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Long-Term Culture of Mesenchymal Stem Cell Increased the Secretion of Exosomes

Xianhui Wei[1, a], Huiqing Liu[1, b], Fujing Liu[1, c], Shengshui Mo[1], Jin Liu[1, d, *]

[1]Shenzhen GUOKE Capital Co., Ltd., China

[a]xianhuiwei@126.com, [b]Huiqing4625@163.com, [c]jingliufu@163.com, [d]natashaliu@163.com

Abstract

Exosomes could be harvested from lots of sources, including mesenchymal stem cells. As biological 'nanocarriers'(30-200nm) they could transport cargo to both nearby and distant cells. These cargoes contain proteins, and RNAs enclosed by a lipid bilayer. Since these cargoes offer opportunities for disease detection and therapeutic applications, they have attracted a great deal of interest to study. Despite significant efforts made in this field, the low yield of isolation of exosomes has still challenged the progress. How to improve the efficiency and quality of exosome isolation remain questions. Most of the exosome studies have been performed without indicating the relation between the yield of exosomes and cell expansion. Therefore, in this study, we aim to investigate whether the exosomes secretion increase or decrease along with long-term cell expansion, and modify an appropriate passage for exosomes isolation. Thus after a long-term expansion of Mesenchymal stem cells, the exosomes were extracted from the culture medium, the total yield of exosomal protein and RNA were compared, and Transmission electron microscopy is applied to above the Morphological characteristics of exosomes. As a result, the concentration of total protein and nuclear acid were increased after long-term expansion. The higher levels of exosome markers, including CD63, TSG101, and exosomal RNA also generate a higher concentration at more upper passages. In summary, our results indicate that late passages result in an increased secretion of exosomes, and reached the highest concentration at eight times passages.

Keywords

Exosomes, Mesenchymal stem cells, Long-term cell culture.

1. INTRODUCTION

Mesenchymal stem cells(MSCs) have the ability of differentiation and self-renewal potential, which are plastic-adherent growth in vitro. A set of markers used for identification since there is no single biomarker is available used for distinguishing. The minimal criteria of MSC include (a) self-renew, (b) Mutipotency of differentiation; (c) express a set of surface markers including CD73, CD90, CD105, while lacking expression of CD14, CD34, CD45, and human leukocyte antigen-DR(HLA-DR). (1,2) MSCs could be harvested from many sources including umbilical cord, although they were initially identified in the bone marrow. (3) stem cell therapy has received ample attention since their multipotency for differentiation and paracrine effect, the capacity of tissue repair. (4)

The therapeutic MSCs highly rely on paracrine factors extracellular vesicles(EVs), which include exosomes and microvesicles that have a structure contain lipids, microRNAs, mRNA and proteins covered by nano-sized bilayer membrane, with a diameter of 30-100nm. (12-13)

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After examination, indicated that these vesicles play critical roles in cell-to-cell communication,(14) cell signalling, and altering cell or tissue metabolism. The miRNA and mRNA which enclosed in the MSCs-derived exosomes are the primary mediators to restore tissue function in numerous diseases models and brings beneficial effects for vitro examination. These microRNA and mRNAs can be delivered to another cell, and functional in the new location (5,6,8) Moreover, their physicochemical and pharmacokinetic characteristics are respected to the cells where they were secreted (7)

Unfortunately, exosomes are small and unstable, how to isolate them quickly and efficiently still challenging the progress. The main methods of exosomes isolation included Ultracentrifugation-based isolation techniques; Size-based isolation techniques or exosome isolation kit;(9-11) But all the current methods generate a low yield which is not satisfying for a large scale therapeutic requirement. (17)Even the standards of optimal cell passage number for exosome isolation is still unestablished. Thus, it is significant to investigate whether there is any relation between exosome production and cell culture passage line. On the other hand, as the cell-based MSCs therapies, the quality, reproducibility, and potency of MSCs exosome must be careful attention to detail during production since the MSC exosome are activate, possible to be altered when MSCs are cultured with tumour cells or in the vivo tumour microenvironment. (15-16)

In this study, we isolated the exosomes from P1-P10 MSCs culture supernatant and investigated the yield od exosomes by measuring total exosomal protein and RNA concentration. providing evidence for subsequent research. Also, Flow cytometric characterisation of MSCs is needed to examine whether the cells are stable.

2. MATERIAL AND METHODS

2.1. Umbilical Cord Mesenchymal Cell Culture

Human Wharton's jelly was separated from the human umbilical cord of healthy volunteer donors. Mesenchymal stem cells(MSCs) were isolated and flushing by culture medium(Gibco, USA),cells were seeding at density of 8000cells/cm2 incubated with the humidified atmosphere of 5%CO2/95% air and digested to a conventional passage when the cells grew to 80% confluence, and the cell supernatant were collected.

2.2. Flow Cytometric Characterisation of MSCs

The cells must express specific cell surface antigen markers to confirm the definition of an MSC. Mesenchymal cells from the umbilical cord should express ≥ 95% of CD 73, CD 90, and CD 105. Collect the10th generation of cells to set database contribution.

2.3. Exosome Isolation

Exosome isolation using Umibo exosome kit (UR52121, Shanghai, China), was performed by the manufacturer's specification. Briefly, 40ml cell culture was mixed with 10ml Umibo solution and incubated for 2h at 4°C. The cell culture complex was centrifuged at 10,000g for 60min at 4°C, and the supernatant was removed, diluted with PBS then the Exosome purification filter was used. The remaining exosome pellet was used for TEM study.

2.4. Transmission Electron Microscopy of Exosome

Exosome obtained after purification and was loaded onto formvar carbon-coated 200mesh copper grids for 10min at room temperature to drain out the excessive fluid. After 5 min stained with 1% phosphotungstic acid, the exosome-containing grids were observed by the transmission electron microscope.

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2.5. BCA Protein Quantification and Western Blot

Protein was quantified using the Easy II Protein Quantitative Kit (Transgen Biotech Assay Kit, China) as recommended by the manufacturer. Briefly, the BCA working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA Reagent B. 20μL of each sample was pipetted into a microplate well with 200μL of working reagent. Then incubated at 37°C for 30 mins. The absorbance of each sample was measured at 562nm in a plate reader.

After the proteins were loadded on sodium dodecyl sulfateopolyacrylamide gel, then transferred to a polyvinylidene flfuoride membrane(Millipore, MA,USA).Antibodies CD63 and TSG101 were used as probe. The relative protein levels were analyzed by image software.

2.6. RNA Isolation

RNA was isolated from exosomes by the mirVana isolation kit (Ambion, TX, USA). Following by the instraction, after exosomes was lysed with lysis buffer and exosomal RNA was suspended with 100ml of DEPC water after purifying by the filter cartridge, concentraction of RNA was quantifified by using Nanp Drop (Nano-Drop Technologies, De, USA)

3. RESULTS

3.1. Mesenchymal Stem Cell Characterization

Mesenchymal stem cell was isolated from the human umbilical cord, and the characterised as described previously, the biological characteristics and surface markers of 10th MSCs agreed with the international society for cellular Therapy 2006, which are plastic-adherent growth in vitro. After investigating under the microscope, the 4th and 10th generation of MSCs were identical, similar to fibroblasts, have a long spindle structure. The 10th generation of cells maintained the same cell morphology and growth characteristics.(Fig 1C) However, the cell body appeared to enlarged, and the cell arrangement was disordered. In addition, flow cytometric analysis was conducted to characterized the cell surface were positive for mesenchymal cell markers such as CD90:99.99%, CD73:99.98%, CD105:99.52%, and negative cell markers such as CD14:1.23%, CD19:1.95% CD34:0.34%, CD45:0.15%, HLA-DR: 0.58%; Collectively, the cells exhibited a typical MSC-like phenotype.(Fig 1A)

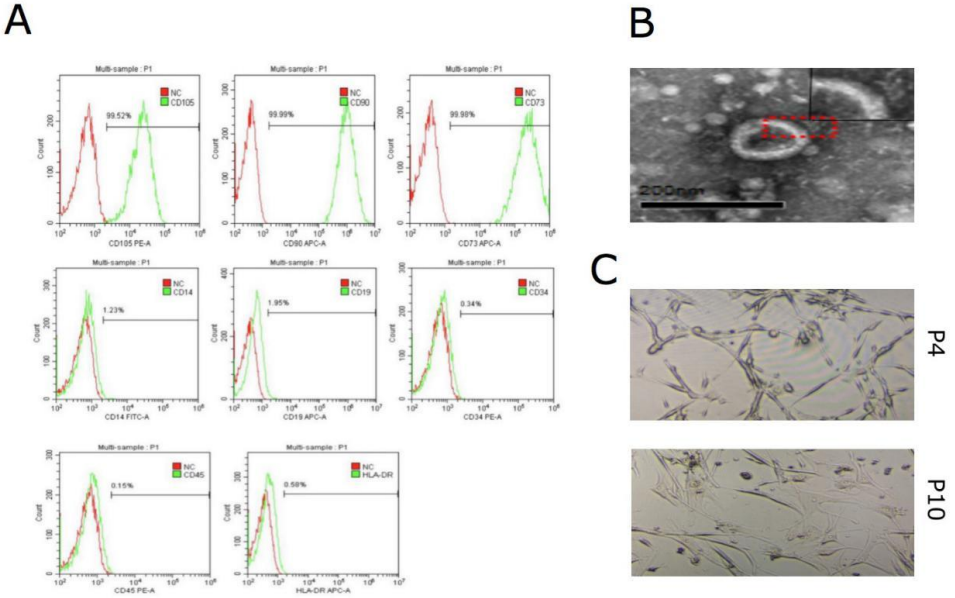

Fig 1. Flow cytometric characterisation analysis of mesenchymal stem cells. (A) Transmission electron microscopy of Exosome.(B) compared passage 4 and passage 10 mesenchmal structure.(C)

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3.2. Exosome Isolation and Morphological Charatcteristics

The small size of exosomes can only be directly visualised with electron microscopy, typical morphological characteristics of exosomes are round-shaped enclosed with lipid bilayer membrane, and the size of 30-100nm was used to demonstrate the presence of exosomes.(Fig 1B)

3.3. Effect of Expansion on the Exosomal-Protein and RNA Concentraction

The number of exosomes isolated from passage 1th to 10th as a result total exosomal protein concentration and RNA concentration rise gently since the passage incresing,(Fig 2B) the experiment repeat three times and generated similar trend. These results indicate that the yield of secretion of representative exosome componments, such as protein and nucleic acids could be increased since the cell expansion.

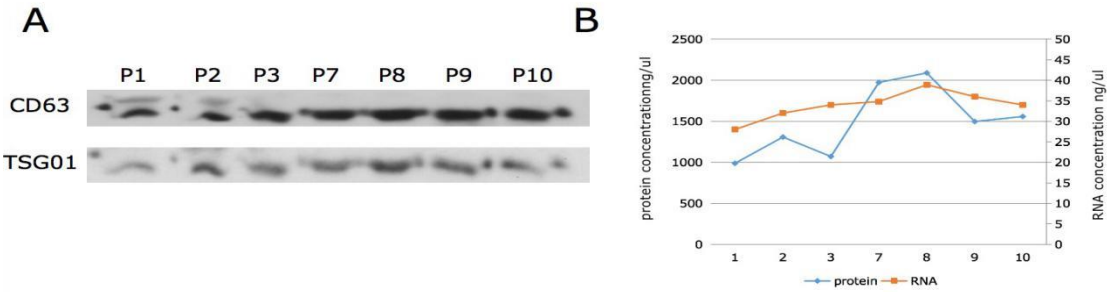

Fig. 2 Western blot analysis with exosome markers. Exosome isolated from exosome-free media were immunoblotted with CD63 and TSG101 (A) Measurement of exosomal protein and RNA concentration. Exosomal protein and RNA were extracted from each generation. Protein concentration was measured by BCA protein quantification and Nano Drop for RNA concentration. (B)

4. DISCUSSION

In this study, we demonstrated that the long-term cell culture in Vitro, resulting in the yield of exosome increased. After a long term Mesenchymal stem cell expansion showed increased exosomal protein and RNA concentration, and showing the highest concentration from both protein and RNA in the passage 8th. The Immunoblot of representative of MSC- drive exosome markers also showed increased in the higher passage. We compared the 4th and 10th generation of MSCs under the microscope, the 10th generation of cells maintained the same cell morphology and growth characteristics, the cell body appeared to enlarged, and the arrangement was slightly disordered, the flow cytometric characterisation on the cells indicated the cells are stable and no differentiation at passage 10.

Base on the research that Long-term cultured leads to mesenchymal stem cells senesced ultimately, caused by a decrease in telomerase activity and shortening of telomere length. (18, 19)Thus, after a ten passage cell expansion, the mesenchymal stem cell might senesce.

To response varieties of cellular stresses, cell senescence as the irreversible state. Senescent cells are not only cycle-arrested cells, but also can secrete bioactive molecules to affect bystander cells, including exosomes which could lead to altering tissue microenvironment, stimulating the organism to clean up senescent cells replace with newly divided cells.

From the aspect of the nucleic acid level, microRNAs covered in exosomes play an important role in many biological processes by regulation of gene expression. Exosomes and miRNAs have a crucial role in the complex networks of cellular senescence. (20, 21) On the other hand, WNT

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is a certain member of the senescence-associated secretory phenotype (SASP), WNT proteins have been shown as a part of exosomes that released to extracellular (18)

To sum up, in this research, both exosomal protein and RNA level show increased after long term cell expansion. Thus, it is possible the senescence cells increase the secretion of exosomes since the number of exosomes is higher in the late passages than in the early passages. Our results show that higher passage increases the yield of both exosomal protein and RNA. However, this needs to be proven by further extensive studies and suggesting that cell senescence of exosome needed to be carefully considered for further exosome research.

5. CONFLICT OF INTEREST STATEMENT

There is no conflict of interest in this manuscript.

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