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Original Article

Evaluation of Human Adipocyte Stem Cells Telomere Length in Early and Late Passages by Quantitative PCR and Southern Blotting

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Abstract

Stem cells are valuable for cell therapy. One of the important disadvantages of stem cells for cell therapy is stem cell senescence. Based on the reports, cell division causes telomere attrition and shortening which this in turn leads to cell senescence. Telomeres are short sequences at the both end of chromosomes that hinder chromosomal attachment. Since pluripotency of stem cells reduces by each cell division, one can conclude that the telomere shortening of stem cell by passaging is inevitable. Here we decided to evaluate the relation between the telomere length and cell passages in human adipocyte stem cells (hAdSCs) by qPCR and Southern blotting. For this purpose, first the AdSCs were isolated from adipocyte tissue by collagenase digestion. Then isolated hAdSCs were subjected to flow cytometry to purify hAdSCs from the rest of adipocyte cells by means of expression of surface markers such as CD105, CD90, CD73 and CD43. Purified hAdSCs were subcultured till appropriate passages (passage 2 and 17). Genomic DNA of appropriate passages were extracted and subjected to both qPCR and Southern blotting for telomere length measurement. Based on the results, hAdSCs telomere length in the passage 2 was much longer than passage 17. Moreover, the longer telomere length in passage 17 was confirmed by Southern blot. Our results indicated that telomeres attrition occur in hAdSCs by each cell division.

Keywords: Human, Adipocyte Stem Cells, Telomere Length, Quantitative PCR, Southern Blotting

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Introduction

Cell therapy is considered as a new advanced method for treatment of many diseases. Bone marrow stem cells (BMSCs) were introduced as an appropriate source for cell therapy. BMSCs derived from mesenchymal tissue, so called mesenchymal stem cells (MSCs), are pluripotent and able to differentiate to all three embryonic layers [1, 2]. There are several advantages utilizing BMSCs for cell therapy, such as reduction of immune response, ease of *in vitro* expansion and their potential to deliver special proteins to specific target tissue [3, 4].

Although BMSCs seems appropriate candidate for cell therapy, quick *in vitro* senescence of BMSCs due to telomere shortening is an important obstacle [5]. There are also MSCs in adipose tissues called adipocyte-derived stem cells (AdSCs) [6]. Recently, there are more attention to AdSCs than BMSCs for cell therapy because of AdSCs advantages such as higher rate of AdSCs isolation with 40 times higher than BMSCs [7], possibility of utilizing autologous stem cells to reduce the immune response and ethic problem regarding of stem cell application [8], and ease of AdSCs isolation and *in vitro* expansion [7, 8].

Telomeres are repetitive DNA sequences, TTAGGG, at the both chromosomal ends which are considered as genome protector against fusion of chromosomes and nucleotide degradation [9]. Replication of telomere sequences take place by telomerase enzyme [10].

Since telomerase expression does not take place in all somatic cells, cells lose about 50 bp of their telomere sequences in each DNA replications [11]. MSCs are very rare in adult tissues; therefore, to be able to utilize MSCs for cell therapy it is necessary to populate them *in vitro*.

Upon isolation and separation of MSCs from natural niche, telomerase expression diminishes which leads to MSCs senescence. Therefore, MSCs can be cultured only for limited times, because senescence causes functional and molecular changes in MSCs such as increase in size and reduction of specific surface markers expression [12]. Moreover, MSCs senescence reduces stem cell pluripotency [5, 13, 14]. Therefore, study of telomere length as a senescence hallmark is essential to harvest *in vitro* cultured MSCs in the best condition for therapeutic application.

Terminal restriction fragment (TRF) is the traditional method of telomere length measurement [15]. This Southern blotting technique requires a high amount of DNA about 5 μg, moreover it takes about 3-5 days to be performed.

Another method of telomere length measurement which has been introduced recently, is a PCR-based method [16]. Unlike TRF, this method requires less DNA amount, about 5 ng, and takes place in one day.

Here, in order to evaluate the accuracy of telomere length alterations among different stem cells passages, human adipocyte stem cells (hAdSCs) were isolated and their te-

lomere length were evaluated by qPCR and Southern blotting method.

Materials and Methods

Isolation of hAdSCs

For hAdSCs isolation, adipose tissue was obtained from pregnant women (25-35 years old) planning on cesarean sections after obtaining written informed consent. Briefly, adipose tissue was cut to the very tiny pieces by a sterile scissor and then embedded in 0.2% collagenase (Gibco, 17100-017) and incubated at 37°C for two hours. Then DMEM containing 10% FBS was added to the digested suspension to inactivate collagenase enzyme. The digested suspension was centrifuged at 1200 rpm for 5 minutes and finally the isolated cells were cultured in a 10 mm culture dish and incubated at 37°C with 5% CO_2 for 72 hours. About 70-80% confluence cells were passaged.

hAdSCs purification by flow cytometry

In this regard, hAdSCs (Passage 1) were trypsinized, and centrifuged at 2000 rpm for 3 minutes at 25°C and resuspended in FACS buffer (PBS, 2% FBS). Then, cells were incubated on ice for 10 minutes. Fluorescence antibodies against CD73, CD44, CD105, CD90, D45 and CD34 were added and cells were incubated with agitation at 4°C for 30 minutes. After removing non-conjugated antibodies by three washes, cells were resuspended in PBS and subjected to flow cytometer (Becton Dickinson, San Jose, CA).

Genomic DNA Extraction

Genomic DNA extraction was done by phenol-chloroform method. Briefly, hAdSCs in passage 2 and passage 17 were lysed by Lysis buffer (10 mM EDTA pH 8.0, 10 mM Tris pH 8.0, 100 mM NaCl, 0.5% w/v SDS, 100 mg/ml Proteinase K), and were incubated for 1.5 hour at 37°C. After proteinase K inactivation via incubation at 75°C for 15 minutes, RNA was removed by treating the sample by 5µl RNase A (10 mg/ml). Then centrifugation was done and the supernatant was transferred to a new tube. Equal volume of phenol: chloroform (1:1) were added to the supernatant and mixed carefully. The aqueous phase was separated and transferred to a new tube. Finally, ethanol precipitation was done to isolate genomic DNA.

Real-Time PCR

Real time PCR was done as previously was explained [16]. Accordingly, three tubes of master mixes containing identical components except primers were prepared; two of them for telomeres of hAdSCs (passage 2 and passage 17) and one for standard 36B4. 36B4 is a single-copy gene that encodes acidic ribosomal phosphoprotein PO and is located on chromosome 12. The components of PCR master mixes were based on TAKARA kit (Japan). Primer concentration for telomere forward primer was 70 nM and for reverse primer 469 nM and template DNA 30 ng. For control 36B4 gene, 300 nM of forward primer and 500 nM of reverse primer were used. DNA concentration was from 12.64 ng to 100 ng in five concentrations by1.68-fold increase. Real time PCR was done utilizing Applied Biosystem (USA) 7500 with 30 cycles. The primer sequences are shown in Table 1.

Relative quantification of gene expression

The Livak method was used for data analysis [17]. For this purpose, first, theΔC_T values of reference and samples were calculated and then the relative telomere length of samples to reference were calculated based on the following formulation:

Relative telomere length = $2^{-(\Delta CT\ Sample\ -\ \Delta CT\ Reference)} = 2^{-\Delta\Delta CT}$.

Table1. Sequence of primers for q-PCR analysis.

Primer	Sequence
TelF	5GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT3
TelR	5TCCCGTATCCCTATCCCTATCCCTATCCCTATCCCTA3
36B4 F	5CAGCAAGTGGGAAGGTGTAATCC3
36B4 R	5CCCATTCTATCATCAACGGGTACAA3

Southern blotting for telomere length evaluation

For telomere length evaluation by Southern blotting, steps were taken based on previous study [18] with a slight modification. Briefly, about 10 µg of the same DNA that was used for qPCR, was double cut by Afa I and Hinf III restriction enzymes during overnight and electrophoresed in 0.5% agarose gel. Gel depurination was done by three different buffers (depurination, denaturation, and neutralization buffer) each for 30 minutes. Then, DNA transferred from gel to nitrocellulose membrane by capillary method. To immobilize DNA, the membrane was baked at 85°C for 2 hours. Hybridization of DNA and probe, CCCTAA, was exerted by 2 mg probe in hybridization solution at 65°C for 20 hours. The DNA–probe hybrid was monitored by chemiluminescence detector.

TRF length analysis

The X-ray films obtained from Southern blotting were scanned with a densitometer. TRF lengths were measured utilizing telo tool following previous report step by step [19]

Statistical analysis

Quantitative data was obtained in triplicate and reported as means ± SEM. Statistical comparisons were performed and a P-value ≤ 0.05 was considered significant.

Results

Isolation and purification of hAdScs

To evaluate telomere length of hAdSCs in two different passages (passage 2 and passage 17), isolated hAdSCs were first subjected to flow cytometry in order to purify hAdSCs from the rest of cells in adipose tissue. The isolated hAdSCs highly expressed CD105, CD90, CD44 and CD73; whereas none of them expressedCD45 and CD34 (Fig. 1). The purified hAdSCs by flow cytometry were cultured to passages 17 and their genomic DNA in passage 2 and passage 17 were isolated and subjected to both q-PCR and Southern blot to evaluate their telomere length.

Real-Time PCR for telomere quantification

To evaluate the relation between the telomere length and cell passages, hAdSCs were subjected to quantitative PCR with specific primers for relative telomere length measurement [16]. Genomic DNA of hAdSCs in passage 2 and 17 and Hela cells genomic DNA as a control were used for real time PCR. Two standard curves, one for samples and one for references, were generated by Plotting CT Values

on Y axis and logarithmic concentrations of samples DNA on X axis (Fig. 2).

Regression values (R^2) of all samples were quite high, about 0.99%. Evaluation of telomere length was performed utilizing this equation: $2^{-(\Delta CT\ Sample\ -\ \Delta CT\ Reference)} = 2^{-\Delta\Delta CT}$. According to the results, telomere length in passage 2 was longer than passage 17 (Fig. 3).

Southern blotting for telomere length evaluation

Southern blot as a golden standard was performed to evaluate the accuracy of telomere length measurement achieved from qPCR. Based on the Sothern blotting results, and compatible with qPCR results, telomere length of hAdSCs in passage 2 was longer than Passage 17 (Fig. 4).

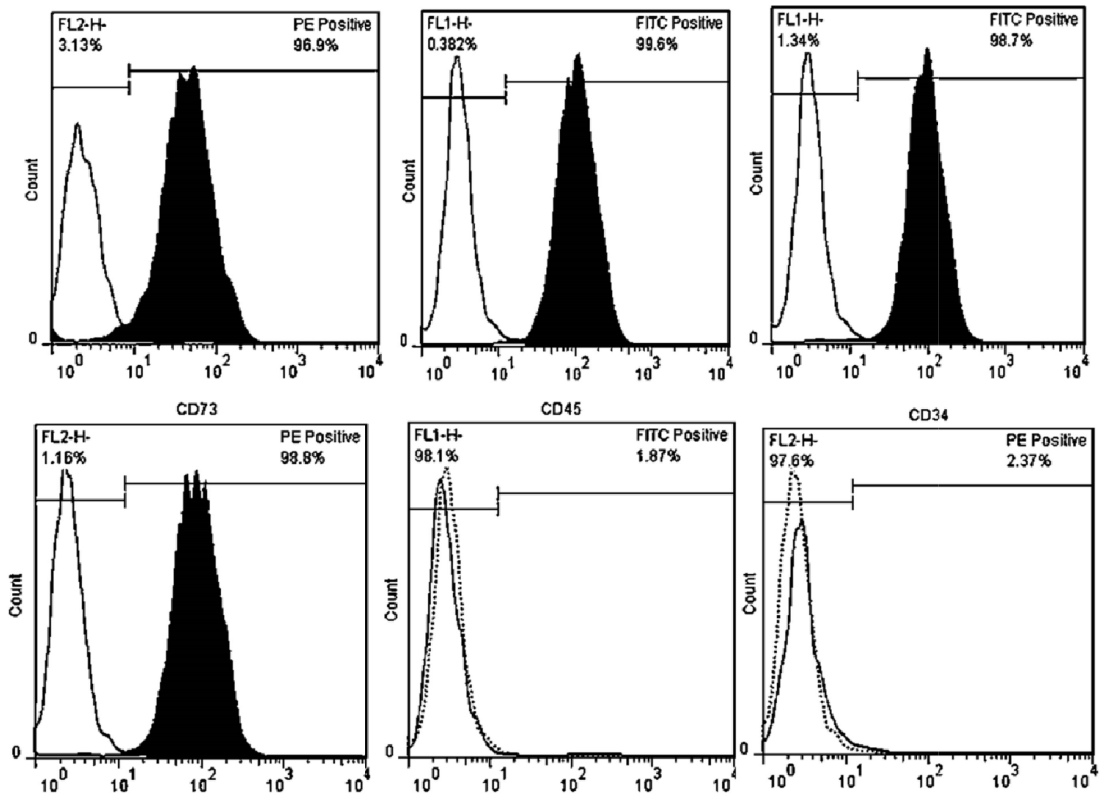

Figure 1. Purification of hAdSCs by flow cytometry. To purify hAdSCs from adipose tissue flow cytometry was done utilizing antibodies against specific surface markers. Isolated hAdSCs highly expressed CD105, CD90, CD73 and CD43. All isolated hAdSCs did not express CD45 and CD34.

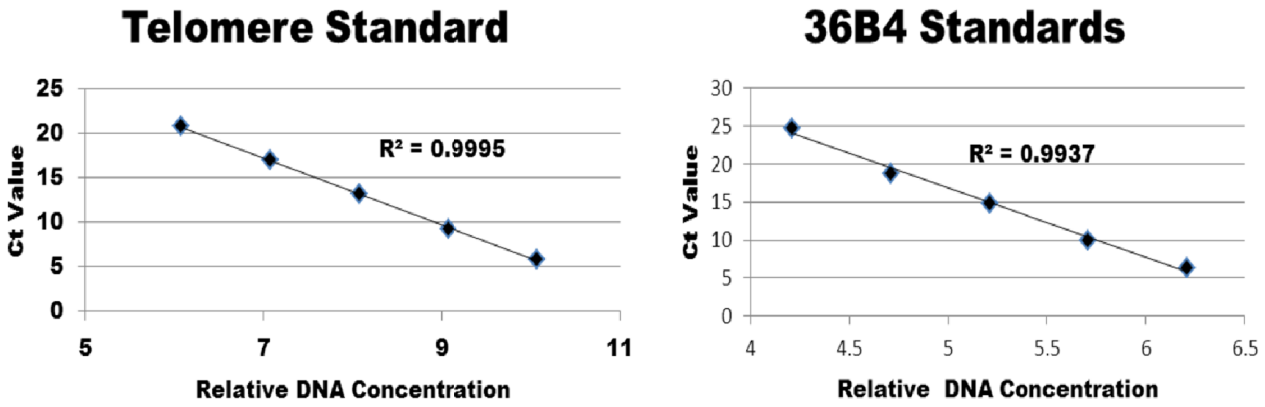

Figure 2. Standard curve of qPCR. Two standard curves were required for each setof q-PCR. The Regression value of both standard curves was more than 0.99.

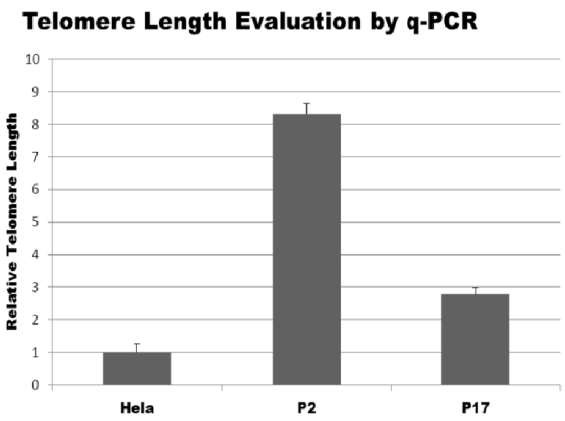

Figure 3. Relative telomere length of samples by qPCR. Relative telomere lengths of samples were calculated by $2^{-\Delta\Delta CT}$ formula. As the results show, telomere length reduced by each cell division. Therefore, the older passages show shorter telomere length.

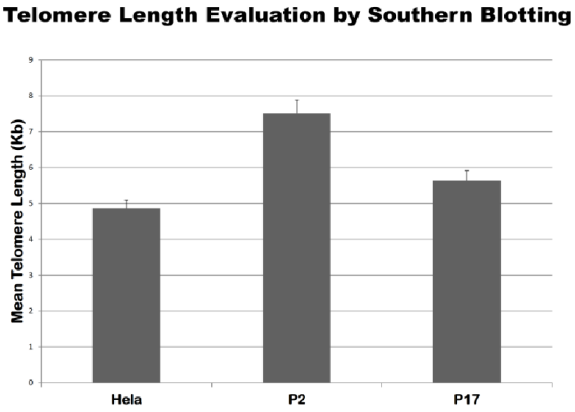

Figure 4. TRFs evolution of hAdSCs by Southern blotting. Evaluation of mean telomere length was performed by Southern blotting. Hela cells were used as control. Based on the results, older passage showed shorter telomere length.

Discussion

Mesenchymal stem cells (MSCs) are pluripotent and able to differentiate into a variety of cells such as pancreatic cells, lung cells, liver cells and endothelial cells [20–22]. Therefore, MSCs were introduced as an appropriate candidate for cell therapy. Adipocyte stem cells are available in adipocyte tissue [6]. These cells are highly similar to MSCs in features such as morphology, high level of cell division, and pluripotent capacity [23–25]. Recently, adipocyte stem cells were introduced as a better candidate for cell therapy than BMSCs due to more advantages including ease of isolation and *in vitro* expansion [7, 8], higher ratio of isolation from adipose tissue [7], and possibility of autologous transplantation to reduce the immune response [8].

It was reported that telomere length shorten by each cell division [11]. Telomere attrition leads to cell senescence. Likewise, *in vitro* passaging of stem cells cause telomere attrition and stem cell senescence. However, it is not the only reason of stem cell senescence; for instance, stress-induced senescence may occur independent of cell division [26]. Senescent stem cells differ with normal stem cells in some features such as morphology, cell size and expression of surface markers [12]. Based on the reports, even stem cells pluripotency decreases by senescence [5, 13, 14]. Since the primary populations of isolated stem cells are rare, before therapeutic application, stem cells must populate which in turn cause telomere attrition and cell senescence. Therefore, to increase therapeutic efficiency of stem cells application, evaluation of senescence is necessary. In this order, evaluation of telomere length as an appropriate hallmark of stem cell senescence is helpful.

In this study, we investigated the telomere length of two different passages of hAdSCs to evaluate the relationship between the hAdSCs passage number and telomere length by real time PCR. The gold standard tool of telomere length measurement is southern blotting analysis of terminal restriction fragments (TRFs) length [18]. Though southern blotting is a gold standard tool for telomere length measurement, qPCR was preferred due to its advantages such as the quicker and easier procedure and less consuming of genomic DNA. Whereas, Southern blotting is quite time consuming, requiring 3-5 days, and needs a high amount of DNA up to 10 µg. In contrast, by qPCR method, telomere length evaluation takes places in one day requiringless than 50 ng of genomic DNA. This method for the first time was introduces by Cawthon *et al*., [16].

Utilizing Cawthon *et al*., procedure and Livak *et al*., calculation methods, telomere length of hAdSCs in passage 2 and 17 were measured and analyzed in the present study.The regression value in the current study was in agreement with previous reports [16]. Analysis of q-PCR showed thatsimilar with somatic cells, attrition of hAdSCs telomere also take place by each cell division (Fig. 3). As a control for qPCR accuracy, the same genomic DNA was applied for Southern blotting. Genomic DNA of Hela cell was utilizedin Southern blotting as control DNA. The mean of Hela telomere length was approximately about 5Kb which was in agreement with previous reports [27, 28] indicating that TRF for Hela cells and therefore for all cells were calculated correctly. Compatible with our results and regardless of the measurement methodology, other studies also have indicated that telomere attrition of MSCs take place *in vitro* [5, 29].

Conclusion

In conclusion, our results indicate that upon isolation of hAdSCs, telomere attrition begun and led to losing some of telomere sequences by each cell division in hAdSCs. Although q-PCR is a quick method for telomere length evaluation, it is not as accurate as TRF method. Therefore, selection of appropriate method is essential in order to achieve the best results.

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