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Re-evaluation of the Relationship between Donor Age and Replicative Lifespan Using ASF-4 Series of Fibroblasts Established from a Single Individual: Application of a Mathematical Model and Examination of Aging Parameters

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Abstract

ASF-4 series of cells were established from tissues collected from a single Japanese individual over the course of 40 years. Previously, we reported that the replicative lifespan of ASF-4 cells was reduced and was dependent on the increasing age of the donor as the telomere length was shortened. In this study, using ASF-4 cells that included cells from donors aged 60 years or older, we confirmed that the replicative lifespan of the cells was negatively correlated with donor age. We created a mathematical model to estimate the replicative lifespan from the experimental data and showed that the replicative lifespan can be determined with high accuracy. Furthermore, we found that the variation in the replicative lifespan increased with a donor age above 60 years, the correlation with donor age was lower, and the parameters of the mathematical model showed a greater variation in cells from a donor aged 60 years or older. These results confirmed that the replicative lifespan of cells decreases depending on the individual's age and suggested that, in the case of humans, there is more diversity in the phenomenon of cellular aging for individuals older than 60 years.

Keywords: individual aging, cellular senescence, replicative senescence, telomere

Introduction

Aging is the process defined as the irreversible and progressive, gradual functional decline of an individual's physiological and reproductive functions over time that is common in many higher organisms. In 1961, Hayflick and Moorhead proposed the concept of cellular senescence by demonstrating that cells collected from an individual had a limited number of divisions within a cell culture, depending on the individual's age[1)] . Subsequently, research on aging at the cellular level confirmed that cells collected from individuals have a

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replicative lifespan, although there are differences between tissue types, and that telomeres at the ends of chromosomes shorten with cellular aging. Nevertheless, the relationship between individual aging and cellular senescence remains to be elucidated.

An ASF-4 series of cells were established from tissues collected from a single individual over a period of 40 years, at 5–10 year intervals, at the donor age, ranging from 36 to 76 years. We previously reported that the replicative lifespan of cells was reduced with increased donor age, as well as shortened telomere length depending on the age of the donor[2]. We further examined the relationship between DNA methylation and donor age using ASF-4 cells[3] and changes in the metabolic systems associated with cellular senescence using comprehensive gene expression analysis through RNA-seq.

In this study, we measured the replicative lifespan of ASF-4 cells collected through the conventional method and re-evaluated the cell culture data by applying a mathematical model to estimate the replicative lifespan of cells from the population doubling level (PDL) data obtained during the cell culture experiments. The relationship between the replicative lifespan of the cells determined by the mathematical model and individual aging is discussed.

Materials and Methods

1. Preparation of ASF-4 cells

ASF-4 cells are fibroblasts that are cultured from skin tissue collected from the same Japanese male donor at different ages; the method for establishing ASF-4 cells and cell culture conditions was previously reported[2]. Briefly, primary cell cultures were initiated from 5 mm^2 of tissue collected from the inner side of the donor's upper arm. The skin tissue was cut into smaller pieces and cultured in a 10-cm dish (Falcon, Franklin Lakes, NJ, USA) in Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum at 37 ° C in a humidified atmosphere with 5% CO_2. After a few days, fibroblasts spread from the edges of the skin tissue, and when confluency was reached, the cells were harvested from the culture dishes by trypsin treatment (0.25% trypsin, 37° C for 10 min) and cultured in passages in a 1:4 dilution at weekly intervals.

This study was approved by the research ethics committee of Nihon Pharmaceutical University (No. 27-04) . The participant signed an informed consent statement prior to participation in the study.

2. Measurement of the cells' replicative lifespan as the cumulative *PDL*

The replicative lifespan (i.e., maximum number of cell divisions) was measured as the PDL. The number of divisions (PDL) x in one passaging culture was calculated as follows:

$$\frac{N_H}{N_I}=2^x$$

or,

$$\log_2 (N_H) - \log_2 (N_I) = x$$

Where N_I is the number of cells at the beginning of passaging, and N_H is the number of cells at the end of the culture passaging. The cells were counted with a Coulter Counter Model D (Coulter Electronics, Hialeah, FL, USA). The PDL of each passaging culture was combined, and the total number of cell divisions was calculated as the *PDLc* (cumulative PDL) at the time point. The limit of cell division was defined as when the cell population did not double after 3 weeks of culture and after two consecutive weeks of passages. The total *PDLc* at this time point was defined as the replicative lifespan (*PDL*) of the cell.

3. Determination of the cell's replicative lifespan by fitting a mathematical model

In one passaging culture, cells in a 1:4 dilution were added to a petri dish and cultured for about one week until the cells were almost confluent, which resulted in an increase in the *PDL* of two for normal cells. For cells that lose their cell division ability due to cellular senescence, the increase of the *PDL* in one passaging culture is assumed to decrease from 2, depending on the total number of *PDLc* cell divisions during the culture, and reach 0 when the cell's replicative lifespan is attained. The decrease in the *PDL* was assumed to be represented by a negative exponential function with an exponent of 2 being low. Based on the above assumptions, the following equation was assumed to represent the change in the increase of the *PDL* in one passaging culture (hereafter referred to as ΔPDL) when the cells were cultured in a 1:4 dilution ratio.

$$\Delta PDL = PDL_i \ (1 - 2^{-(PDLmax - PDLc)})$$

Where ΔPDL is the amount of change in the *PDL* in the passaging culture, *PDLi* is the initial value of ΔPDL for each cell type (assuming that *PDLc* is zero, the number of cell divisions in a 1:4 dilution ratio passaging culture is expected to be approximately [2]). PDL_{max} is the replicative lifespan of that cell type, and *PDLc* is the total *PDL* at that time point.

Furthermore, from the data on changes in cell number during the cell culture, we observed that ΔPDL decreased linearly with cell culture time for some ASF-4 cells; therefore, we considered this and assumed the following formula, multiplying the overall formula by $(a \times PDLc + 1)$.

$$\Delta PDL = PDLi \ (1 - 2 - ^{(PDLmax - PDLc)}) \cdot (a \times PDLc + 1)$$

In this equation, a represents the slope of the linear decrease in the number of divisions during passaging culture dependent on *PDLc*, which is expected to be between -0.002 and -0.01, based on the experimental data. This equation was fitted to ΔPDL and *PDLc* obtained from the data of the cell numbers during passaging culture using the nonlinear least squares method with PDL_i, PDL_{max}, and a as target parameters to be optimized, and each parameter in the model equation was calculated for each cell type. The nonlinear least squares method was performed using the statistical software R[4]. The initial values for the

least squares method calculations of PDL_i was set to 2.0, PDL_{max} was set to the replicative lifespan obtained by method 2 described in Materials and Methods, and a was set to −0.002.

Results

The cell names, donor age, and measured replicative lifespan of the ASF-4 cells collected to date are presented in Table 1. There is a strong negative correlation between the donor age and the replicative lifespan of the cells, with a correlation coefficient of −0.832 ($p = 1.28 \times 10^{-6}$). The replicative lifespan (PDL_{max}) obtained by fitting the mathematical model was similar to the replicative lifespan calculated from the number of cells using method 2 described in the Materials and Methods section. The correlation coefficient between the replicative lifespans that were calculated experimentally and the PDL_{max} that were calculated using the mathematical model was −0.992, and the mean difference was −1.02 (standard deviation (SD), 0.93). The advantages of the method using a mathematical model for the estimation of the replicate lifespan are that changes in the number of cells in the cell culture process can be considered to an extent, and the least squares method allows for the evaluation of errors in the calculating PDL_{max} (Table 1). The standard error of estimated PDL_{max} values using the nonlinear least squares method ranged from 0.16 to 1.92, indicating that the least squares method was able to estimate PDL_{max} with high accuracy.

Table 1. Donor age and replicative life span (population doubling level; PDL) of ASF-4 cells obtained to date.

The table also shows indicates the values of PDL_i, PDL_{max}, and a values. The standard errors of PDL_i, PDL_{max}, and a obtained by the least-squares method are shown in parentheses. "Donor Age" means the age of sampling from one individual.

No	Cell Name	Donor Age	Lifespan (PDL)	PDLi	PDLmax	a	
1	ASF-4-1	36.2	66.5	2.08 (0.14)	67.1 (0.37)	−3.0E−3	(1.4E−3)
2	ASF-4-2	47.5	62.1	2.05 (0.12)	63.4 (0.28)	−2.5E−3	(1.4E−3)
3	ASF-4-3L	56.9	52.1	2.16 (0.20)	52.7 (0.30)	−4.6E−3	(2.5E−3)
4	ASF-4-3R	56.9	46.5	2.15 (0.21)	45.6 (0.28)	−4.7E−3	(3.1E−3)
5	ASF-4-4L1	62.6	41.0	2.63 (0.33)	41.4 (0.39)	−1.1E−2	(3.4E−3)
6	ASF-4-4L2	62.6	48.2	2.12 (0.18)	49.9 (0.78)	−4.3E−3	(2.5E−3)
7	ASF-4-4R1	62.6	48.7	2.55 (0.30)	50.5 (1.55)	−9.7E−3	(2.7E−3)
8	ASF-4-4R2	62.6	45.3	2.18 (0.19)	48.0 (1.92)	−5.5E−3	(2.7E−3)
9	ASF-4-5L1	67.4	45.5	2.04 (0.10)	46.5 (0.26)	−2.8E−3	(1.7E−3)
10	ASF-4-5L2	67.4	39.7	2.31 (0.14)	41.1 (0.57)	−8.4E−3	(2.1E−3)
11	ASF-4-5R1	67.4	55.1	2.14 (0.16)	57.0 (0.39)	−3.6E−3	(1.9E−3)
12	ASF-4-5R2	67.4	39.7	2.04 (0.14)	39.0 (0.29)	−2.9E−3	(2.8E−3)
13	ASF-4-6L1	72.8	41.5	2.35 (0.11)	42.4 (0.40)	−9.8E−3	(1.5E−3)
14	ASF-4-6L2	72.8	41.0	2.35 (0.11)	42.1 (0.41)	−1.0E−2	(1.4E−3)
15	ASF-4-6R1	72.8	41.4	2.33 (0.12)	42.8 (0.37)	−9.0E−3	(1.6E−3)
16	ASF-4-6R2	72.8	48.0	2.26 (0.10)	48.8 (0.21)	−5.9E−3	(1.3E−3)
17	ASF-4-7L1	76.0	41.5	2.18 (0.18)	42.1 (0.27)	−5.9E−3	(2.7E−3)
18	ASF-4-7L2	76.0	40.0	2.17 (0.14)	40.3 (0.16)	−6.0E−3	(2.3E−3)
19	ASF-4-7L3	76.0	43.5	2.35 (0.11)	44.0 (0.24)	−8.7E−3	(1.4E−3)
20	ASF-4-7R1	76.0	44.7	2.32 (0.11)	45.4 (0.28)	−7.4E−3	(1.5E−3)
21	ASF-4-7R2	76.0	43.3	2.39 (0.23)	44.7 (0.98)	−9.9E−3	(2.8E−3)
22	ASF-4-7R3	76.0	37.9	2.11 (0.14)	41.3 (0.23)	−4.6E−3	(2.5E−3)

Fig. 1 shows the dependency of ΔPDL and the estimated ΔPDL using the mathematical model of *PDLc* for the ASF-4-1, ASF-4-3L, ASF-4-5L1, and ASF-4-7L1 cells. As shown in Fig. 1, the estimated ΔPDL values using the mathematical model (gray line) and the ΔPDL values calculated from the experimental data (black line) showed good agreement. Fig. 2 shows the relationship between *PDLc* and the difference in the ΔPDL values obtained from the experimental data and mathematical models for the same ASF-cells presented in Fig. 1. No dependence on residuals of *PDLc* was observed, and no systematic errors were indicated. Similar results were obtained for other cell lines, thus demonstrating the validity of the mathematical model. Fig. 3 shows the relationship between the PDL_{max} calculated using the mathematical model and donor age. Similar to the replicative lifespan obtained using method

2 described in the Materials and Methods section, PDL_{max} also revealed a strong negative correlation with donor age, with a correlation coefficient of -0.801 ($p = 4.46 \times 10^{-6}$).

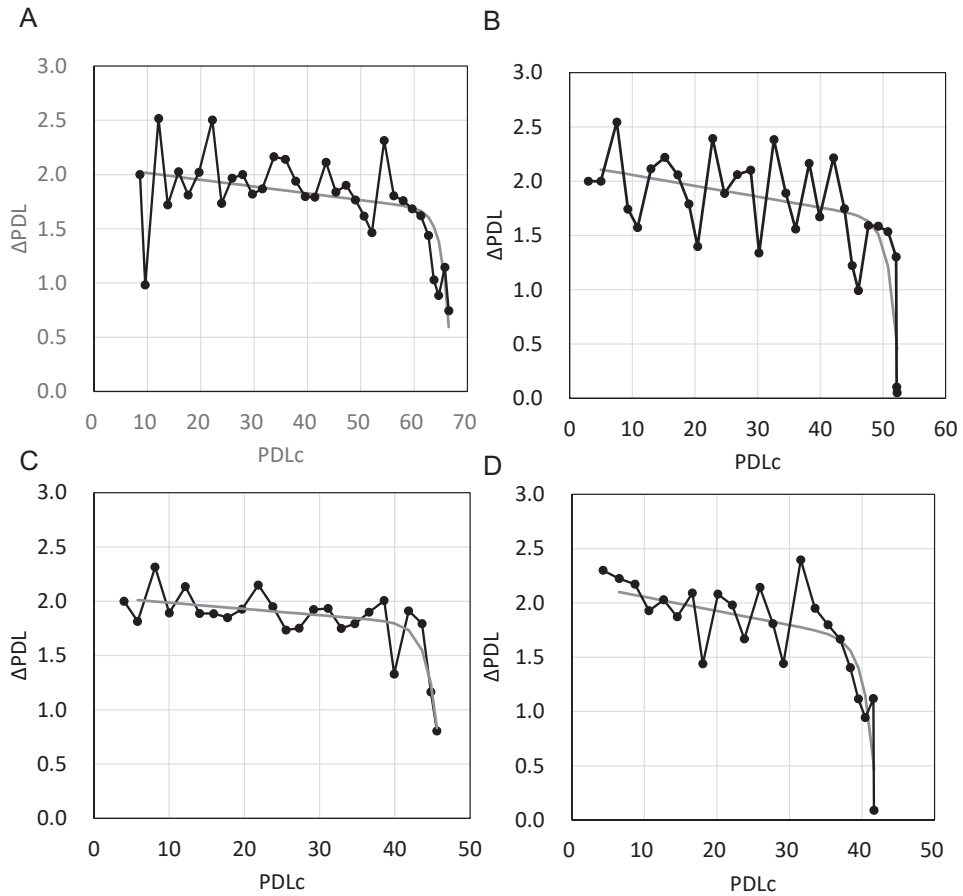

Fig. 1. Relationship between the number of cell divisions per passaging culture (ΔPDL) and the total number of cell divisions (PDL_c).

Relationship between the number of cell divisions per passaging culture (ΔPDL) and the total number of cell divisions (PDL_c) for the ASF-4-1 (A), ASF-4-3L (B), ASF-4-5-L1 (C), and ASF-4-7-L1 (D) cell cultures (black line) and the theoretical curve obtained using the mathematical model (gray line).

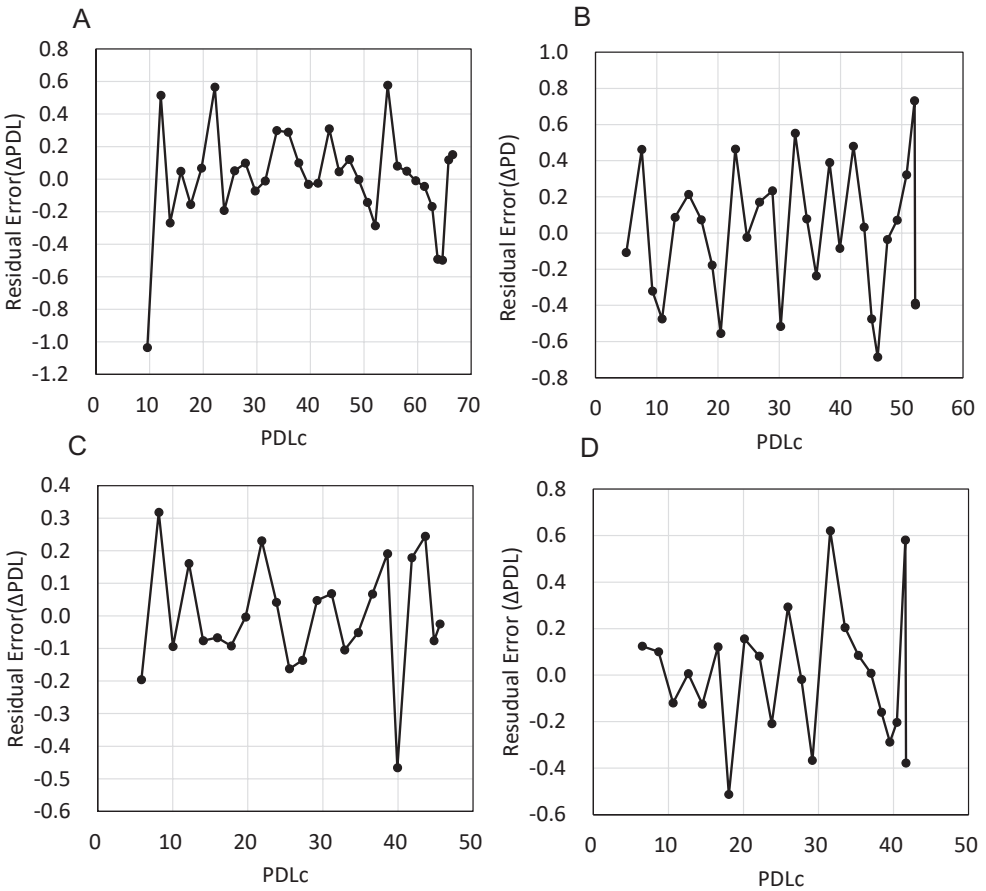

Fig. 2. Relationship between residuals of the number of cell divisions per passaging culture (Δ *PDL*) and the total number of cell divisions (PDL_c).

Relationship between the residuals of ΔPDL and the total number of cell divisions (PDL_c) for the ASF-4-1 (A), ASF-4-3L (B), ASF-4-5-L1 (C), and ASF-4-7-L1 (D) cell cultures. No dependency on PDL_c was observed, and the residual error was mostly random.

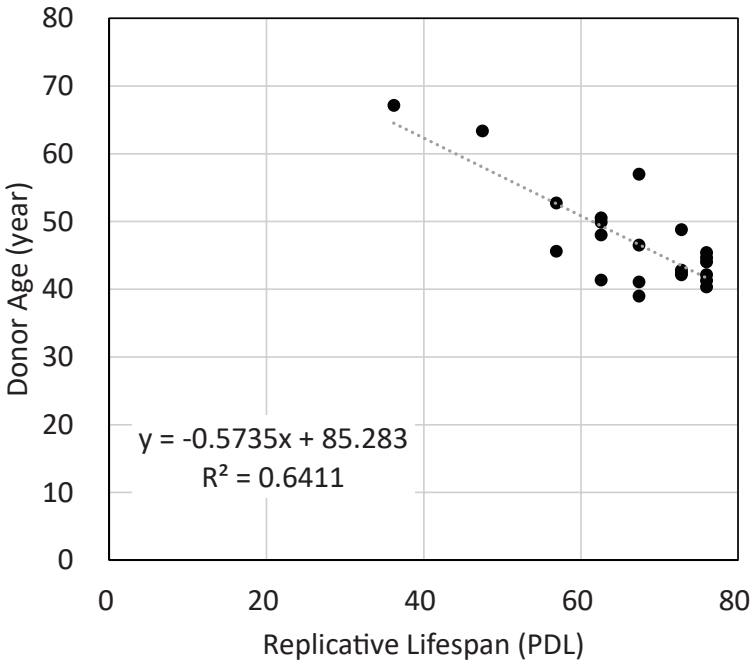

Fig. 3. Relationship between the replicative lifespan calculated using the mathematical model and donor age.
The linear regression equation and coefficient of determination (R^2) are shown. The replicate lifespan decreased with the increasing donor age, and the correlation coefficient was −0.801.

Discussion

1．Relationship between the replicative lifespan of cells and individual aging

The concept of cellular senescence through replicative lifespan was proposed by Hayflick and Moorhead（1961）and the importance of the cellular replicative lifespan has become widely accepted in aging research. Individual aging and cellular senescence have been linked in many animal species, and in some mammalian species, a link between species longevity and cellular replicative lifespan has been suggested[5]. However, in recent years, reports have questioned the link between aging and cellular replicative lifespan, especially in humans[6, 7, 8]. This is due to many studies that have investigated individual and cellular aging using cells from multiple human subjects, and it was found that individual and environmental differences in aging are considerable because humans adapt to various environments and are more capable of surviving the aging process than other organisms due to the effects of civilization. Using ASF-4 fibroblasts, which were established from a single human donor at different ages, the effects of individual and environmental differences can be eliminated, and the relationship between individual aging and cellular senescence can be precisely evaluated. In addition to measuring the replicative lifespan through the accumulation of PDL during cell culture, we were able to evaluate the replicative lifespan

more precisely by creating a mathematical model (Table 1).

For the ASF-4-1 to ASF-4-4 cell types, we previously reported on the shortening of the replicative lifespan and telomeres, and a strong negative correlation between donor age and replicative lifespan was observed[2]. In the present study, we confirmed the negative correlation between donor age and replicative lifespan for the ASF-4 cells established from tissues collected from the donor between the ages of 36 and 76 years, including the ASF-4-5 to 7 cell types that were collected at the age of 60 years and older. However, in the previous analysis of ASF-4-1 to ASF-4-4, the correlation between donor age and replicative lifespan was greater, with correlation coefficients ranging from −0.92 to −0.98. Table 1 and Fig. 3 show that the variation in replicative lifespan was greater for the cells established from the donor at the age of 60 years and older, which may be the reason for the lower correlation coefficient in this study.

This could be attributed to the larger number of cells that were established at age 60 years and older. In general, the diversity of the effects of aging on each cell increases even at the individual level, and it is possible that the diversity of senescent cells increases with advancing age, even within an individual. If this is true, then it is plausible that increasing the number of samples collected would have resulted in cells with diverse replicative lifespans that reflect the diversity in the state of aging.

Another notion is that after a certain age, the degree of increase in the replicative lifespan of a cell may decrease with respect to the increasing age of the individual, or that a given replicative lifespan does not decrease from a certain value. It has been reported that the replicative lifespan of cells collected from humans over 90 years of age tends to be rather long[9].

Although research on a single participant over a long period of time makes it challenging to conclude due to the small number of early cell samples, Fig. 3 shows that the decrease in replicative lifespan appears to slow down after the donor age of 60 years. This may indicate that the relationship between individual age and the replicative lifespan of cells is not linearly related and that there is a limit to the replicative lifespan of cells to the decline in divisional aging, or at least that the replicative lifespan of cells does not considerably decline beyond the age of 60.

In addition to the correlation coefficient between donor age and the replicative lifespan, Fig. 3 indicates the degree of reduction in the replicative lifespan with respect to the progression in donor age. The overall value of the reduction of replicative life span per donor age for the 22 ASF-4 cells was 0.59 PDL/year, which, when calculated using only the ASF-4-1 to ASF-4-4 data, was 0.81 PDL/year. This suggests that a reduction in the replicative lifespan of cells per year is on average 0.59–0.81 PDL in humans. However, as mentioned above, the correlation between donor age and replicative lifespan tended to be less for donors at ages above 60 years; therefore, further investigation is required on the relationship between the replicative lifespan and individual aging.

2．Biological significance of the parameters obtained from the mathematical model

In addition to the ΔPDL and PDL_c measurements in the cell culture experiments, the mathematical model used in this study included PDL_i and a as parameters. These were constants for each cell type that were introduced for convenience in fitting the data, but it is possible that they may indicate characteristics that are related to cellular senescence. To investigate this, we examined the dependence of these two cell-type constants on the donor age of the ASF-4 cells, and the results are shown in Fig. 4. The value of PDL_i was almost 2, as expected, but tended to increase slightly with donor age. The a showed minimal variation with donor age when lower than 60 years, with a value between −0.002 and −0.005, but showed considerable variation when the donor age was above 60 years.

Because there was no significant correlation between the a value and estimated replicative lifespan (PDL_{max}) from Table 1, it is possible that the value of a represents a cell property other than the replicative lifespan. Conversely, since it is estimated from the variation in the replicative lifespan that diversity increases as donor age increases above 60 years, it may be related to the previously mentioned change in cell diversity above 60 years of age.

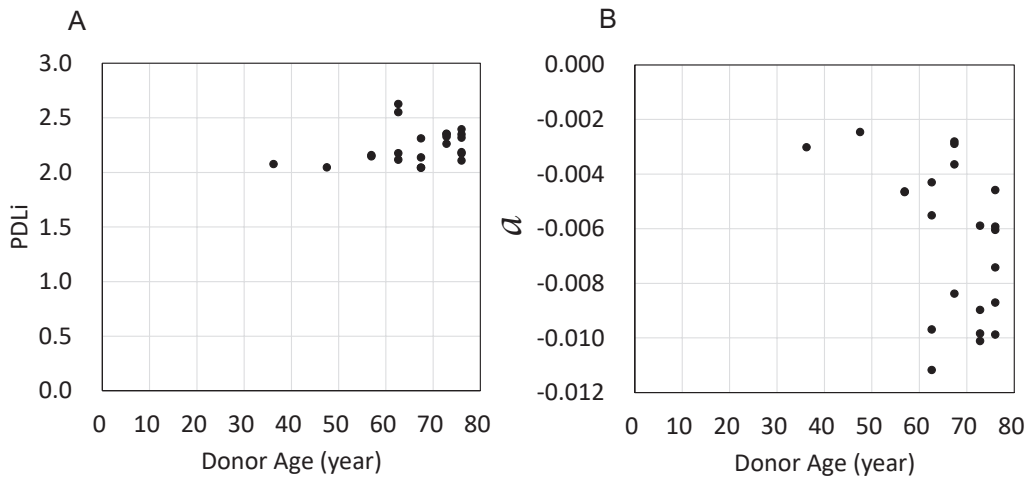

Fig. 4. Relationship between the donor age of ASF-4 cells and the values of PDL_i (A) and a (B) in the mathematical model.

The PDL_i value was almost 2 and tended to increase slightly with donor age. The a parameter showed a value ranged from −0.002 to −0.004 when the donor age was less than 60 years old, but tended to vary more when the donor age was over 60 years old, with values ranging from −0.003 to −0.01.

Conclusion

The relationship between individual aging and cellular senescence remains to be elucidated. In this study, ASF-4 cells, fibroblasts collected from a single Japanese male over a period of 40 years were used, and we confirmed a strong negative correlation

between donor age and the replicative lifespan of cells. We created a mathematical model to determine the replicative lifespan from experimental data and confirmed the correlation by applying the mathematical model. The results obtained from analyzing the ASF-4 cells that were collected at the age of 36 to 76 years suggest that for cells established from donors aged 60 years or older, there was increased diversity in terms of replicative lifespan and that the dependence of the replicative lifespan on donor age was reduced.

抄録

ASF-4系列の細胞は、単一の個人から40年にわたり、５－10年間隔、提供年齢で、36歳から76歳にわたり採取された組織から作られた細胞である。以前、我々はこの細胞系を用いて、提供年齢に応じて細胞の分裂寿命が短縮していること、テロメア長が短縮していることなどを報告した。本研究では、以前の報告後に得られた60歳以上の提供年齢の細胞を含むASF-4系列の細胞を用いて、提供年齢と細胞の分裂寿命が負の相関性を示すことを確認するとともに、実験データから分裂寿命を推定する数理モデルを作成し、再現性良く分裂寿命が求められることを示した。さらに、60歳以上の提供年齢では、分裂寿命についてのばらつきが大きくなり、提供年齢との相関性が低くなること、また数理モデルのパラメーターも60歳以上の提供年齢の細胞でばらつきが大きくなることを見出した。以上から、Hayflickらの提唱した、個体老化により細胞の分裂寿命が減少することを再度確認するとともに、ヒトの場合は、60歳を超える個体について細胞の老化現象に多様性が顕著にあらわれることを示唆する結果を得た。

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