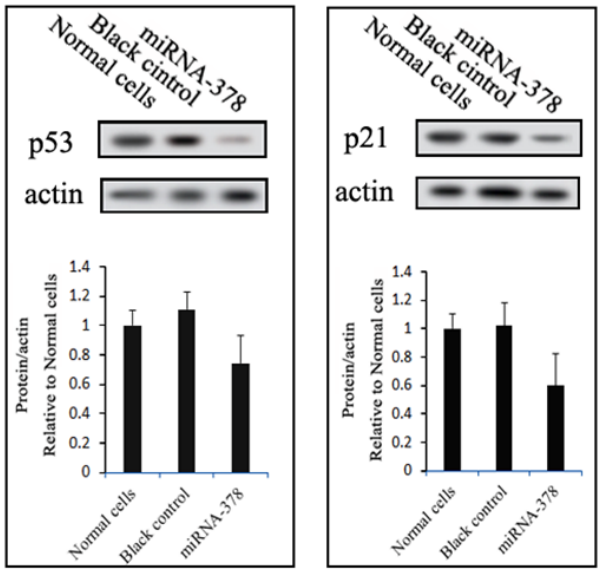

Figure 5. p21 and p53 expression after miRNA-378 transfection in rabbit UCMSCs. A and B Western blot analysis of p21 and p53 protein levels after miRNA-378 transfection respectively, the result showed p21 and p53 were significantly down-regulated.

pagation of DNA-damaged cells, and deficiencies in these factors enhances cell viability. To understand a potential mechanism associated with cell proliferation after transfected miRNA-378, expression of p53 and p21 was assessed using western blotting and found to change significantly after transfected miRNA-378 in rabbit UCMSCs ($p<0.05$; Figure 5).

Discussion

Cell therapy has emerged as a strategy for the treatment of many human diseases. At present, tissue stem cells were tentatIVely expanded and orientationally induced *in vitro* to some cells that are needed, which are then implanted into patients to repair damage, to replace regressIVe tissue and improve the function of hereditarily defectIVe tissue. The transplantation of hematopoietic stem cell has been widely used in the treatment of hematopoietic malignent diseases, which provides an exemplification for the study of tissue stem cells. It was reported that MSCs could be used to repair and reconstruct some tissues such as bone cartilage, lung, brain, etc. Human MSCs cultured *in vitro* were implanted into rat brains. At present, the number of MSCs in mononuclear cells is not enough for tissue engineering. Accordingly, it is necessary to expand and purify MSCs *in vitro*.

MiRNAs are small noncoding RNAs that are cleaved from 70-100 nucleotide (nt) hairpin pre-miRNA precursors in the cytoplasm by RNaseIII Dicer into their mature form of 19-25 nt. Single-stranded miRNAs bind messenger RNAs of potentially hundreds of genes at the region of 3'UTR with perfect or near perfect complementarity, resulting in degradation or inhibition of the target messenger RNA (21). MiRNAs are fundamental biological molecules that have been shown to play important roles in biological development(22). miRNA-378 is one of the small noncoding RNA molecules able to regulate gene expression at posttranscriptional level. Its two mature strands, miR-378a-3p and miR-378a-5p, originate from the first intron of the peroxisome proliferator-actIVated receptor gamma, coactIVator 1 beta (ppargc1b) gene encoding PGC-1β. Embedding in the sequence of this transcriptional regulator of oxidatIVe energy metabolism implies involvement of miR-378 in metabolic pathways, mitochondrial energy homeostasis, and related biological processes such as muscle development, differentiation, and regeneration. On the other hand, modulating the expression of proangiogenic factors such as vascular endothelial growth factor, angiopoietin-1, or interleukin-8, influencing inflammatory reaction.

Fused gene encodes a cytoplasmic protein which contains a regulation of G-protein signaling (RGS) domain and a dishevelled and axin (DIX) domain. The encoded protein interacts with adenomatosis polyposis coli, catenin (cadherin-associated protein) beta 1, glycogen synthase kinase 3 beta, protein phosphatase 2, and itself. This protein functions as a negatIVe regulator of the wingless-type MMTV integration site family, member 1 (WNT) signaling pathway and can induce apoptosis. Suppressor of fused (Sufu) Sufu acts as a central negatIVe regulator of Hedgehog signaling by sequestering the Glioma-associated oncogene homolog transcription factors in an inactIVe complex. Sufu deletion in mice leads to the continuous actIVation of Hedgehog signal and embryonic lethality at day 9.5. In our research, we demonstrated that miRNA-378 could directed target Sufu in rat UCMSCs, promote cell proliferation via down-regulated expression of p53 and p21.

In summary, we detected the role of miRNA-378 in cell proliferation of rabbit UCMSCs. The results demonstrated that miRNA-378 could promote proliferation of rabbit UCMSCs via targeting Sufu. We have also obtained evidence that the anti-apoptosis induced by miRNA-378 is related, at least in part, to inhibition of the p53-p21 pathway. Amplification of rabbit UCMSCs *in vitro* may represent an ideal candidate resource for cellular transplant therapy in tissue engineering.

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