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MicroRNA profiling analysis revealed different cellular senescence mechanisms in human mesenchymal stem cells derived from different origin

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ABSTRACT

Mesenchymal stem cells (MSCs) from human umbilical cord (UC) and cord blood (CB) share many common properties and exhibit promising clinical potential. Cellular senescence, which induces the loss of stem cells characters and disrupts their therapeutic functions, has been demonstrated to be under the regulation of microRNAs (miRNAs). In this study, we compared the miRNA profiles in early and late passage UCMSCs and CBMSCs based on deep sequencing. 224 and 170 miRNAs were significantly altered in UCMSCs and CBMSCs respectively. A functional annotation of the predicted miRNA targets revealed a series of common senescence pathways. However, Functional enrichment analysis revealed different bioprocesses involved in cellular senescence of UC- and CB-MSCs. The common miRNAs shared by the two kinds of MSCs also exert different function in terms of GO enrichment analysis. Our results supported MSCs derived from different origin may undergo senescence through different path.

1. Introduction

Mesenchymal stem cells are defined as somatic stem cells with the ability to differentiating into various lineages of cells. Owning to their plasticity, MSCs have been taken as ideal candidates for the treatment of tissue injury such as muscle, neural, skeletal and myocardial [1]. In addition, MSCs have a remarkable immune modulation capacity, exhibiting powerful therapeutic effect on immune disorder [2]. In comparison to MSCs derived from other adult tissues, MSCs from human umbilical cord (UC) and cord blood (CB) can be harvested shortly after birth with no harm to the donors and are attractive sources for clinical use. Though these two kinds of MSCs shared a bunch of same characters, previous researchers have demonstrated their distinctive differentiation potential and immune modification properties [3–5].

MSCs cannot escape from Hayflick limit, which are different from embryonic stem cells [6]. That means, after long period of time in culture, MSCs will inevitably come into a senescent process, which is characterized by an irreversible cell cycle arrest, significant morphology change and positive senescence markers including marked β-galactosidase activity, short telomere length and up-regulated tumor suppressor genes [7,8]. Moreover, senescent MSCs suffer a notable function deterioration. MSCs gradually lose their differentiation potentials during the senescent process [9]. Cellular senescence can also strongly impair the immune regulatory capacity of MSCs both in vitro and in vivo [10,11]. Therefore, understanding the senescence process of MSCs during ex vivo culture would be of vital importance to help improve MSC's expansion yields and preserve their therapeutic effect. In our hypothesis, the impact of passive culture stress on UCMSCs and CBMSCs can be different, which would significantly determine their clinical potential. A comparison of the senescence mechanism of UCMSCs and CBMSCs is important to help evaluate their therapeutic function.

Senescent MSCs undergo significant genetic and epigenetic changes [12–14], including microRNA (miRNA) dependent post transcription regulations. MiRNAs are small non-coding RNAs with 19–25 nucleotides. By a perfect or non-perfect base-pairing interaction, one miRNA can repress hundreds of mRNA targets. Thus miRNAs in the cell orchestrate molecular regulation [15]. Abundant evidence demonstrated important roles of miRNAs in regulating functions of stem cells [16]. Senescence of stem cells also linked to dysregulated expression of miRNAs. MiR-335 and miR-195 have been found play key roles in senescence of bone marrow MSCs and changing their expression can inverse MSCs' therapeutic effect [17,18]. Two studies have characterized senescence-related miRNA expression of long-term cultured bone marrow MSCs by a microarray or quantitative PCR method [9,19]. However, these studies only focused on a limit number of significant miRNAs and their specific targets, and a proper understanding of the global function of miRNAs during senescence remains elusive. Considering umbilical cord and cord blood derived MSCs as promising sources for cell therapy,

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it is necessary to investigate the role of miRNAs in senescence of UC- and CB-MSCs.

Previously we have performed a comprehensive comparison of the miRNA profiles of early cultured UC- and CB-MSCs [20]. In this paper, we further investigated the senescence-related changes of miRNA profiles of UC- and CB-MSCs after a long-term culture. By means of the previously built method, the miRNA deep sequencing results of MSCs were processed and their comprehensive effects on senescence were evaluated. Our study revealed though a group of common miRNAs were dysregulated in both UC- and CB-MSCs, the global regulation roles in senescence process of UCMSCs and CBMSCs are different in terms of GO enrichment analysis. Our study proved MSCs underwent different senescent path base on the analysis on miRNAs deep sequencing. This study contributed to understanding the senescence mechanisms of MSCs and persevering their therapeutic potentials.

2. Materials and methods

2.1. Isolation and culture of human mesenchymal stem cells

A total of five pairs of human umbilical cord (UC) and cord blood (CB) samples were derived from Zhongda hospital with informed consent as previously described [20]. Within 24 h, the samples were processed as below.

For UCMSCs, the umbilical cord was washed in PBS to remove residual blood. Then three vessels were removed and the remaining cord was cut into small pieces and washed in PBS again. Finally, the pieces were transferred to 10 cm culture dishes containing 5 ml EGM-2 medium (Lonza, Walkersville, MD, USA) supplemented with 10% FBS and incubated at 37 °C in 5% CO_2. Medium was changed every three days. When 80% the cell confluence was reached in approximately 2–3 weeks, the monolayer was digested with trypsin and subcultured further.

For CBMSCs, cord blood sample was lysed with Hetasep (Stem Cell Technologies, Vancouver, British Columbia, Canada). Then the supernatant was layered on the surface of Ficoll-Paque Plus (GE Healthcare, Milwaukee, WI, USA) in 50 ml tubes. Monocytes were separated by centrifuging at 400*g* for 40 min. The monocytes were then washed twice in PBS and seeded into a six-well plate at a density of 5×10^6/well cultured in EGM-2 medium supplemented with 10% FBS at 37 °C in 5% CO_2. Medium was changed every three days. After 10–14 days, when 80% the cell confluence was reached, the monolayer was digested with trypsin and subcultured further.

2.2. Senescence-associated β galactosidase staining (SA-β-Gal)

Human UCMSCs and CBMSCs were cultured in six-well plates. SA-β-Gal activity was detected using a SA-β-Gal staining kit (Beyotime, Shanghai, China) according to the manufacturer's protocol.

2.3. RNA isolation

Total RNAs from UCMSCs and CBMSCs at early (passage 4) and late (passage 11) passages were extracted with mirVana miRNA isolation kit (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's protocol. Integrity of RNA was checked with electrophoresis.

2.4. MiRNA deep sequencing analysis

To prepare for deep sequencing, four small RNA libraries (UCMSC-P4, UCMSC-P11, CBMSC-P4, CBMSC-P11) were constructed using Small RNA Expression Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Briefly, RNA samples were hybridized and ligated with adapter mix. Then, reverse transcription was performed followed by RNase H digestion and PCR amplification. The PCR products were finally size-selected and purified. Each pool of libraries included three biological repeats. Then, the SOLID V2 sequencing system (Applied Biosystems, Foster City, CA, USA) was performed to generate read counts of nucleotide sequences.

The sequencing results were analyzed by SOLiD system small RNA analysis pipeline tool (RNA2MAP). The raw data were first filtered to remove low quality reads. Then the data were mapped to the Genbank (http://www.ncbi.nlm.nih.gov/genbank) and sequences matching to rRNAs, tRNAs, snoRNAs and scRNAs were discarded. Finally, by mapping to the miRBase database (http://www.mirbase.org), known and novel miRNAs were identified. The total read counts of each sample were normalized to reads per million (RPM).

To perform differential expression analysis, a DEGseq package tool [21] was applied and the significantly differential miRNAs were identified according to the p-value (<0.05).

2.5. Quantitative real-time PCR

To validate the deep sequencing results, a quantitative real-time PCR (qRT-PCR) analysis was performed. First-strand cDNA synthesis was carried out with 1 μg of total RNAs using specific stem-loop RT primers. Then a qRT-PCR was performed using EvaGreen (Biotium, Hayward, CA, USA) in the ABI 7500 System (Applied Biosystems). The data were normalized using U6 siRNA as an endogenous control. Relative expression of each miRNAs was compared using a $2^{-\Delta\Delta CT}$ method.

2.6. Target gene analysis and functional annotation

A previously developed method was performed to investigate miRNA interaction with its targets [22]. Briefly, the repression level of one target mRNA (REα) can be calculated by accumulating the inhibitory efficacy (IE) of all interacted miRNAs:

$$\mathrm{RE}\alpha = \sum_{i=1}^{n} IE_{i\alpha} \tag{1}$$

where n is the number of regulating miRNAs. The inhibitory efficacy of one specific miRNA to mRNA α ($IE_{i\alpha}$) can be calculated as:

$$IE_{i\alpha} = D_{i\alpha} \cdot RS_{i\alpha} \tag{2}$$

where $RS_{i\alpha}$ is the repression score of miRNA *i* to mRNA α, obtained from TargetScan Database (http://www.targetscan.org); D_i is defined as the distribution of miRNA *i*, and is calculate as:

$$D_{i\alpha} = A_{i\alpha} \cdot W_{i\alpha} \tag{3}$$

where A_i is the abundance of miRNA *i* to the total reads of sequencing ($\frac{Read\ of\ miRNA\ i}{Total\ reads}$), $W_{i\alpha}$ is the weight of miRNA binding to specific target ($\frac{RS_{i\alpha}}{\sum RS_i}$).

Collectively, the repression score of an individual target gene is calculated as:

$$\mathrm{RE}\alpha = \sum_{i=1}^{n} \frac{RS_{i\alpha}{}^2}{\sum RS_i} \cdot \frac{Read\ of\ miRNA\ i}{Total\ reads} \tag{4}$$

Thereafter, the target genes data between early and senescent MSCs were further compared using DEGseq package tool and the most differential target genes were identified ($p < 0.05$). To characterize the functional roles affected by these miRNA targets, the differential genes were inputted into DAVID, an online functional annotation tool [23,24] and the associated biological processes were generated. Then, these biological processes were enriched using Enrichment map [25].

To figure out individual miRNA's function, we engaged to identify miRNAs corresponding to each clusters of biological processes. Target genes of each cluster were analyzed by miRWalk [26]. To keep the most significant miRNAs, we limit the *p*-values below to 0.005.

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