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Discovery and characterization of miRNA during cellular senescence in bone marrow-derived human mesenchymal stem cells

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ABSTRACT

Cellular senescence is an irreversible cell cycle arrest in which specific mRNAs and miRNAs are involved in senescence progression. miRNAs interact with specific mRNAs to regulate various cellular mechanisms, including metabolism, proliferation, apoptosis, senescence and differentiation. In this study, we identify and characterize miRNAs during cellular senescence in mesenchymal stem cells (MSCs). Using previously reported miRNAs, expression profiling of 23 miRNAs was performed using real-time PCR analysis. Among these miRNAs, 19 miRNAs showed upregulated expression patterns in senescent MSCs compared with young MSCs, and 5 miRNAs were downregulated. These miRNAs have not been previously identified as being related to cellular senescence but seem to be related. miR-103-2*, miR-140-5p and miR-330-5p are highly upregulated, while miR-29b and miR-199b-5p are significantly downregulated in senescent MSCs. We identify unique functions of 5 miRNAs and predict putative target genes of 5 miRNAs using our previous report. Among them, miR-199b-5p directly suppressed LAMC1 expression, as shown in a luciferase assay. miR-199b-5p significantly regulates translational activity but does not control post-transcriptional activity. Likewise, miR-199b-5p modulates LAMC networks, which demonstrates the resulting phenomenon during cellular senescence, namely, that miR-199b-5p indirectly regulates cellular senescence in MSCs.

1. Introduction

Mesenchymal stem cells (MSCs) are adult stem cells known to be capable of self-renewal and divergence into different and multiple lineages, including bone, cartilage, adipose tissue, muscle, tendon, and stroma (Tuan et al., 2003). Therapeutic systems of MSCs are restricted to practical development because they have a shortened life span under in vitro culture systems (Stenderup et al., 2003). Bone marrow-derived MSCs reach 40 passages and do not progress to proliferation before reaching the senescence stage (Mori et al., 2005). In spite of their short life span, MSCs are useful in regenerative medicine (Baxter et al., 2004). The ability of MSCs to overcome cellular senescence demonstrates a resolution to limited life span and supports the study of MSC biogenesis (Liu et al., 2013).

Cellular senescence is an irreversible cell cycle arrest that occurs at the G0 phase of cell cycles; the theory of Hayflick limits states that most cells finitely divide into daughter cells (Hayflick, 1965). Senescent cells demonstrate increased expression levels of p16INK4 and pRB (Narita et al., 2003) and enlarged cell size (Hayflick, 1965), and the phenomenon of senescence-associated β-galactosidase (SA-β-Gal) has been described (Dimri et al., 1995). The extracellular matrix (ECM) provides cell growth, survival and motility (Bissell et al., 1982). During senescence, ECM components undergo progressive changes (Sell and Monnier, 1989) that modify cell-to-cell and cell-to-matrix interaction (Pagani et al., 1991). Expression levels of integrin and collagen (Mancini et al., 2012) decrease during senescence, while those of fibronectin and laminin (Yoo et al., 2013) increase, suggesting the phenomenon of cytoskeletal remodeling (Mancini et al., 2012). Some senescent related genes in MSC are described in our previous study (Yoo et al., 2013). In addition, it has been previously reported that some miRNAs are associated with cellular senescence in several different cell types (Bonifacio and Jarstfer, 2010; Dellago et al., 2013; Faraonio et al., 2012; Glud et al., 2011; Ito et al., 2010; Kim et al., 2012; Lee et al., 2011; Li et al., 2009; Maes et al., 2009; Ohdaira et al., 2012; Rippe et al., 2012; Tzatsos et al., 2011; Ukai et al., 2012; Y. Wang et al., 2011).

Abbreviations: ECM, extracellular matrix; MSC, mesenchymal stem cell; miRNA, microRNA; RISC, RNA-induced silencing complex.

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MicroRNAs (miRNAs) are small non-coding RNAs and are approximately 22 nt in their mature miRNA form (Lee et al., 2004). miRNAs have highly conserved sequences and negatively suppress target genes in the regulation of cellular functions, such as metabolism, proliferation, apoptosis, differentiation and senescence (Bartel, 2004). Mature miRNA is excised from precursor miRNA via stepwise processes and the Dicer enzyme (Lee et al., 2004). Mature miRNAs form part of an RNA-induced silencing complex (RISC) that binds the 3′-untranslated region (3′-UTR) of messenger RNA (mRNA) and regulates post-transcriptional or translational activity (Yoo et al., 2012).

In this study, 43 miRNAs were identified and characterized in MSCs. Expression profiles were examined using cloned miRNAs in young and senescent MSCs. Target gene miRNAs were selected from our previous database (Yoo et al., 2013). Among them, miR-199b-5p is downregulated in senescent MSCs and directly regulates LAMC1. This result demonstrates differential expression of cloned miRNA during cellular senescence, suggesting that they may play critical roles in the senescence of MSCs, directly or indirectly.

2. Materials and methods

2.1. Cell culture and construct of senescent human mesenchymal stem cells

Human bone marrow-derived mesenchymal stem cells were purchased from Cambrex Bio Science (Walkersville, MD, USA), and HeLa (CCL-2™) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MSCs were cultured in α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 20 μg/ml gentamicin (Invitrogen). HeLa cells maintained in RPMI-1640 (Welgene, Korea) supplemented with 10% FBS and 1% antibiotic (100 U/ml penicillin and 100 μg/ml streptomycin, Welgene). All cells were maintained according to the manufacturer's instructions.

2.2. MicroRNA cloning

The total RNA was isolated from human mesenchymal stem cells (hMSCs) using TRIzol reagent (Molecular Research Center Inc., Cincinnati, OH, USA). The miRNAs were cloned into a vector using a ^{Dyna}Express miRNA Cloning Kit (BioDynamics Laboratory Inc., Japan) according to the manufacturer's instructions with modifications. The total RNA was separated on denaturing polyacrylamide gel to isolate small RNAs of 18–28 nts in length. Purified 18–28 nt long RNAs were dephosphorylated by alkaline phosphatase and subjected to phenol/chloroform extraction with ethanol precipitation. The 3′-linker was ligated to the dephosphorylated RNAs. The ligated products were blocked at the 3′-end to prevent circularization via 5′-linker ligation and purified on a polyacrylamide gel. The resulting small RNAs 36 to 46 nts in length were isolates from the gel. The products were ligated with ribonucleotide 5′-end after their phosphorylation, followed by polyacrylamide gel electrophoresis purification. The resulting small RNA with 2 linkers ranging from 53 to 63 nts were reverse-transcribed to make cDNA. After amplification, the PCR product was analyzed on 3% agarose gel and cloned into T vector (Promega, Madison, WI, USA). The isolated putative clones were transformed into DH5α strain using a Dokdo Mini-prep Kit (ELPIS-Biotech, Korea), sequenced, and analyzed (Macrogen, Korea).

2.3. Analysis of miRNA expression using a miScript miRNA assay

Total RNA (1 μg) was prepared and reverse transcribed according to the manufacturer's instructions (Qiagen, Hilden, Germany). PCR reactions were carried out in a total volume of 20 μl using 1 μl reverse-transcribed samples under the following cycling conditions: 15 min at 94 °C for initial denaturation, followed by 35 cycles of 94 °C for 10 s, 65 °C for 10 s and 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min. Data were generated using the CFX Manager™ software (Bio-Rad, Hercules, CA, USA). The data were representative of three independent experiments performed on different days.

2.4. Target prediction and dual luciferase assay

The putative target gene of miR-199b-5p was predicted using three different web-based programs (Target Scan, Pictar, microRNA.org). The 3′UTRs of the target genes were cloned into the pmiRGLO vector (Promega) at the *Xba*I site. Site-directed mutagenesis was performed using a Dokdo™ Site-Specific Mutagenesis Kit (ELPIS-Biotech) to mutate seven base pairs in the predicted seed region that was targeted by miR-199b-5p in the target 3′UTR. Next 200 ng of plasmid and 20 nmol of either miR-199b-5p or NC were co-transfected into HeLa cells for 24 h. Luciferase activity was measured using Dual-Luciferase Reporter Assay System Kit (Promega) and luminescence intensity was measured using an analyzer VICTOR3 (PerkinElmer, Foster City, CA, USA). The data were representative of three independent experiments performed on different days.

2.5. Western blotting

hMSCs transfected mimics of NC, miR-199b-5p, and ASO-199b-5p during 48 h. Protein was extracted with RIPA cell lysis buffer (ELPIS) and detected by Western blotting using anti-mouse LAMC (1:1000 dilution in 5% skim milk, Santa Cruz Biotechnology, Santa Cruz, CA, USA). GAPDH (1:5000 dilution in 5% skim milk, Santa Cruz) was used as an internal control. The data were representative of three independent experiments performed on different days.

2.6. Preparation of synchronized MSCs

MSCs were seeded at a density of 1×10^6 cells/100 mm culture dish in a normal growth medium. When the cells were 70% confluent, cells were grown in the presence of 2 mM thymidine (Sigma) for 18 h, washed in PBS, and grown in fresh medium without thymidine for 8 h. Thymidine was added again to 2 mM to block cells at G1/S. After 18 h, cells were harvested and fixed with 70% ethanol. Harvested cells were stained with propidium iodide (PI) and analyzed for cell cycle using flow cytometry. E.g. Details are described in a previous study (Fang et al., 1998).

2.7. BrdU incorporation

ELISA was used to measure cell proliferation by quantitating BrdU incorporated into the newly synthesized DNA of cells undergoing replication. BrdU offers a nonradioactive alterative to [H^3]-thymidine. MSCs and transfected mimics were transferred to a 96-well plate. Cell proliferation was determined using a BrdU assay kit (Roche) according to the manufacturer's instructions. The data were representative of three independent experiments performed on different days.

2.8. Statistical analysis

The data are presented as the mean ± S.E.M. from at the least three independent experiments. The significant differences were analyzed using the Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. MicroRNA cloning in human MSCs

In this study, we identify and characterize miRNAs in human mesenchymal stem cells (h-MSCs). Approximately 100 small RNA sequences were obtained from h-MSCs. After sequence analysis, a total of 100 putative small RNA clones in the range of 21–23 nt were identified using