Journal of Biosciences and Medicines, 2024, 12, 55-70
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Direct Correlation among Telomere Length, Cellular Aging, and Rejuvenation Effects of Honey Child Powder

Naofumi Shiomi[1*], Keiko Watanabe[2], Yuki Fujiwara[2], Takae Yamasaki[2], Hideto Matsuyama[1,3]

[1]Research Center for Membrane and Film Technology, Kobe University, Kobe, Japan
[2]School of Human Sciences, Kobe College, Nishinomiya, Japan
[3]Department of Chemical Science and Engineering, Kobe University, Kobe, Japan
Email: *n-shiomi@people.kobe-u.ac.jp

How to cite this paper: Shiomi, N., Watanabe, K., Fujiwara, Y., Yamasaki, T. and Matsuyama, H. (2024) Direct Correlation among Telomere Length, Cellular Aging, and Rejuvenation Effects of Honey Child Powder. *Journal of Biosciences and Medicines*, **12**, 55-70.
https://doi.org/10.4236/jbm.2024.127006

Received: May 26, 2024
Accepted: July 7, 2024
Published: July 10, 2024

Abstract

Purpose: Telomere length (TL) is an indicator of age; however, hormonal influences complicate individual aging. It remains unclear whether TL shortening is a direct factor in both individual and cellular aging. Therefore, we examined the direct relationship between TL and cellular senescence at the cellular level. Methods: Telomerase activity, TL, and gene expression were measured in cultured human lung-, fetal-, and skin-derived fibroblasts, human skin keratinocytes, and telomerase reverse transcriptase (TERT) gene-immortalized cells using detection kits, Cawthon's method, and reverse transcription-quantitative polymerase chain reaction, respectively. Novel substances that elongate telomeres were screened to confirm cell rejuvenation effects. Results: Long-term cell culture of TIG-1-20 normal human fibroblasts resulted in TL shortening, decreased division rate, and senescence progression, whereas in OUMS-36T-2 cells, TL elongation via TERT gene transfer increased the division rate, reduced endoplasmic reticulum stress, and upregulated genes associated with young individuals, indicating that cellular rejuvenation occurs via TL elongation. In addition, a honey child powder (HCP) extract was found through screening, and the HCP extract strongly suppressed the menin gene, resulting in increased telomerase activity and extended cell lifespan. Upon addition of the HCP extract to skin fibroblasts, gene expression of moisturizing components, including collagen, hyaluronic acid, and elastin, increased, and exhibited a rejuvenating effect with an increase in elastin amount. Conclusions: TL elongation or shortening is involved in cell proliferation rate and cellular aging, and TL elongation rejuvenates cells. In addition, HCP extract has a rejuvenating effect on cells and is

expected to be a rejuvenating compound.

Keywords

Telomere, Cellular Aging, Telomerase Reverse Transcriptase Gene, Rejuvenation, Honey Child Powder

1. Introduction

Telomere length (TL) has garnered increasing attention as an indicator of aging [1]. Several statistical studies on individuals have shown a correlation between TL and lifespan [2] [3], and TL is now considered to be one of the important biomarkers of age-related pathologies [4]. Furthermore, although TL may not be associated with cellular longevity in *Caenorhabditis elegans* [5], several studies have suggested that TL elongation delays aging in mice [6]-[8]. For example, cells possess telomerase, an enzyme that elongates telomeres. However, telomerase reverse transcriptase (TERT), one of the components of telomerase, is rarely expressed in somatic cells, resulting in little telomerase activity and shortened TL with each division [9]. In contrast, when an adenoviral vector carrying the TERT gene was administered to 1- and 2-year-old mice to activate telomerase, rejuvenating effects were observed through TL elongation [7]. In another study, knock-in mice were produced by introducing the TERT gene into mice with short TLs; after the mice aged, the TERT gene was expressed using a drug for telomere elongation, and rejuvenating effects were observed in the immune system and other organs [8]. These results suggest that TL shortening may promote individual aging, and elongating TL may promote individual rejuvenation.

Telomeres are single-stranded DNAs at the ends of chromosomes that possess a repeating sequence of six bases (TTAGGGG) in mammals and have a T-loop structure. TL shortens with each division; if the T-loop structure cannot be formed (M1 stage), telomere capping proteins cannot bind and inhibit cyclone-dependent kinase 2 via the p53 and p21 pathways, resulting in cell division arrest [10]. Therefore, TL just serves as a molecular clock in determining mitotic lifespan, and the shortening of TL before reaching the M1 stage hasn't been reported to induce individual aging and cellular senescence yet. As many physiologically active substances intricately affect the aging of an individual, it cannot be ruled out that individual aging is not caused by TL shortening but by many other factors, including hormones.

Therefore, we examined whether TL directly causes cellular senescence. We found that TL is related to cell division rate and cellular senescence and that TL elongation has a rejuvenating effect on aging cells. We also found that honey child powder (HCP) extract, a traditional Japanese herbal medicine that is expected to be effective against aging, contains a novel compound that increases TL and promotes rejuvenation of skin fibroblasts.

2. Methods

2.1. Cell Lines and Media

The following cell lines were used: a human lung-derived fibroblast, TIG-1-20 (JCRB050, PDL20); two human fetal-derived fibroblasts, OUMS-36 (JCRB1006.0) and OUMS-36T-2 (JCRB1006.2); a TERT gene-immortalized OUMS-36 cell line; five human skin-derived fibroblasts, TIG103 (JCRB0528, female, Age: 69 years), TIG102 (JCRB0527, female, Age: 69 years), TIG121 (JCRB0536, male, Age: 8 years), TIG107 (JCRB0532, male, Age: 81 years), and TIG114 (JCRB0534, male, Age: 36 years); and a human skin keratinocyte, HaCaT. Fibroblasts were obtained from the JCRB Cell Bank (Osaka, Japan), and all cells except OUMS-36T-2 were normal diploid fibroblasts. HaCaT keratinocytes were obtained from Cell Line Service (Eppelheim, Germany).

Modified Eagle's medium (MP Biomedicals Inc., IIIkirch, France) containing sodium bicarbonate and a penicillin/streptomycin solution (FUJIFILM Wako Pure Chemical Corp., Richmond, VA, USA, REF 168231-91) was mixed with fetal bovine serum at a ratio of 9:1 (hereafter referred to as MEM) and used for cell culture. All cultured cells were incubated in a CO_2 incubator at 5% CO_2 and 37˚C.

2.2. Measurement of Telomerase Activity and TL

Cells ($1 \times 10^5 - 1 \times 10^6$ cells) were suspended in 50 µL of CHAPS Lysis Buffer to lysate the cells, then centrifuged at 1000 × g for 1 min and the supernatant was used as enzyme solution for telomerase activity measurement. Telomerase activity was measured using a TRAPEZE® XL Telomerase Detection Kit (Sigma-Aldrich, St. Louis, MO, USA REF S7707). TL was measured after the purification of chromosomal DNA from 10^4 - 10^5 cells using NucleoSpin Tissue (MACHEREY-NAGEL GmnH & Co. KG REF 740952.50) according to Cawthon's method [11]. Telomere primers tel1, tel2, 36B4u, and 36B4d were used and a primer mix of 1.25 µM was prepared. A Rotor-Gene SYBR Green polymerase chain reaction (PCR) Kit was used for real-time PCR. Reaction mixtures (5 µL 2× SYBR Green, 1 µL chromosomal DNA solution, 4 µL 1.25 µM primer mix) were prepared and subjected to 50 cycles of 95˚C for 15 s and 58˚C for 2 min. TL (telomere reaction fragment length) were calculated using the equations based on the ΔCt values of telomere and 34b4 genes. The TL of HaCaT cells was 10.0 kb and used as a control.

2.3. Measurement of Gene Expression Using Reverse Transcription-Quantitative PCR (RT-qPCR)

Total mRNAs were purified from cells using the RNeasy Lipid Tissue Mini kit (Qiagen, Hilden, Germany, REF 74804) and complementary DNAs were synthesized using the QantiTect Reverse Transcription Kit (Qiagen, REF 205311). Gene expression was examined using real-time PCR. Reaction mixtures were prepared using the Rotor-Gene SYBR Green PCR Kit, and commercially available DNA

primers (Qiagen, QuantiTect Primer Assays; Table 1) were used as DNA primers for gene detection. Real-time PCR was performed using the Rotor-Gene Q device (Qiagen), and the reaction was carried out at 95˚C for 5 s and 65˚C for 10 s for 50 cycles. The ratio of gene expression to the control was calculated using the ΔΔCt method based on ΔCt values that were calculated using the β-actin gene as a control.

Table 1. DNA primers are used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Symbol	Official name	Entrez Gene ID	Gene Globe ID
ACTB	Actin, beta	60	QT01680476
SOD2	Superoxide dismutase 2	6684	QT01008693
CAT	Catalase	847	QT00079674
POLG	Polymerase (DNA directed), gamma	5428	QT00097895
KL	Klotho	9365	QT00246232
LMNA	Lamin A/C	4000	QT01678495
WRN	Werner syndrome, RecQ helicase-like	7486	QT00074809
SIRT1	Sirtuin 1	23,411	QT00051261
SIRT2	Sirtuin 2	22,933	QT00069531
EGF	Epidermal growth factor	1950	QT00051646
IGF	Insulin-like growth factor 1	3479	QT00029785
MEN	Multiple endocrine neoplasia I	4221	QT00064848
MYC	v-Myc avian myelocytomatosis viral oncogene homolog	4609	QT00035406
TGFB	Transforming growth factor, beta 1	7040	QT00000728
TERT	Telomerase reverse transcriptase	7015	QT00073409
HAS2	Hyaluronan synthase 2	3037	QT00027510
HAS3	Hyaluronan synthase 3	3038	QT00014903
COL1A1	Collagen type I alpha 1	1277	QT00037793
COL1A2	Collagen type I alpha 2	1278	QT00072058
ELN	Elastin	2006	QT00034594

2.4. Long-Term Culture of TIG-1-20 Cells and Culture of Skin Fibroblasts

The TIG-1-20, skin fibroblasts (TIG102, TIG103, TIG107, TIG114, and TIG121),

and HaCaT keratinocytes were inoculated at 5×10^4 cells and cultured for 7 days in 25 cm^2 culture flasks containing 6 mL MEM. The obtained cells were washed once with phosphate-buffered saline (PBS), detached with 1.25% trypsin solution (Thermo Fisher Scientific, Waltham, MA, USA, REF15090046), collected by centrifugation (800 × g, 3 min), and suspended in 2 mL of MEM. The total cell number was calculated using a cell counter (WAKEN Tech Co. Ltd., Kyoto, Japan, REF WC2-100) and the doubling time and the population doubling number (PDL) were calculated. TL was measured in a portion of the cells. TIG-1-20 cells (5×10^4 cells) were subcultured under the same culture conditions and the measurements were repeated. Furthermore, TIG-1-20 cells at PDL37 and PDL56 were respectively cultured in 24-well plates containing 2 mL of MEM for 24 h. The cells were observed using microscopy and senescence-associated β-galactosidase (SA-β-gal) was measured with a Senescence Detection Kit (BioVision, Milpitas, CA, USA, REF K320-250).

Gene silencing in TIG-1-20 cells was conducted as follows: two small interfering RNAs (siRNA), HS TERF1_8 and HS TERF2_6 (Qiagen, REF si03149048 and si04238507), were used for the gene silencing of telomere repeat binding factor (TRF) 1 and TRF2. TIG-1-20 cells (1×10^5 cells) at PDL30 and PDL45 were inoculated into 24-well plates containing 2 mL of MEM and cultured for 16 h. The medium was replaced with 0.5 mL of Opti-MEM I reduced serum medium (Thermo Fisher Scientific, REF 31985062), and gene silencing was conducted using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, REF13778030). Cells containing siRNA were incubated for 5 days, washed with 2 mL of PBS, detached with 1.25% trypsin solution, collected by centrifugation (800 × g, 3 min), and suspended in 2 mL of MEM. The obtained cells (2×10^4) were inoculated into 6-well plates containing 4 mL of MEM and cultured for 1 week, and the cell number was counted with a cell counter.

2.5. Culture of OUMS36 and OUMS-36T-2 Cells

OUMS36 and OUMS-36T-2 cells (5×10^4 cells) were respectively cultured in a 25 cm^2 culture flask containing 6 mL MEM for 4 days. The cells were washed once with PBS, detached with trypsin, and suspended in 2 mL MEM. The number of cells was counted using a cell counter and the doubling time was calculated. The size distribution of 10,000 cells was measured using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and the cells were observed using optical microscopy. A portion of the cells was used for TL measurement, the remaining was used for mRNA purification, and gene expression levels were measured using RT-qPCR.

Furthermore, OUMS36 and OUMS-36T-2 cells (1×10^4 cells) were respectively cultured in 24-well culture plates containing 2 mL of MEM to measure endoplasmic reticulum stress. SA-β-gal activity was measured using a Senescence Detection Kit, and autophagy was measured using a CYTO-ID Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA, REF ENZ-51031-0050).

Immunostaining of glucose-regulated protein 78 (GRP78) was conducted as previously described [12], with the exception of anti-GRP78 rabbit polyclonal antibody (Abcam, Cambridge, UK, REF ab21685) as a primary antibody, and fluorescence measurement using anti-rabbit Immunoglobulin G goat polyclonal antibody conjugated with cyanine 3 as a secondary antibody was conducted using a fluorescence microscope (EVOS FL Cell Imaging System ThermoFisher Scientific, Tokyo, Japan).

2.6. Rejuvenation Effects of HCP Extracts on TIG-1-20 and TIG121 Cells

Screening of extracts in preliminary experiments was conducted as follows: 15 kinds of food items (ginger, garlic, green onion, onion, spinach, carrot, natto, mozuku seaweed, wakame seaweed, turmeric, broccoli sprouts, shiitake mushroom, clams, and oysters) were mashed in a blender. The mashed food and 13 kinds of herbal medicine powders (kikyo, ougon, rengyo, gomishi, annin, hakka, placenta [bovine, equine, sheep], soft-shelled turtle, pit viper, and honey child) were used for the test. The mashed food or herbal medicine powder was mixed with distilled water to 1 mg/mL, autoclaved at 120˚C for 1 min, and sterilized by a 0.2-μm sterile filter. TIG-121 cells (5×10^4 cells) cultured to near mitotic arrest were grown in 25 cm^2 flasks containing 5 mL of MEM, then 20 μL of the extract was added and cultured for 6 days. After cells were collected, cell counts were measured. Samples with the fastest growth rates were selected based on TL length.

HCP was purchased from Ebiya Wholesaler (Tokyo, Japan), which was produced by freeze-drying and grinding 21-day-old male bee larvae; no additional compounds were present. HCP (100 mg) and 1 mL of water were placed in a 2.0 mL micro tube, autoclaved at 120˚C for 1 min to extract active ingredients, centrifuged at 10,000 × g for 10 min, and the supernatant was filtered through 0.2 μm sterile filter. The resulting solution was used as the HCP extract.

TIG-1-20 cells (1×10^5 cells) were cultured in 25 cm^2 culture flasks containing 6 mL MEM with or without 40 μL HCP extract for 1 week. The obtained cells were detached using 1.25% trypsin solution, collected by centrifugation (800 × g, 3 min), and suspended in 2 mL of MEM. The total cell number was counted using a cell counter, and the ratio of increased cell number per week (number of cells in 1 week/number of cells at the beginning) and the integrated PLD value were calculated. A similar culture was repeated using the subculture method for approximately 2 months. The cell sizes were observed using an optical microscope, and gene expression was examined using RT-qPCR in cells cultured for 1 week. Telomerase activity was measured in cells cultured for 1 and 4 weeks.

TIG121 skin-derived fibroblasts (1×10^5 cells) were placed in a 25 cm^2 culture flasks containing 6 mL of MEM with or without 40 μL of HCP extract and cultured for 7 days. The cells were detached using 1.25% trypsin solution, collected by centrifugation, and suspended in 2 mL of MEM. The size of the cells was ob-

served under a microscope and gene expression was examined using RT-qPCR. The cells (1×10^4) were also cultured in 6-well culture flasks containing 2 mL of MEM with or without 40 μL HCP extract for 7 days. The amount of collagen on the cell surface was measured using a Collagen Quantification kit (COSMO BIO, Tokyo, Japan, REF COL-001), and the amount of intracellular elastin was measured using the Fastin Elastin Assay (Biocolor, Carrickfergus, UK, REF UK F2000) after the cells were disrupted by sonication.

2.7. Statistical Analysis

More than three independent experiments were performed for each test, and means, standard deviations (SD), and p-values based on a two-tailed Student's t-test were calculated using KaleidaGraph (Hulinks, Tokyo, Japan). Results with a p-value > 0.05 are not shown in the figures. Results are presented as means ± SD.

3. Results

3.1. Relationship between TL and Cellular Senescence

First, we examined whether TL shortening by repeated cell division directly causes cellular senescence. Several TIG cell lines, which had been studied in many aging studies, were used for the study. **Figure 1(a)** shows the relationship between PDL and doubling time when TIG-1-20 fibroblasts (PDL20) were cultured for a long time by repeated subculture. The doubling time was not constant until division ceased at the M1 phase (approximately 57 divisions) and gradually increased with an increase in PDL; a correlation (R2 = 0.812) was observed between the two. The relationship between TL and doubling time in TIG-1-20 cells and the other cells (HaCaT and OUMS cells) is shown in **Figure 1(b)**. TL in TIG-1-20 cells correlated with doubling times, and a similar correlation was observed in other cell lines (R2 = 0.866), suggesting that doubling time may be controlled by TL length. Thus, TRF1 and TRF2 proteins were silenced using siRNAs to decrease their release. **Figure 1(c)** shows that the decrease in these proteins by silencing caused a decrease in doubling time, suggesting that the release of TRF1 and TRF2 from the telomere may be an important factor in the decrease in doubling time.

Figure 1(d) shows the characteristics of cells with a relatively low mitotic frequency (PDL37) and those with a high mitotic frequency (PDL57). **Figure 1(d) (i)** shows micrographs of the cells; PDL37 cells were uniform and regularly fibrous, whereas PLD57 cells had a large and uneven cell morphology, and an accumulation of undesirable material in the cells was observed. **Figure 1(d) (ii)** shows SA-β-gal activity [13].

The cells stained with a Senescence Detection Kit stained blue when SA-β-gal was activated by cellular senescence; PDL57 cells stained blue more times than PDL37 cells, suggesting that a decrease in the cell division rate by TL shortening progresses cell senescence.

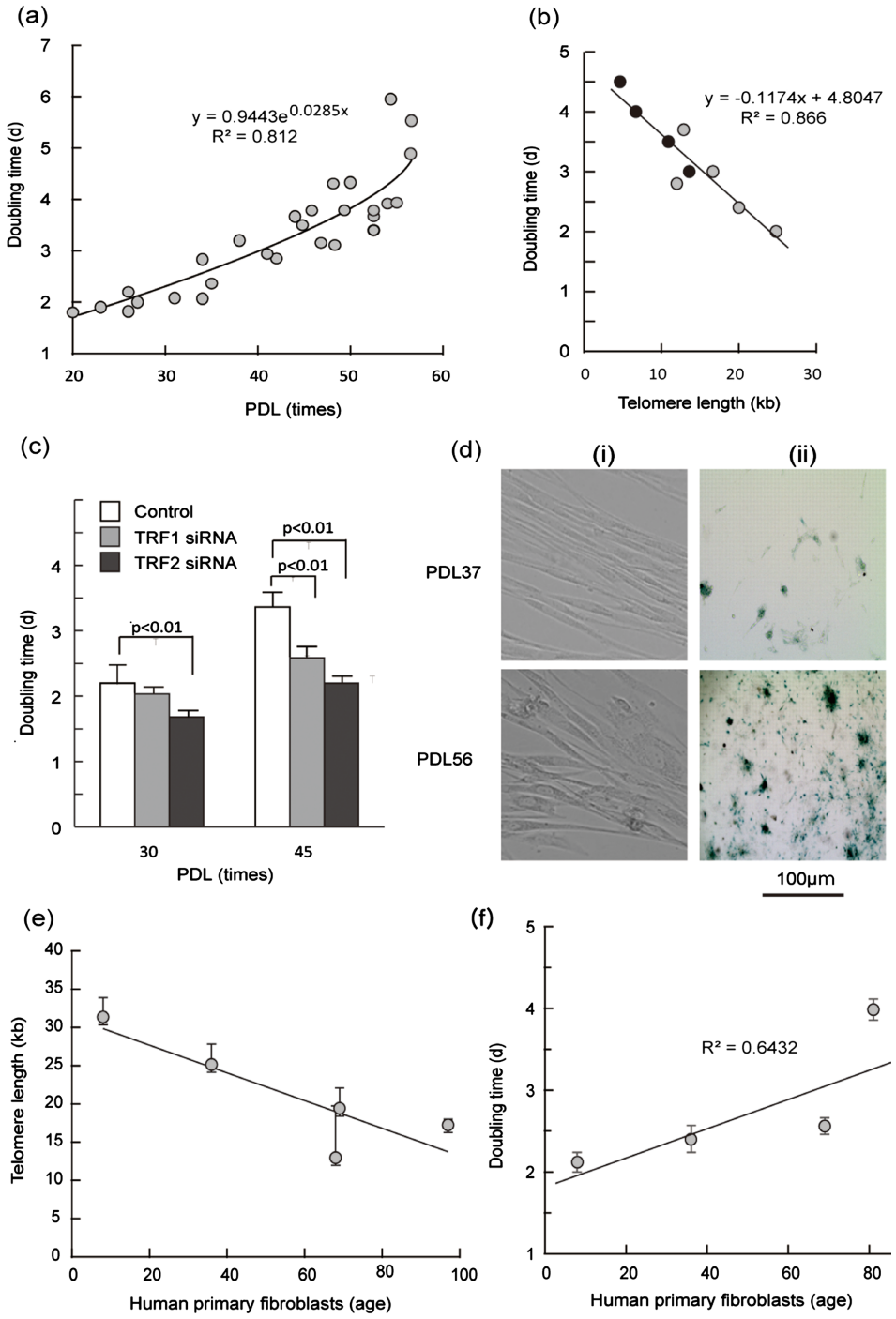

Figure 1. Relationship between telomere length (TL), proliferation rate, and cellular senescence in long-term cultured cells and skin fibroblasts. (a) Relationship between population doubling number (PDL) and doubling time in long-term culture of TIG-1-20 fibroblasts. Three independent experiments with two samples were conducted, and each average value is shown. (b) Relationship between TL and TIG-1-20 cells (gray circles) and the other cells (HaCaT and OUMS cells, black circles). Nine independent experiments with two samples were conducted and each average value is shown. (c) Doubling time when the mRNA of telomere repeat binding factor (TRF)1 and TRF2 proteins in TIG-1-20 cells (PDL30 and PDL45) were silenced using a small interfering RNA (siRNA) method (mean ± standard deviation [SD], n = 3). (d) Characteristics of TIG-1-20 fibroblasts in PDL37 and PDL56: (i) Micrographs; (ii) senescence-associated β-galactosidase (SA-β-gal) activities. (e) Relationship between age and TL of five human skin fibroblast strains taken from different aged individuals (mean ± SD, n = 3). (f) Relationship between age and doubling time of human skin fibroblasts taken from different aged individuals (mean ± SD, n = 3).

Furthermore, we examined whether the relationship between TL and doubling time observed in TIG-1-20 cells could be applied to skin fibroblasts obtained from different people of various ages. **Figure 1(e)** and **Figure 1(f)** show the relationships between age, TL, and doubling time. The cells had short telomeres in relation to age, and the doubling time increased with increasing age, even in primary cells taken from individuals of various ages (R2 = 0.6432). As TIG-1-20 and skin cells were normal and showed little telomerase activity, telomerase is not thought to be involved in the results obtained in **Figures 1(a)-(f)**, suggesting that the TL shortening reduces the proliferation rate and induces cellular senescence.

3.2. Effect of TL Elongation on Cell Rejuvenation

Considering that cell division occurred many times owing to long-term culture, cellular senescence may not only be caused by TL shortening but also by changes in cellular components such as proteins and membranes. To eliminate this possibility, we compared the characteristics of the same cell line with different TLs. The average TLs in the two cell lines are shown in **Figure 2(a)**. The OUMS-36 fetal-derived fibroblasts had shortened telomeres (7 kb on average) as a result of dividing them a dozen times, whereas the OUMS-36T-2 cells had elongated telomeres (78 kb on average) as a result of transforming the TERT gene into OUMS-36 cells.

The characteristics of the two cell lines with different TLs were examined. **Figure 2(b)** shows the doubling time; the average doubling time of OUMS-36 cells was 3.2 days, whereas that of OUMS-36T-2 cells was 2.3 days. **Figure 2(c)** shows the forward scatter height values, which correspond to the size of the cells, using flow cytometry; the curve of the OUMS-36T-2 cells showed a sharp peak, indicating that the cells were very uniform in size. **Figure 2(d) (i)** shows micrographs of these cells; the OUMS-36T-2 cells were more uniform and smaller than the OUMS cells, thereby supporting the flow cytometry results. The results in **Figure 2(b)**, **Figure 2(c)** and **Figure 2(d) (i)** indicate that the division of OUMS-36T-2 cells was notably smoother than that of OUMS-36 cells.

Furthermore, endoplasmic reticulum stress was examined by testing SA-β-gal activity, autophagy activity, and expression of GRP78. **Figure 2(d) (ii)** shows the SA-β-gal activity; the blue color was less prominent in OUMS-36T-2 cells than in OUMS-36 cells, indicating that cellular senescence was notably suppressed by telomere elongation. **Figure 2(d) (iii)** and **Figure 2(d) (iv)** show the autophagy activity and immunoassays of GRP78, which emits green fluorescence when autophagy is active and red fluorescence when GRP78 is expressed. In OUMS-36T-2 cells, autophagic activity (**Figure 2(d) (iii)**) and GRP78 expression (**Figure 2(d) (iv)**) were very weak, indicating that the state of cellular stress in OUMS-36T-2 cells was considerably lower than that in OUMS36 cells.

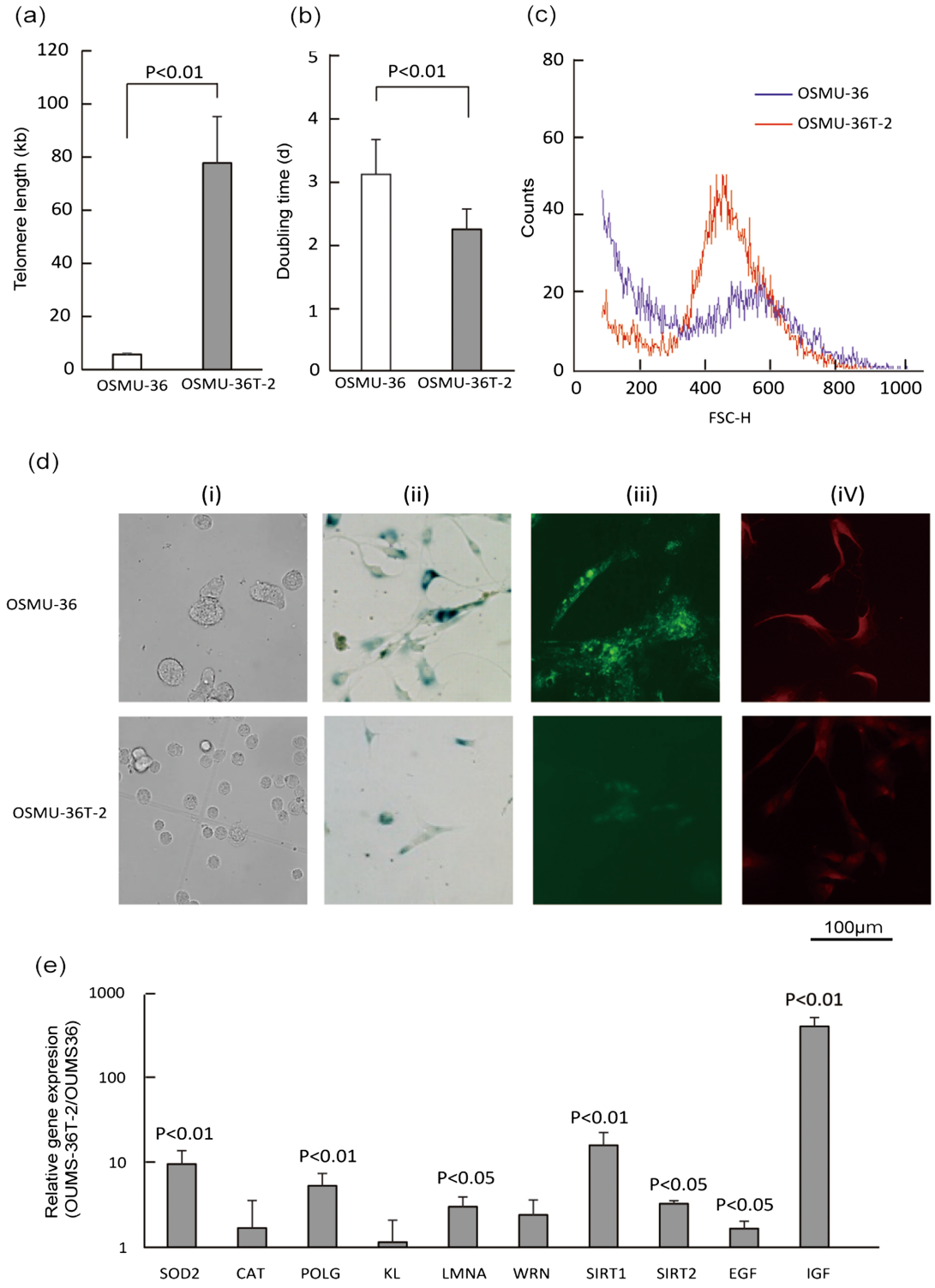

Figure 2. Relationship between telomere length (TL) and senescence using OUMS cells and OUMS-36T-2 cells. (a) Telomere lengths of OUMS-36 and OUMS-36T-2 cells (mean ± standard deviation [SD], n = 4). (b) Doubling times of OUMS-36 and OUMS-36T-2 cells (mean ± SD, n = 4). (c) Flow cytometry of OUMS-36 and OUMS-36T-2 cells. (d) (i) micrographs of cell suspension; (ii) senescence-associated β-galactosidase (SA-β-gal) activities; (iii) autophagy activities stained using a CYTO-ID Autophagy Detection Kit; and (iv) immunostaining using anti-glucose-regulated protein 78 (GRP78) antibody. (e) Ratio of OUMS-36T-2 cell to OUMS-36 cell gene expression (mean ± SD, n = 4); SOD2, superoxide dismutase 2; CAT, catalase; KL, klotho gene; LMNA, lamin A/C; WRN, Werner protein; SIRT, sirtuin; EGF, epidermal growth factor; IGF, insulin-like growth factor.

Figure 2(e) shows the ratio of gene expression in OUMS-36T-2 cells to that in OUMS-36 cells. Two indicator genes for mitochondrial aging (SOD2, CAT), a DNA plolymerase γ gene (POLG), a klotho gene (KL), two genes associated with premature aging (LMNA and WRN), two sirtuin genes (SIRT1 and SIRT2), a epidermal cell growth factor gene (EGF), and an insulin-like growth factor gene (IGF) were used as indicators of cellular aging. As shown in Figure 2(e), the expression of most of the genes, except for, CAT1, KL, and WRN, in OUMS-36T-2 cells was significantly higher than that in OUMS36 cells, indicating that OUMS-36T-2 cells were in a considerably younger state than were OUMS36 cells.

Taken together, these results suggest that 1) TL elongation or shortening is involved in cell proliferation rate and cellular senescence; 2) Elongation of TL rejuvenates cells; 3) TL is an adequate biomarkers of cellular aging.

3.3. Cell-Rejuvenating Effects of HCP Extract

We next searched for novel compounds that enhance telomerase activity to confirm the cell-rejuvenating effects of TL elongation. As a result of screening traditional Japanese foods and herbal medicines (28 compounds in total) that were expected to have rejuvenating effects, extracts of garlic, HCP, pit viper powder, and placenta that had telomerase activation effects were selected as candidates, and HCP, one of the most effective extracts, was used for the following tests.

To examine the effect of the HCP extract, TIG-1-20 cells that had been cultured until PLD42 were repeatedly cultured in MEM with or without HCP extract. Figure 3(a) shows the ratios of cell increase per week; when cultured with the HCP extract, the average growth ratio was significantly higher at 1 - 4 weeks than it was without HCP. However, the growth ratio decreased after the 4th week despite the addition of the HCP extract. The mitotic limit of the cells cultured without HCP extract (control) was 52 times, whereas that of cells cultured with HCP was 60 times, an increase of 8 times in cell lifespan (Figure 3(b)). Figure 3(c) shows the morphology of cells with and without HCP extract 1 week later. The cells cultured with HCP extract were more uniform and smaller in size than those cultured with the control, indicating that cellular senescence was suppressed. Figure 3(d) shows the telomerase activity at 1 and 4 weeks. Telomerase activity in cells cultured with HCP extract significantly increased 1 week later compared with that in control cells; however, 4 weeks later, telomerase activity decreased, and no significant difference was observed. Figure 3(e) shows the ratio of gene expression related to the regulation of the TERT gene in the cells treated with HCP extract to that in the control; HCP extract induced a significant increase in TERT gene expression by approximately 10-fold and significantly suppressed the expression of the multiple endocrine neoplasia (MEN) gene.

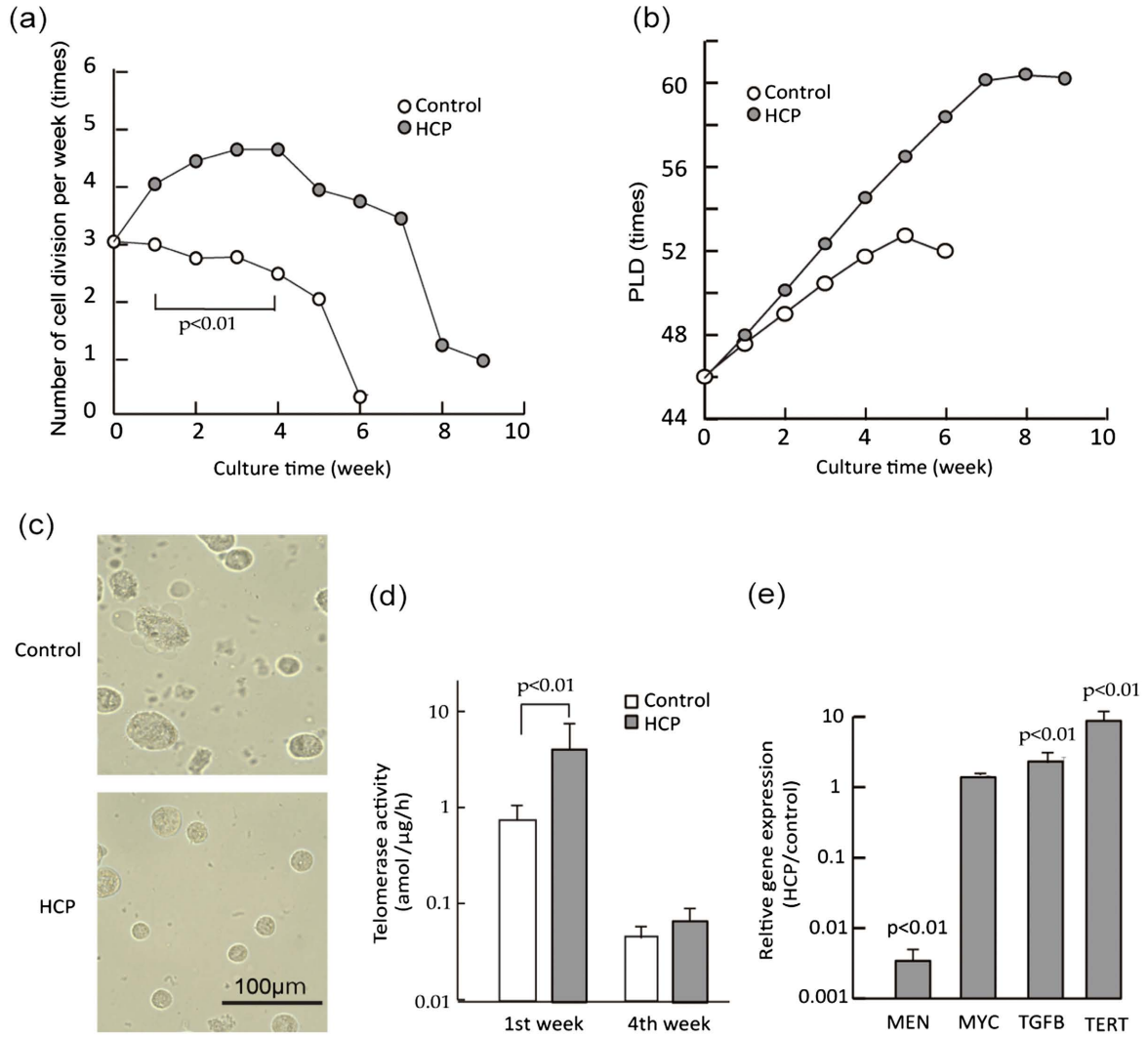

Figure 3. Effect of honey child powder (HCP) extract on rejuvenation of TIG-1-20 fibroblasts. (a) Growth ratio of TIG-1-20 cells cultured in the medium with HCP (gray circles) and without HCP (white circles) every week (n = 3). (b) Population doubling number (PDL) value calculated from the results of **Figure 3(a)**. (c) Micrographs of cells suspension cultured for 1 week with and without HCP. (d) Telomerase activity at the first and fourth week of cells without HCP (white bars) and with HCP [gray bars] (mean ± standard deviation [SD], n = 3). (e) Ratio of gene expression in cells with HCP extract cultured for 1 week to that in cells without HCP (control; mean ± SD, n = 3); MEN, multiple endocrine neoplasia; MYC, v-Myc avian myelocytomatosis viral oncogene homolog; TGFB, transforming growth factor, beta; TERT, telomerase reverse transcriptase

We also examined the rejuvenating effects of HCP extract on skin moisturization. **Figure 4(a)** shows the expression of two hyaluronic acid synthase genes (HAS2, HAS3), two type I collagen synthase genes (COL1A1, COL1A2), and elastin (ELN) genes, when TIG121 human skin-derived fibroblasts were cultured in MEM medium with and without HCP extract. The expression of the HAS3, COL1A1, and ELN genes in cells cultured with HCP extract was significantly higher than that in control cells. **Figure 4(b)** shows the amount of collagen on the cell surface and elastin in the cells cultured with and without HCP extract; no significant difference was observed in the amount of collagen on the cell surface, and the amount of extracellular elastin did not increase (data not shown).

However, the amount of intracellular elastin significantly increased in cells treated with HCP extract. These results suggest that the HCP extract has partial rejuvenating effects on skin moisturization.

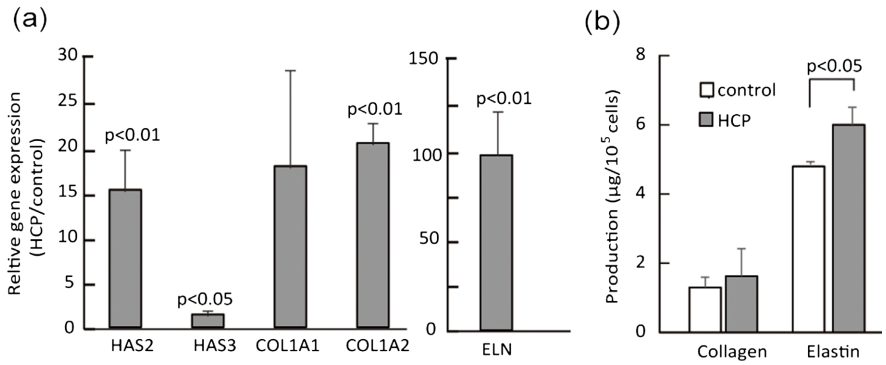

Figure 4. Moisturizing effects of honey child powder (HCP) extract on TIG121 skin fibroblasts. (a) Ratio of gene expression of TIG121 skin fibroblasts cultured in the medium with HCP extract for 1 week to that in cells cultured without HCP (mean ± SD, n = 3). (b) Collagen content at cell surface (left side) and intracellular elastin content of cells (right side) when TIG121 skin fibroblasts were cultured in the medium without HCP (white bars) and with HCP (gray bars; mean ± SD, n = 3).

4. Discussion

In this study, we investigated whether TL is directly related to cellular senescence. TL shortening during cell division by long-term subculture of human fibroblasts directly inhibited the cell division rate and induced cell senescence (**Figure 1**). Additionally, when TL was elongated by transformation with TERT, the division rate increased, ER stress due to cellular aging was alleviated, and the expression of a group of genes that were highly expressed in young individuals increased (**Figure 2**). When the endoplasmic reticulum is stressed by aging, autophagy activity generally increases because of an increase in abnormal proteins [14]; this results in excessive expression of GRP78 [15]. Moreover, superoxide dismutase 2 and catalase activities are reduced in elderly mice [16], and *C. elegans* in which these activities are enhanced by genetic mutations have an extended lifespan [17]. Klotho is involved in calcium homeostasis, and transgenic mice with enhanced Klotho levels are rejuvenated [18]. DNA polymerase γ is involved in DNA stabilization, and sirtuin 1 and sirtuin 2 are involved in mitochondrial activation and chromosome stabilization [19]; low expression of these genes induces aging. In addition, the growth factors EGF and IGF are generally used as indicators of fibroblast senescence, because their expression is significantly decreased in cells with a high mitotic frequency [20]. The results obtained in this study indicate that TL is related to the regulation of cellular senescence and can be used as an indicator of cellular senescence.

In addition, silencing TRF2 mRNA using siRNAs temporarily restored the mitotic rate, suggesting that TRF1 and TRF2 released by telomere shortening may control the cell growth rate (**Figure 1(c)**). TRF1 and TRF2 bind to telomere

repeats (TTAGGG) and play an important role in cell division [21]; shortening of TL causes the release of these proteins. TRF1 and TRF2 bind to and stabilize telomeres [22], and TRF2 is associated with senescence [23]; however, the effects of TRF1 and TRF2 on cell growth rate have not been reported. Therefore, our results indicate a new function for TRFs. The detailed mechanism is under investigation, and will be reported in the future.

Furthermore, we found that the HCP extract increased telomerase activity, elongated TL, and improved expression of HAS3, COL1A1, and ELN, which are important for skin moisturization [24]. HCP extract suppresses menin, a regulator of the TERT gene, resulting in enhanced telomerase activity. Lin *et al.* reported that the MEN mutant strain was similar to immortalized cells [25]. Therefore, telomerase activation by the HCP extract may be due to repression of the MEN gene. TA-65 [26], sapogenin [27], and epitalon [28] have already been found to elongate telomeres, and their effects when administered to mice have been investigated, but no compounds that inhibit menin have been found. Therefore, HCP extract is a novel telomerase-active compound.

Notably, the HCP extract effects were high during the first week of addition but decreased after the second week. This trend was similar to that observed for pit viper powder and placenta, which showed increased telomerase activity. The TERT gene is managed by multiple regulatory factors in normal somatic cells [25]. Therefore, activation of telomerase by the repression of menin may had led to the activation of another regulatory mechanism that triggers a rapid decrease in telomerase activity. To continuously extend the telomeres of cells in the future using telomere-lengthening compounds, it is necessary to elucidate the mechanism underlying this regulation and identify any additional regulatory factors.

This study has a number of limitations. In general, most animals have sufficiently long telomeres, suggesting that relationship between TL and cellular senescence in human cells may not be applied to the other animals. Further investigation is needed in non-human organisms. We identified a possible relationship between TL and cellular senescence, which is implicated in TRF. However, the mechanism has not yet been proven, and further investigation is needed. Additionally, we identified telomere elongation and rejuvenation effects in HCP. However, our investigation had an insufficient number of cell types and samples, and the phenomenon of telomerase activity reduction has not yet been fully elucidated. Further investigation is necessary to improve the usefulness of HCP.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Sanders, J.L. and Newman, A.B. (2013) Telomere Length in Epidemiology: A Biomarker of Aging, Age-Related Disease, Both, or Neither? *Epidemiologic Reviews*,

35, 112-131. https://doi.org/10.1093/epirev/mxs008

[2] Heidinger, B.J., Blount, J.D., Boner, W., Griffiths, K., Metcalfe, N.B. and Monaghan, P. (2012) Telomere Length in Early Life Predicts Lifespan. *Proceedings of the National Academy of Sciences of USA*, **109**, 1743-1748. https://doi.org/10.1073/pnas.1113306109

[3] Njajou, O.T., Cawthon, R.M., Damcott, C.M., Wu, S., Ott, S., Garant, M.J., *et al.* (2007) Telomere Length Is Paternally Inherited and Is Associated with Parental Lifespan. *Proceedings of the National Academy of Sciences of USA*, **104**, 12135-12139. https://doi.org/10.1073/pnas.0702703104

[4] Fasching, C.L. (2018) Telomere Length Measurement as a Clinical Biomarker of Aging and Disease. *Critical Reviews in Clinical Laboratory Sciences*, **55**, 443-465. https://doi.org/10.1080/10408363.2018.1504274

[5] Raices, M., Maruyama, H., Dillin, A. and Karlseder, J. (2005) Uncoupling of Longevity and Telomere Length in C. Elegans. *PLOS Genetics*, **1**, e30. https://doi.org/10.1371/journal.pgen.0010030

[6] Tomás-Loba, A., Flores, I., Fernández-Marcos, P.J., Cayuela, M.L., Maraver, A., Tejera, A., *et al.* (2008) Telomerase Reverse Transcriptase Delays Aging in Cancer-Resistant Mice. *Cell*, **135**, 609-622. https://doi.org/10.1016/j.cell.2008.09.034

[7] Bernardes de Jesus, B.B., Vera, E., Schneeberger, K., Tejera, A.M., Ayuso, E., Bosch, F., *et al.* (2012) Telomerase Gene Therapy in Adult and Old Mice Delays Aging and Increases Longevity without Increasing Cancer. *EMBO Molecular Medicine*, **4**, 691-704. https://doi.org/10.1002/emmm.201200245

[8] Jaskelioff, M., Muller, F.L., Paik, J.-H., Thomas, E., Jiang, S., Adams, A.C., *et al.* (2010) Telomerase Reactivation Reverses Tissue Degeneration in Aged Telomerase-Deficient Mice. *Nature*, **469**, 102-106. https://doi.org/10.1038/nature09603

[9] Greider, C.W. and Blackburn, E.H. (1987) The Telomere Terminal Transferase of Tetrahymena Is a Ribonucleoprotein Enzyme with Two Kinds of Primer Specificity. *Cell*, **51**, 887-898. https://doi.org/10.1016/0092-8674(87)90576-9

[10] Kumari, R. and Jat, P. (2021) Mechanisms of Cellular Senescence: Cell Cycle Arrest and Senescence Associated Secretory Phenotype. *Frontiers in Cell and Developmental Biology*, **9**, Article 645593. https://doi.org/10.3389/fcell.2021.645593

[11] Cawthon, R.M. (2002) Telomere Measurement by Quantitative PCR. *Nucleic Acids Research*, **30**, e47. https://doi.org/10.1093/nar/30.10.e47

[12] Shiomi, N., Ito, M. and Watanabe, K. (2016) Characteristics of Beige Adipocytes Induced from White Adipocytes by *Kikyo* Extract. *Journal of Biomedical Science and Engineering*, **9**, 342-353. https://doi.org/10.4236/jbise.2016.97029

[13] Itahana, K., Campisi, J. and Dimri, G.P. (2007) Methods to Detect Biomarkers of Cellular Senescence: The Senescence-Associated Beta-Galactosidase Assay. *Methods in Molecular Biology*, **371**, 21-31. https://doi.org/10.1007/978-1-59745-361-5_3

[14] Qi, Z. and Chen, L. (2019) Endoplasmic Reticulum Stress and Autophagy. *Advances in Experimental Medicine and Biology*, **1206**, 167-177. https://doi.org/10.1007/978-981-15-0602-4_8

[15] Pfaffenbach, K.T. and Lee, A.S. (2011) The Critical Role of GRP78 in Physiologic and Pathologic Stress. *Current Opinion in Cell Biology*, **23**, 150-156. https://doi.org/10.1016/j.ceb.2010.09.007

[16] Tsay, H.J., Wang, P., Wang, S.L. and Ku, H.H. (2000) Age-Associated Changes of Superoxide Dismutase and Catalase Activities in the Rat Brain. *Journal of Biomedical Science*, **7**, 466-474. https://doi.org/10.1007/bf02253362

[17] Johnson, T.E. (1990) Increased Life-Span of Age-1 Mutants in *Caenorhabditis ele-*

gans and Lower Gompertz Rate of Aging. *Science*, **249**, 908-912. https://doi.org/10.1126/science.2392681

[18] Kurosu, H., Yamamoto, M., Clark, J.D., Pastor, J.V., Nandi, A., Gurnani, P., *et al.* (2005) Suppression of Aging in Mice by the Hormone Klotho. *Science*, **309**, 1829-1833. https://doi.org/10.1126/science.1112766

[19] Lee, S.-H., Lee, J.H., Lee, H.-Y. and Min, K.-J. (2019) Sirtuin Signaling in Cellular Senescence and Aging. *BMB Reports*, **52**, 24-34. https://doi.org/10.5483/bmbrep.2019.52.1.290

[20] Shiraha, H., Gupta, K., Drabik, K. and Wells, A. (2000) Aging Fibroblasts Present Reduced Epidermal Growth Factor (EGF) Responsiveness Due to Preferential Loss of EGF Receptors. *Journal of Biological Chemistry*, **275**, 19343-19351. https://doi.org/10.1074/jbc.m000008200

[21] Sarek, G., Kotsantis, P., Ruis, P., Van Ly, D.V., Margalef, P., Borel, V., *et al.* (2019) CDK Phosphorylation of TRF2 Controls T-Loop Dynamics during the Cell Cycle. *Nature*, **575**, 523-527. https://doi.org/10.1038/s41586-019-1744-8

[22] Broccoli, D., Smogorzewska, A., Chong, L. and de Lange, T. (1997) Human Telomeres Contain Two Distinct Myb-Related Proteins, TRF1 and TRF2. *Nature Genetics*, **17**, 231-235. https://doi.org/10.1038/ng1097-231

[23] Muñoz, P., Blanco, R. and Blasco, M.A. (2006) Role of the TRF2 Telomeric Protein in Cancer and Aging. *Cell Cycle*, **5**, 718-721. https://doi.org/10.4161/cc.5.7.2636

[24] Mawazi, S.M., Ann, J., Othman, N., Khan, J., Alolayan, S.O., Al thagfan, S.S., *et al.* (2022) A Review of Moisturizers; History, Preparation, Characterization and Applications. *Cosmetics*, **9**, Article 61. https://doi.org/10.3390/cosmetics9030061

[25] Lin, S.-Y. and Elledge, S.J. (2003) Multiple Tumor Suppressor Pathways Negatively Regulate Telomerase. *Cell*, **113**, 881-889. https://doi.org/10.1016/s0092-8674(03)00430-6

[26] de Jesus, B.B., Schneeberger, K., Vera, E., Tejera, A., Harley, C.B. and Blasco, M.A. (2011) The Telomerase Activator TA-65 Elongates Short Telomeres and Increases Health Span of Adult/Old Mice without Increasing Cancer Incidence. *Aging Cell*, **10**, 604-621. https://doi.org/10.1111/j.1474-9726.2011.00700.x

[27] Küçüksolak, M., Yılmaz, S., Ballar-Kırmızıbayrak, P. and Bedir, E. (2023) Potent Telomerase Activators from a Novel Sapogenin via Biotransformation Utilizing *Camarosporium laburnicola*, an Endophytic Fungus. *Microbial Cell Factories*, **22**, Article No. 66. https://doi.org/10.1186/s12934-023-02069-3

[28] Yue, X., Liu, S.-L., Guo, J.-N., Meng, T.-G., Zhang, X.-R., Li, H.-X., *et al.* (2022) Epitalon Protects against Post-Ovulatory Aging-Related Damage of Mouse Oocytes *in Vitro*. *Aging*, **14**, 3191-3202. https://doi.org/10.18632/aging.204007