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Nicotinamide extends replicative lifespan of human cells

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Summary

We found that an ongoing application of nicotinamide to normal human fibroblasts not only attenuated expression of the aging phenotype but also increased their replicative lifespan, causing a greater than 1.6-fold increase in the number of population doublings. Although nicotinamide by itself does not act as an antioxidant, the cells cultured in the presence of nicotinamide exhibited reduced levels of reactive oxygen species (ROS) and oxidative damage products associated with cellular senescence, and a decelerated telomere shortening rate without a detectable increase in telomerase activity. Furthermore, in the treated cells growing beyond the original Hayflick limit, the levels of p53, p21WAF1, and phospho-Rb proteins were similar to those in actively proliferating cells. The nicotinamide treatment caused a decrease in ATP levels, which was stably maintained until the delayed senescence point. Nicotinamide-treated cells also maintained high mitochondrial membrane potential but a lower respiration rate and superoxide anion level. Taken together, in contrast to its demonstrated pro-aging effect in yeast, nicotinamide extends the lifespan of human fibroblasts, possibly through reduction in mitochondrial activity and ROS production.

Key words: cellular senescence; NAD^+; nicotinamide; population doubling; ROS; telomere.

Introduction

Normal cells cultured *in vitro* proliferate for a limited number of population doublings (the 'Hayflick limit') and enter a stage of replicative senescence. At this stage, cells are arrested at the G1 phase of the cell cycle and express a specific cellular and biochemical phenotype (Hayflick & Moorhead, 1961; Campisi, 2001).

Telomere shortening is believed to be an intrinsic mechanism that sets a limit to the cellular doubling capacity, at least in human cells (Campisi, 2001). Activation of telomerase by introduction of the hTERT gene in primary human fibroblasts enables cells to proliferate indefinitely without the expression of the senescence phenotypes (Bodnar *et al.*, 1998). Recently it has been proposed that attrition of telomeres that failed to be protected by the telomere cap structure also induces senescence (Karlseder *et al.*, 2002). Telomere shortening and/or telomere attrition is probably registered as DNA damage, thereby, inducing a DNA damage checkpoint response (d'Adda *et al.*, 2003; Reaper *et al.*, 2004). Without being repaired, this will cause continuous activation of the p53-growth inhibitory pathway, and therefore drive cells into a state of irreversible growth arrest. Activation of the p53-growth inhibitory pathway itself, either through DNA-damaging treatments or overexpression of p53, p21WAF1, p16INK4a, or Rb, induces an acute state of senescence in a variety of cancer cells (Hwang, 2002). Considering all the different senescence-inducing conditions (Toussaint *et al.*, 2000), the activation of the growth-inhibitory pathway itself likely plays a key role in the manifestation of cellular senescence.

The level of reactive oxygen species (ROS) increases as cells approach the Hayflick limit (Hutter *et al.*, 2002). ROS are continuously produced by electrons leaking from the mitochondrial electron transport chain and other sources (Loschen & Azzi, 1975; Genova *et al.*, 2004). In addition, continuous treatment of cells with sublethal levels of ROS not only increases the level of oxidative damage products such as lipofuscin (Sitte *et al.*, 2001), but also causes permanent growth arrest accompanied by the activation of the p53-growth inhibitory pathway (Toussaint *et al.*, 2000). Furthermore, it has been shown that oxidative stress accelerates telomere shortening (von Zglinicki, 2002; Serra *et al.*, 2003). Therefore, at the cellular level, ROS play a key role in inducing senescence. Understanding more about the nature and interplay of telomere attrition, the p53-growth inhibitory pathway, and ROS would greatly enhance our understanding of the mechanics of aging.

Nicotinamide, a precursor of the coenzyme NAD^+, serves to rapidly synthesize NAD^+ through the salvage pathway once taken up by cells (Liu *et al.*, 1982; Jackson *et al.*, 1995). It has been shown to positively affect cell survival in a variety of cell types. Nicotinamide promotes maturation of fetal cells (Sakai *et al.*, 2002) and induces proliferation and differentiation of embryonic stem cells to yield insulin-producing cells (Vaca *et al.*, 2003). It also enhances an adaptive response to physical and chemical damage (Guruprasad *et al.*, 2002), protects brain cells from oxidative damage caused by reperfusion after ischemic infarction (Sun & Cheng, 1998; Mokudai *et al.*, 2000; Klaidman *et al.*, 2003), and prevents injury of pancreatic islet cells during free radical exposure (Kallmann *et al.*, 1992). Protection of cells from oxidative stress during reperfusion is believed to be attributed to the nicotinamide-driven supply of NAD^+ and energy, which would otherwise be depleted by heavy poly (ADP-ribosyl) polymerization of nuclear proteins by PARP-1 (Klaidman *et al.*, 2003; Skaper, 2003). However, the underlying mechanisms

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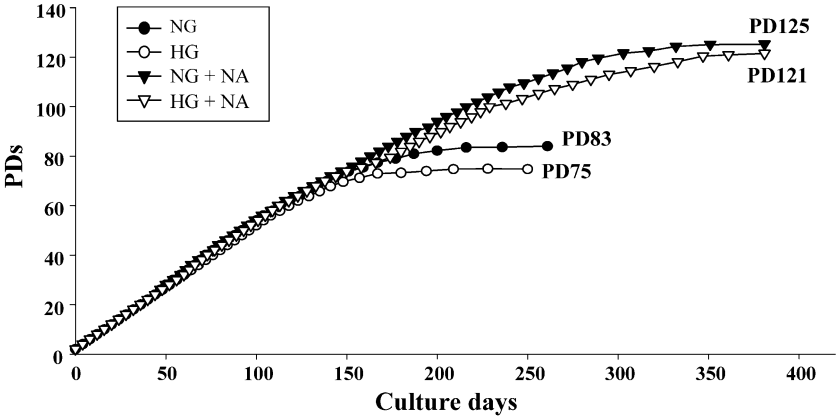

Fig. 1 Expansion of the population doubling capacity of normal human fibroblasts by nicotinamide. NHFs were continuously passaged in the DMEM containing glucose either of 25 mM (high glucose; HG; —○—); or 5.5 mM (normal glucose; NG; —●—). Or, starting from PD30, the cultures were continuously treated with 5 mM nicotinamide (NAM) (high-glucose medium; —▽—) (normal-glucose medium; —▼—). At each passage, cells were counted, and the number of population doublings was calculated.

for other effects are largely undetermined. Nicotinamide was also shown to temporarily delay expression of certain aspects of the senescence phenotype in human fibroblasts (Matuoka *et al.*, 2001), without the accompaniment of a significant extension of cellular lifespan. Furthermore, nicotinamide was shown to inhibit the activities of yeast Sir2, a NAD^+-dependent histone deacetylase (Luo *et al.*, 2001; Bitterman *et al.*, 2002) involved in gene silencing and genome stabilization (Guarente, 1999; Tanny *et al.*, 1999) as well as the doubling capacity (Kaeberlein *et al.*, 1999; Kim *et al.*, 2004). Sir2-mediated lifespan extension in yeast has been shown to be abrogated by nicotinamide treatment (Anderson *et al.*, 2003).

In this study, the long-term effects of nicotinamide treatment on the doubling capacity (cellular lifespan) of human primary fibroblasts were examined. Unexpectedly, nicotinamide treatment caused a huge increase in the population-doubling capacity of the cells. This effect was accompanied by a substantial decrease in telomere shortening and ROS accumulation as well as a delay in activation of the p53-growth inhibitory pathway. ATP levels and cellular respiration rate were substantially reduced but stably maintained during the extended lifespan suggesting a decreased but stable mitochondrial function and concomitant reduction of ROS production by the nicotinamide treatment.

Results

Lifespan extension in normal human fibroblasts by continuous treatment of nicotinamide

The effect of nicotinamide was examined in two different culture conditions. Human fibroblasts were continuously passaged until reaching senescence in a medium containing glucose at concentrations of either 25 mM (high glucose) or 5.5 mM (normal glucose). It has been reported that cells proliferate to a higher number of population doublings (PD) at a lower glucose concentration (Blazer *et al.*, 2002). Indeed, as shown in Fig. 1, cells proliferated up to PD83 in the presence of 5.5 mM glucose, while at 25 mM they proliferated for a shorter lifespan (PD75). Beyond these PD points, cell numbers did not increase for 21 days of subsequent incubation. At 5–6 PD prior to these points, growth rates were significantly reduced (from approximately 2 days/PD to over 10 days/PD). During this slow-growth phase, most cells expressed the senescence phenotype, being enlarged and highly positive for autofluorescence (data not shown) and senescence-associated β-galactosidase (SA β-gal) activity (see Fig. 2A). Furthermore, the cells expressed high level of p21WAF1 and contained Rb proteins mostly in hypophosphorylated state (Fig. 2B, lanes 6 and 14). Therefore, we concluded that the fibroblasts tested in this study reached senescence at PD75 and PD83 in the presence of 25 mM and 5.5 mM glucose, respectively.

To determine the effect of nicotinamide on population-doubling capacity, the fibroblasts were continuously cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5 mM nicotinamide beginning at PD30. Treatment with nicotinamide did not have any effect on the cell proliferation rate at early passages nor did it rescue cells from a growth-arrested state induced by serum starvation (data not shown). However, as shown in Fig. 1, cells cultured in the presence of nicotinamide divided many times beyond the original Hayflick limits; from PD75 to PD121 in high glucose and from PD83 to PD125 in normal glucose media (a 1.6- and 1.5-fold increase, respectively). Therefore, the lifespan of human fibroblasts in terms of population doublings is significantly extended by nicotinamide treatment. The similar increase in the PD numbers of the cultures at the different glucose concentrations suggests a mechanism that is independent of those governed by the levels of glucose fed to the cells.

In further studies using cells cultured in both the high and normal glucose conditions, almost identical results were obtained, and therefore only the results from the cells cultured in normal glucose medium will be presented unless a difference should be mentioned.

Nicotinamide delayed expression of the senescence phenotypes

Figure 2A shows that the increase in population doublings was accompanied by a delay in the expression of senescence

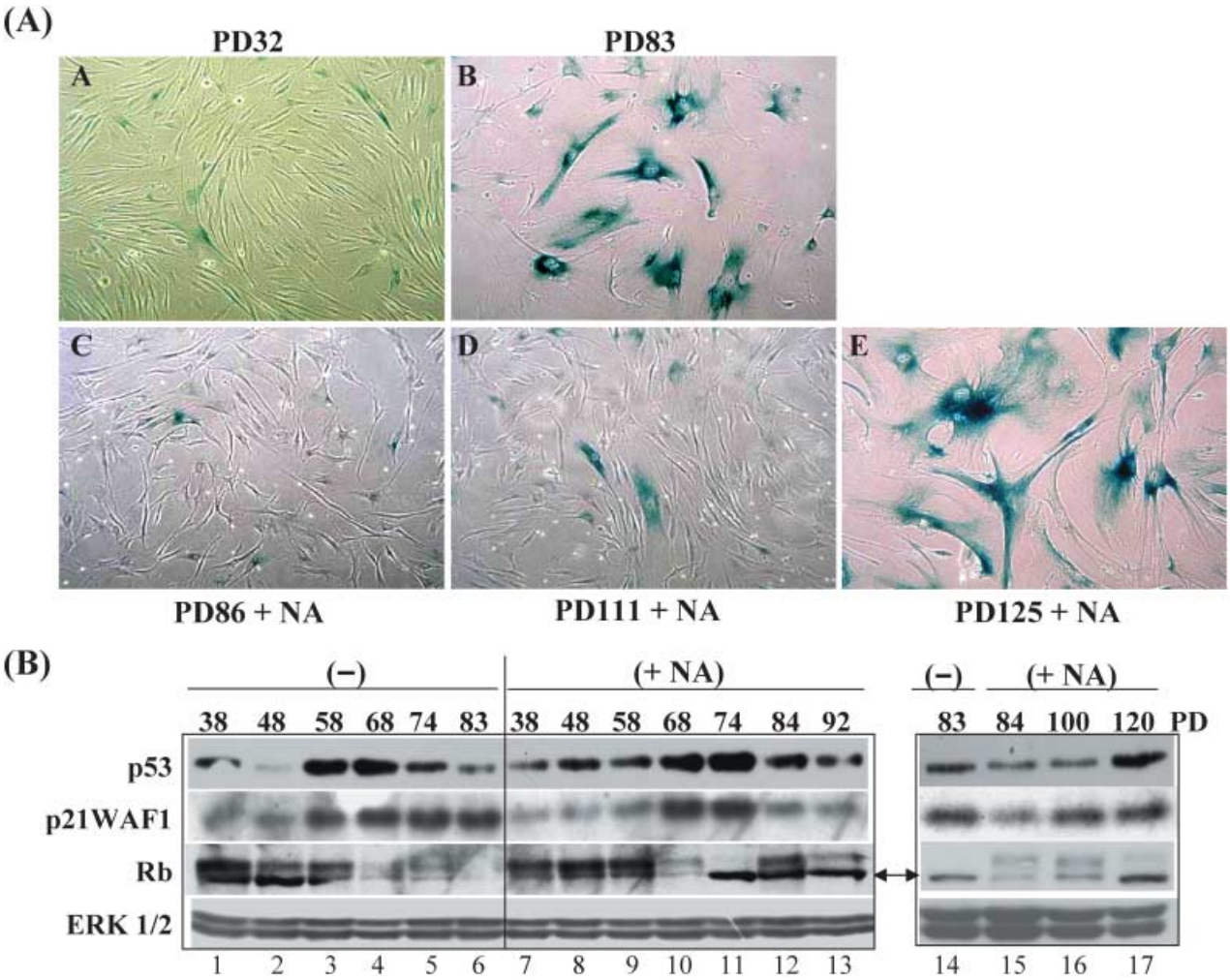

Fig. 2 Attenuation of SA β-gal activity expression and biochemical rejuvenation by nicotinamide treatment. (A) NHFs were passaged in a normal glucose medium until senescence (PD83) or in the medium containing 5 mM nicotinamide (PD86 + NA, PD111 + NA, and PD125 + NA) starting from PD 30 until senescence. At the indicated PDs, cells were fixed and stained for SA β-gal activity. (B) Cells cultured either in the absence (–) or presence of nicotinamide (+ NA) were collected at the indicated PD points, and applied to Western blot analysis for p53, p21WAF1, Rb, and ERK 1/2. The arrow in the Rb blots points the hypophosphorylated form. Expression of ERK 1/2 did not change during the passage, and therefore serves as a loading control. The right panel represents the protein patterns in the cells cultured at a different experiment, showing the patterns of the cells proliferated more closely to the delayed senescence points. In both (A) and (B), almost identical results were obtained with cells cultured in the high glucose medium (data not shown).

phenotypes. At PD83, most cells cultured in the normal glucose medium had enlarged and flattened cytoplasm and high SA β-gal activity, as with typical senescent cells. However, the nicotinamide-treated cells at a similar PD (PD86) exhibited phenotypes similar to those of cells at a much lower PD value (PD32 in the Fig. 2A), most being much smaller and poorly positive for SA β-gal activity. This apparently young phenotype was maintained well over PD111 and finally replaced by the senescent phenotype only as the PD points approached 125. Lipofuscin accumulation, another marker for cellular senescence (von Zglinicki *et al.*, 1995), was apparently high in cells cultured in the high-glucose medium, but was delayed in cells cultured in both the nicotinamide-supplemented medium and the normal-glucose medium (data not shown). These results strongly suggest that nicotinamide treatment delays the expression of senescence phenotypes and extends cellular lifespan in primary fibroblasts. However, the fibroblasts eventually entered a state of cellular senescence (at PD125 and PD121), indicating that the treatment does not immortalize cells.

The nicotinamide-treated population was biochemically rejuvenated

Next, we determined whether the activation of the p53-p21WAF1-Rb growth-inhibitory pathway was affected in cells treated with nicotinamide. The presence of nicotinamide had little effect on the levels of p53, p21WAF1, and Rb proteins until cells approached a subsenescent stage. One exception was that the hit-and-run type early increase of the p53 level was delayed by the nicotinamide treatment (Fig. 2B, lanes 7–11). However, in the cells fed with nicotinamide and growing beyond the normal Hayflick limit (cells at PD84, 92, and 100), the levels of

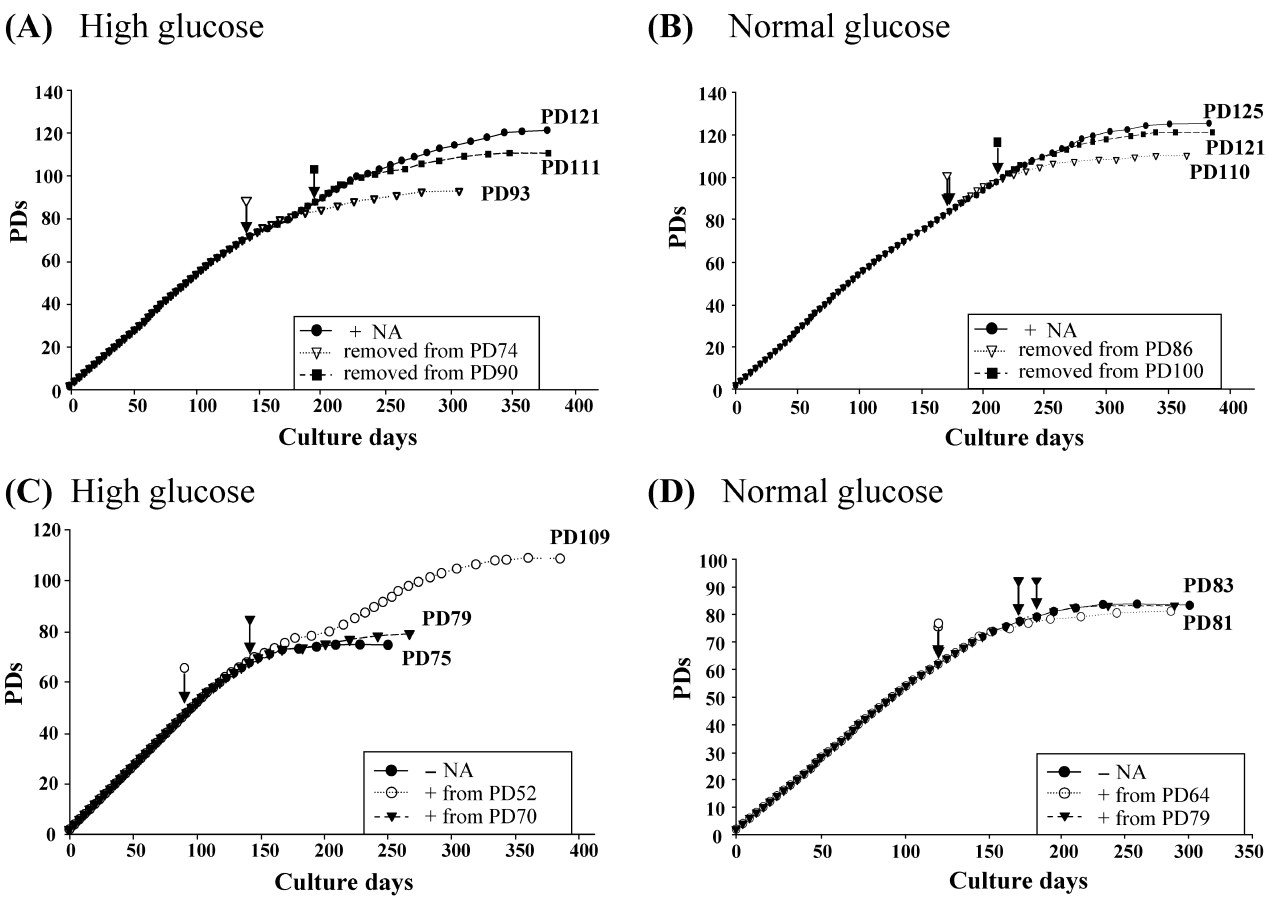

Fig. 3 Treatment duration-dependent extension of cellular lifespan by nicotinamide. (A) and (B) NHFs were cultured in medium of high (A) or normal glucose (B) in the presence of 5 mM nicotinamide starting from PD30. Addition of nicotinamide in media was terminated at either PD74 (···▽···) or PD90 (- ■ -) in the case of (A) and PD86 (···▽···) or PD100 (- ■ -) in the case of (B), and cells were further passaged until senescence (PD93 and PD111 for the high-glucose culture, and PD110 and PD121 for the normal-glucose culture). Arrows indicate the point when nicotinamide was withdrawn from the culture medium. (C) and (D) NHFs were treated with 5 mM nicotinamide starting from PD52 (···○···) or PD70 (- ▼ -) in a high-glucose medium (C) and PD64 (···○···) or PD79 (- ▼ -) in a normal-glucose medium (D). Without nicotinamide (–), cells doubled to PD75 and 83, respectively. Arrows indicate the point when nicotinamide was added to the culture medium.

p53 and p21WAF1 were substantially lowered, and the hyperphosphorylated Rb proteins reappeared (compare lanes 11 vs. 12–13 and 14 vs. 15–16 in Fig. 2B). This 'young cell-like' status was maintained until the cells finally approached the delayed senescence (PD120; lane 17). Overall, it appears that the p53-p21WAF1-Rb growth inhibitory pathway was suppressed in the cells approaching the original Hayflick limit and growing beyond it. The sudden appearance of the young cell-like state of p21-WAF1 and Rb at PD84 suggests that, at the onset of the initial senescence, there might be a selective outgrowth of cells that had such a biochemical status. Indeed, when the nicotinamide-treated cells at PD80 were sparsely plated and their clonal proliferation was observed for a 22-day period, one-third of cells continued to actively proliferate and developed into colonies (data not shown). In contrast, when cells cultured in the absence of nicotinamide were plated, not a single colony over the size of four cells developed during the 22-day period. These results suggest that, upon nicotinamide treatment, some cells failed to enter a senescence state and outgrew the senescing cells becoming a majority in the subsequent culture.

The extent of nicotinamide's effect was dependent on the treatment duration

We next determined whether continued presence of nicotinamide is required for the lifespan extension effect. First, nicotinamide was removed in the middle of the cell culture after having been introduced starting at PD30, and cell culture was continued until the cells senesced. In the case of cells grown in the high-glucose medium (Fig. 3A), when nicotinamide was removed at PD90, cells proliferated for 21-more doublings and senesced at PD111, while when removed at PD74, they proliferated until PD93. The cells cultured in the normal-glucose medium, also, proliferated significantly further after removal of nicotinamide (cells senesced at PD121, when removed at PD100, and at PD110 when removed at PD 86) (Fig. 3B). It appears that cell proliferation stopped at 20 or so PDs after the removal of nicotinamide. These results indicate that lifespan extension was induced not by nicotinamide itself but by certain changes caused by nicotinamide, and the effect of this change lasts for a substantial number of population doublings.

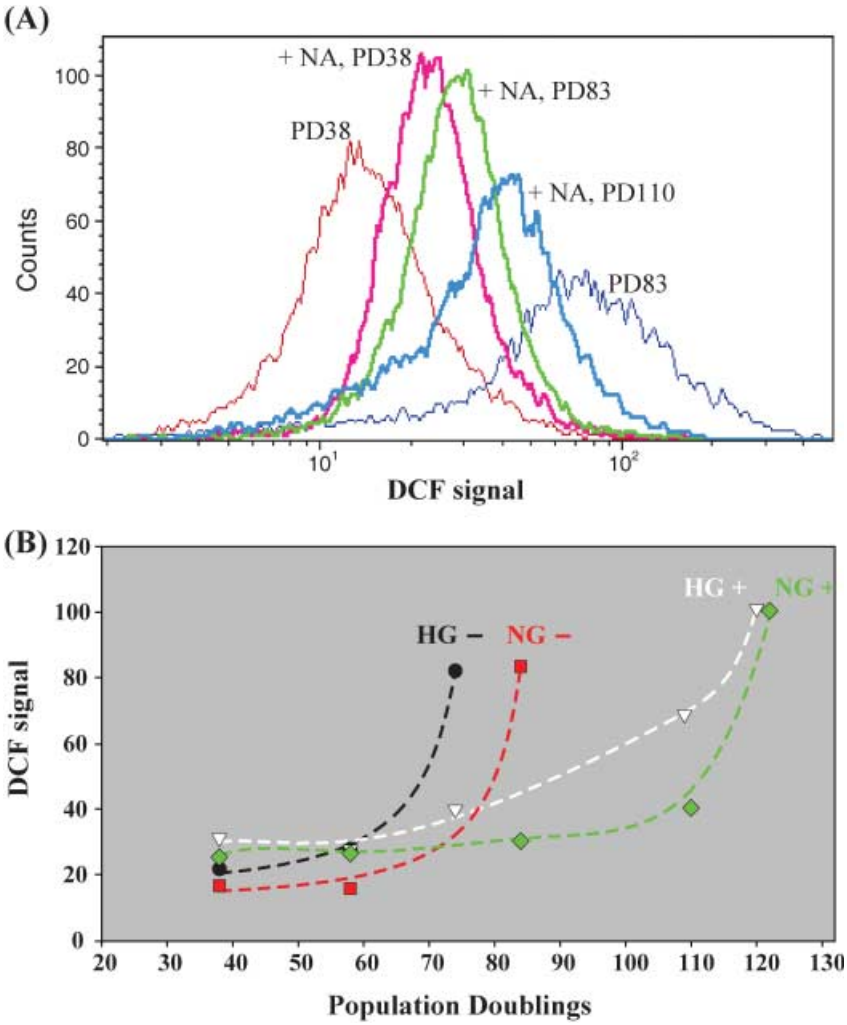

Fig. 4 Decreased ROS accumulation in cells cultured in the presence of nicotinamide. (A) The untreated NHFs at PD38 and PD83, and the nicotinamide-treated NHFs at PD38, 83, and 110, NHFs (+ NA, PD38; + NA, PD83; + NA, PD110) were incubated with DCFH-DA and applied to flow cytometry. A representative histogram for the ROS level vs. cell distribution is presented. (B) Regression curves for the changes in ROS level during cell cultures were plotted by using cells at PD38, 58, and 83 for normal glucose (■); PD38, 58, 83, 110, and 122 for normal glucose supplemented with nicotinamide (◆); PD38, 58, and 74 for high glucose (●); PD38, 58, 74, 110, and 119 for high glucose supplemented with nicotinamide (▽). Each point was mean of the measurements of the cells from two different culture plates.

In the second experimental setting, cells were fed nicotinamide starting at later points in the passage. The cells that were cultured in the high-glucose medium proliferated until PD109 when fed nicotinamide starting from PD52, while those fed starting from PD70 proliferated only to PD79 (Fig. 3C). Therefore, the earlier the cells were treated with nicotinamide, the longer the lifespan was extended. This result demonstrates that the duration and timing of nicotinamide treatment affects the extent of prolongation of cellular lifespan. However, in cells cultured in the normal-glucose medium, similar delayed treatments showed no effect from nicotinamide treatment (Fig. 3D). Therefore, for the cells cultured in normal level glucose, longer term nicotinamide treatment is necessary to be effective. Nonetheless, overall, these results strongly suggest that the effect of nicotinamide is exerted through an accumulation of certain cellular changes throughout the continued proliferation rather than by an acute one-time alteration of certain cellular activities.

Reduced ROS accumulation in nicotinamide-treated cells

It has been frequently observed that the intracellular ROS level increases as cells progress toward replicative senescence (Hutter *et al.*, 2002). Furthermore, oxidative stress such as H_2O_2 treatment induces the senescence phenotype in fibroblasts as well as many different types of cancer cells (Hwang, 2002). Nicotinamide has been reported to exert an antioxidant effect in certain physiological states (Kamat & Devasagayam, 1999). To test whether the cellular ROS (hydroxyl radical) level was altered by treatment with nicotinamide, cells at various passages were stained with 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and subjected to flow cytometry (Fig. 4A). As predicted, the ROS level in cells at senescence (PD83) was much higher than in early passage cells (PD38). There was approximately an eightfold increase in the ROS level between cells at PD38 and PD83. However, much lower levels of ROS accumulated in cells cultured in the presence of nicotinamide; even at PD110, cells had a ROS level lower than the cells at PD84 (senescent without nicotinamide). This suggests that nicotinamide lowers ROS production or accelerates ROS removal during cell proliferation. Interestingly, at lower PDs, cells cultured in the presence of nicotinamide had higher levels of ROS (+ NA, PD38) than cells at the same number of PD in the absence of nicotinamide (PD38). It is apparent, as shown in the regression curve, that in the presence of nicotinamide, ROS levels are high at early

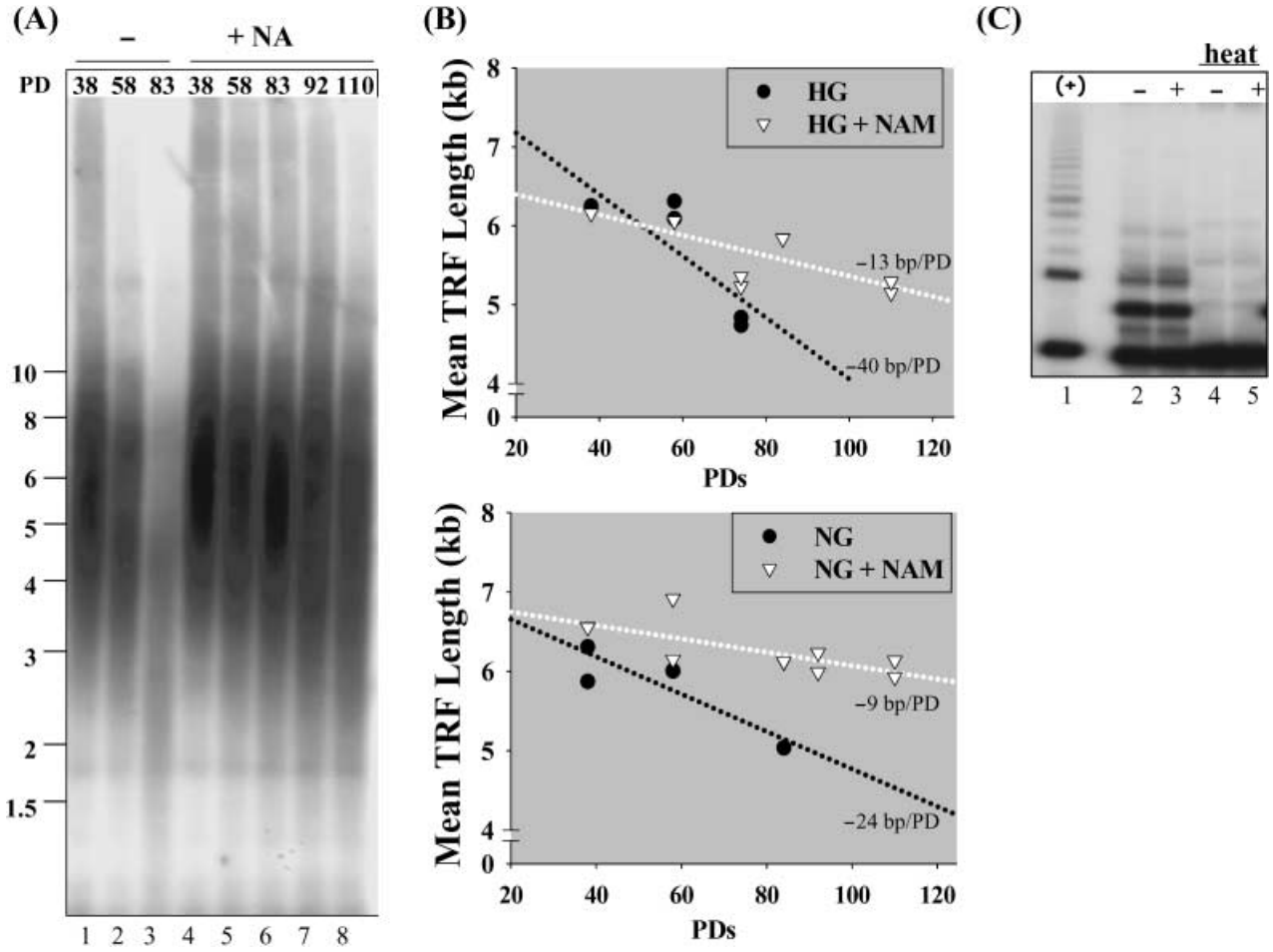

Fig. 5 Reduced telomere shortening rates in cells cultured in the presence of nicotinamide. (A) TRF analysis of genomic DNA isolated from NHFs cultured in the absence (lanes 1–3) or presence (lanes 4–8) of 5 mM nicotinamide, and collected at the indicated PD points. Bars on *Y* axis indicate the position of 1 kb DNA ladder bands. The analysis of the cells independently cultured resulted in the same TRF pattern (second TRF Southern blot; data not shown). (B) TRF length values from the two TRF Southern blots (one shown in (A) and the second TRF Southern blot which is not shown) were plotted together against PD numbers (for PD38, 58, and 83 of the untreated culture, and for PD38, 58, 83, 92, and 110 of the nicotinamide-treated culture) using SigmaPlot 8.02 software, and the slope from each regression curve was adopted as the telomere shortening rate (base pair/PD), which is 40 bp/PD and 13 bp/PD for cells cultured in high-glucose medium without and with nicotinamide, respectively (the upper box); 24 bp/PD and 9 bp/PD for cells cultured in normal-glucose medium without and with nicotinamide, respectively (the lower box). (C) Telomerase activity in NHFs cultured in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of nicotinamide as measured by TRAP assay. Lane 1 represents telomerase activity of MCF-7 cells as a positive control. In lanes 4 and 5, samples were treated at 90 °C for 10 min before being applied to TRAP assay. The patterns similar to (A) and (C) were obtained with the cells cultured in high-glucose medium (not shown).

passages but stabilized thereafter until far beyond the initial senescence point in both the high- and normal-glucose conditions (Fig. 4B). Therefore, nicotinamide has a ROS-lowering effect in long-term culture, although not as an antioxidant.

Decreased telomere attrition in cells treated with nicotinamide

It is generally believed that the population doubling of human cells is primarily determined by telomere length, and cells stop dividing once their telomeres reach a certain critical length (Allsopp, 1996; Campisi, 2005). It is therefore likely that cells growing in the presence of nicotinamide maintained their telomeres in a different manner. In the absence of nicotinamide, telomere length was gradually shortened with a rate of 24 bp PD^{-1} (Fig. 5A, lanes 1–3, and 5B, lower box), and the telomeres became quite short and heterogeneous in the senescent cells (lane 3). Meanwhile, for the cells cultured in the presence of nicotinamide, telomere shortening rate was significantly reduced, to 9 bp per division (Fig. 5A, lanes 4–8, and 5B, lower box). It appeared that telomeres in the cells at PD110 (in the presence of nicotinamide) were significantly longer with a stronger signal than in the cells undergoing senescence at PD83 in the absence of nicotinamide (lanes 8 vs. 3). This reduced telomere shortening rate was not due to an activation of telomerase. As shown in Fig. 5C, the results of a TRAP assay demonstrate that, in the cells treated with nicotinamide, there was no activation of telomerase in the untreated cells (lanes 2 vs. 3).

These results confirm the current widely accepted hypotheses regarding telomere maintenance and senescence. First, the

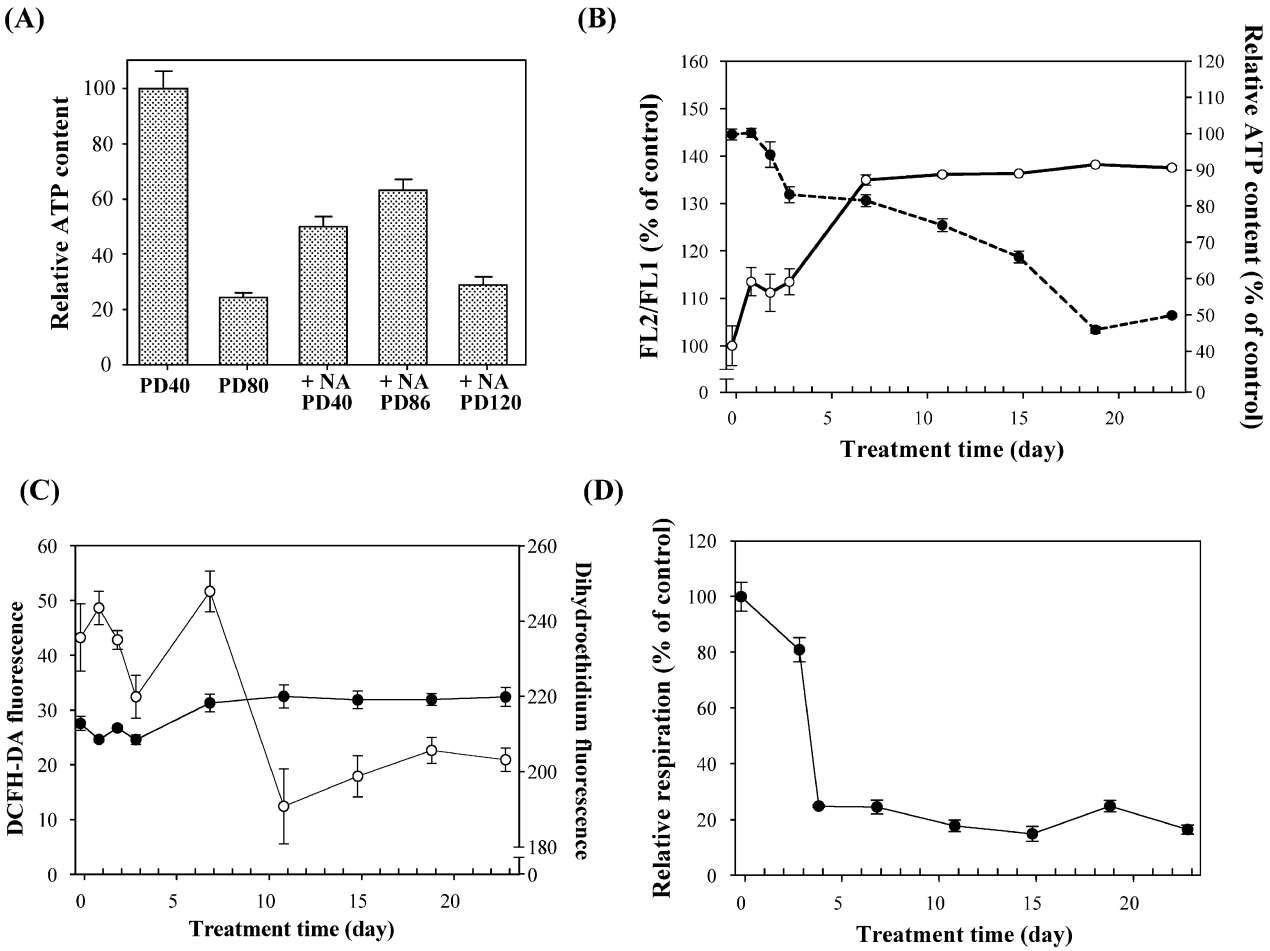

Fig. 6 Decreased ATP level and cellular respiration and increased $\Delta\psi_m$ in the nicotinamide-treated cells. (A) 2×10^4 cells at PD40 and PD80 (untreated), and PD40 (PD40 + NA), PD86 (PD86 + NA), and PD120 (PD120 + NA) (cultured in the presence of 5 mM nicotinamide) were lysed and assayed for ATP content. Relative ATP level was indicated as percentage of that in the untreated cells at PD40. The numbers are mean values of six measurements from two independent experiments. (B–D) NHFs at PD30 were treated with 5 mM nicotinamide and an acute effect on ATP level (filled circle) and $\Delta\psi_m$ (open circle) (B), levels of hydroxyl radicals (filled circle) and superoxide anion (open circle) (C), and cellular respiration rate (D) were determined. Cells that had been passaged 5, 4, 3, 2, or 1 times (days 23, 19, 15, 11, and 7), or incubated for 3, 2, or 1 days (days 3, 2, and 1) in the presence of nicotinamide were applied to different assays. Cells were passaged every 4 days at a ratio of 1 : 4, and collected 3 days after passage. % values relative to those from untreated cells were plotted as indicated by (% to control).

delay of senescence and extension of cellular lifespan certainly accompanied telomere length maintenance, suggesting that telomere shortening plays a key role in determining cellular lifespan. Second, in cells devoid of detectable increase in telomerase activity, telomere length can still be modulated.

Nicotinamide treatment altered mitochondrial function and superoxide anion levels

During aging, ATP content and mitochondrial function decrease in tissues of human and animals (Marcinek *et al.*, 2005; Short *et al.*, 2005) as well as in human fibroblasts (Zwerschke *et al.*, 2003). We examined the effect of nicotinamide treatment on the ATP content in the cells. First, as has been similarly reported, cellular ATP content in cells approaching senescence (PD80) was only 24% of that in early passage cells (PD40) (Fig. 6A). Interestingly, in the cells cultured in the presence of nicotinamide (+ NA, PD40), the ATP level was much lower, reaching only 50% of that in the untreated cells. However, the ATP content did not further decrease in the subsequent passages (63% at PD86) until they reached the delayed senescence point at PD120, in which the ATP content dropped to a level close to that of the untreated senescent cells (PD80). This effect of nicotinamide was similarly reproduced in cells cultured in high-glucose medium (data not shown).

Next, using cells at PD30, any acute effect of nicotinamide was examined daily for a 3-day period and at each passage for the subsequent five passage-culture period in the presence of nicotinamide. As shown in Fig. 6B, cellular ATP levels decreased steadily, reaching 94% and 83% by 48 and 72 h, respectively. During the subsequent passage, the ATP level further decreased reaching 46% after the fourth passage and then plateaued. Treatment of 10 and 20 mM nicotinamide caused a big decrease in ATP levels resulting in a drop of 12% and 29%, respectively,

at 24 h, when the treatment at 5 mM caused only a marginal decrease (data not shown). These results suggest that nicotinamide might down-regulate cellular ATP levels. The decrease in ATP level did not appear to be due to the decrease in the mitochondrial membrane potential ($\Delta\psi_m$) since the $\Delta\psi_m$ as determined by using JC-1 changed in rather an inverse correlation with the change of ATP level. $\Delta\psi_m$ reached nearly 140% after the fourth passage when the ATP level was 46% of the untreated level (Fig. 6B). During this five-passage time course, the cellular hydroxyl radical level was stably maintained as demonstrated by the relatively constant DCFH-DA-originated fluorescence levels (Fig. 6C). However, the level of superoxide anion was not. The dihydroethidium-originated fluorescence signal representing the superoxide anion level fluctuated heavily until the second passage, but thereafter remained at decreased levels around 20–40% of the untreated cells (Fig. 6C). Therefore, the nicotinamide treatment induced a decrease in ATP and superoxide anion levels and an increase in $\Delta\psi_m$ during the five-passage culture period. These changes were accompanied by a dramatic decrease in cellular respiration rate, which dropped rapidly at around day 4 of nicotinamide treatment, and thereafter was maintained constantly at the 20% level (Fig. 6D). During the five-passage culture period in the presence of nicotinamide, the changes in the respiration rate, ATP level, and the $\Delta\psi_m$ preceded the decrease in superoxide anion level. In particular, the decrease in the respiration rate occurred most dramatically and rapidly, suggesting that at least some of the noted changes were possibly outcomes of the drop in the mitochondrial respiration rate.

Nicotinamide treatment altered the effect of mitochondrial inhibitors, rotenone and oligomycin

NAD^+ at millimolar concentrations inhibits the NADH:ubiquinone oxidoreductase (Complex I), which constitutes the entry point for electrons to the respiratory chain, and thereby suppresses ATP generation and cellular respiration (Hatefi *et al.*, 1969). Furthermore, the treatment of 5 and 10 mM nicotinamide for 24 h caused an increase in cellular [NAD^+] by 0.3 and 0.6 mM, respectively, along with a parallel increase in the [NAD^+]/[NADH] level, although both decreased upon treatment at 20 mM (Fig. 7A). Furthermore, during the five-passage culture period in the presence of nicotinamide, the [NAD^+] increased 150% reaching 2.7 mM from 1.8 mM (data not shown). Therefore, it is possible that nicotinamide caused the decrease in ATP levels and cellular respiration by inhibiting the NADH:ubiquinone oxidoreductase. This possibility was tested by comparing the effect of nicotinamide to that of rotenone, which is a widely used Complex I inhibitor (Hatefi, 1968). As shown in Fig. 7B, rotenone treatment for 24 h caused a 30% drop in ATP level as well as a 70% drop in $\Delta\psi_m$, while nicotinamide treatment (20 mM instead of 5 mM to get more dramatic effect within 24 h) increased the $\Delta\psi_m$ by sixfold although the ATP level was similarly decreased. Furthermore, cotreatment with nicotinamide in the rotenone-treated cells resulted in a slight recovery of $\Delta\psi_m$, but a further drop in ATP level. These results indicate that the effect of nicotinamide is

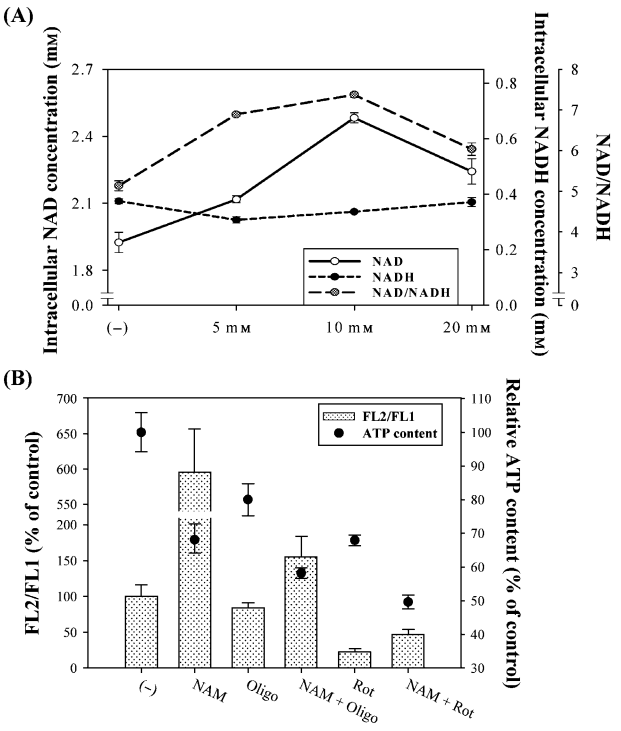

Fig. 7 Increased cellular [NAD^+] level and alteration of the effect of mitochondrial inhibitors on ATP level and $\Delta\psi_m$ in the nicotinamide-treated cells. (A) Cells at PD30 were incubated in the medium containing 5, 10, 20 mM nicotinamide for 24 h before being assayed to determine [NAD^+] and [NADH]. In the extract of the 1×10^6 untreated cells (–), there was 5.391 nmole of total NAD^+. Therefore, [NAD^+] per cell was 1.925 mM assuming the volume of a fibroblast is 2.8 picoliter based on a previously reported measurement (James *et al.*, 1996). (B) 3×10^5 cells were incubated in the presence of 20 mM nicotinamide (NAM), or 8 μg mL^{-1} mM oligomycin (Oligo), 20 μM rotenone (Rot) alone, or together with nicotinamide (NAM + Oligo) and (NAM + Rot) for 24 h and assayed for ATP level (●) or $\Delta\psi_m$ (bars). In (A) and (B), the numbers are mean values of three measurements of cells prepared from two independent experiments.

not identical to that of rotenone. Therefore, nicotinamide probably does not work through inhibiting the Complex I of the electron transport chain. Another possible way to increase $\Delta\psi_m$ and decrease ATP level is by activation of mitochondrial ATP synthase in reverse mode. Treatment with oligomycin, an inhibitor of the F0 component of mitochondrial ATP synthase (Fisher & Hodges, 1969), caused a marginal decrease in $\Delta\psi_m$ and a substantial decrease in ATP level. However, in cells treated with both oligomycin and nicotinamide, the ATP level was lowered while $\Delta\psi_m$ increased significantly compared with the cells treated with oligomycin alone. The simplest interpretation of these results is that nicotinamide drove protons back to the mitochondrial intermembrane space by consuming ATP. This would be possible by activating ATP synthase in a reverse mode.

p21WAF1 gene induction was attenuated in the nicotinamide-treated cells

NAD^+ was shown to directly down-regulate p53 activity (McLure *et al.*, 2004). Therefore, in nicontinamide-treated cells, the level

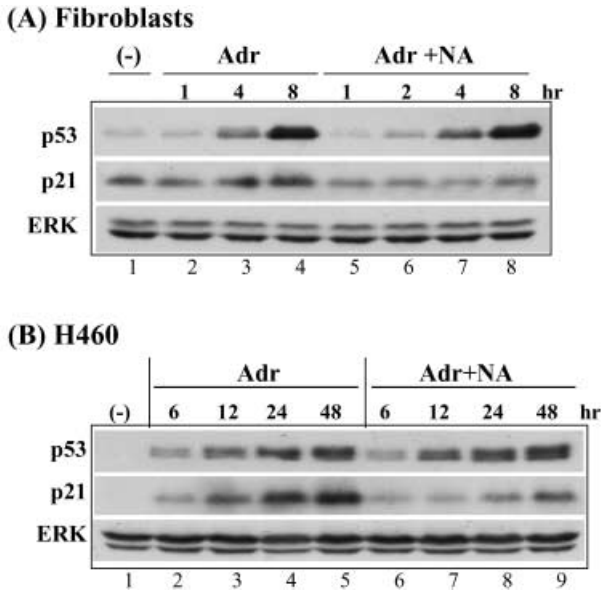

Fig. 8 Down-regulation of p21WAF1 expression in cells cultured in the presence of nicotinamide. (A) NHFs at PD12 were treated with 0.5 μM adriamycin for 0, 1, 4, or 8 h in the absence (lanes 1–4) or for 1, 2, 4, 8 h in the presence (lanes 5–8) of 10 mM nicotinamide. Cell extracts were applied to Western blot analysis for p53, p21WAF1, and ERK 1/2. (B) NCI-H460 cells were treated with 0.5 μM adriamycin alone (lanes 2–5) or with 5 mM nicotinamide (lanes 6–9) for 6, 12, 24, and 48 h. In lane 1, extract from untreated cells was loaded.

of p21WAF1 induced by DNA damage is expected to be low. Following nicotinamide treatment of cells suffering DNA damage, p53 acetylation level increased acutely (H. T. Kang & H. E. Lee, unpublished result), possibly through a direct inhibition of SIRT1 (Bitterman *et al.*, 2002), a decetylase targeting p53 (Luo *et al.*, 2001). Therefore, we determined how p21WAF1 induction was acutely affected by nicotinamide treatment. As shown in Fig. 8, treatment with nicotinamide caused a substantial decrease in the level of p21WAF1 induced in adriamycin-treated fibroblasts (A) and NCI-H460 lung carcinoma cells (B), while having no apparent effect on the level of p53 (compare lanes 2–4 vs. 5–8 in Fig. 7A, and lanes 2–5 vs. 6–9 in Fig. 7B). These results demonstrate that an acute increase in p53 acetylation could not be linked to an increase in the transcriptional activity of p53 in these cells. This decreased inducibility of p21WAF1 might confer an enhanced proliferative potential to cells in which the senescence-associated DNA damage signaling is activated.

The proliferative lifespan of normal keratinocyte was also extended by the nicotinamide treatment

Next, we checked other types of normal human cells for the effect of nicotinamide. A pooled line of normal human keratinocyte was divided into two at PD3, and continuously passaged in either the presence or absence of 5 mM nicotinamide. In both cases, keratinocytes proliferated with a doubling rate of approximately 36–48 h per doubling until PD16. However, from PD18, the untreated keratinocytes became enlarged and their growth was severely retarded. The plate was largely taken over by other types of cells including fibroblasts, which had been a minority in the previous passages (Fig. 9A). At PD22, keratinocytes were barely noticeable due to the rapidly proliferating fibroblasts (Fig. 9B), and passage was terminated. In contrast, keratinocytes in the nicotinamide-treated culture continuously proliferated, forming tight cellular adhesions (Fig. 9C). At PD24, their doubling rate decreased and other types of cells started emerging, but the majority of the keratinocytes kept the young-cell morphology and constituted a major population (Fig. 9D). This

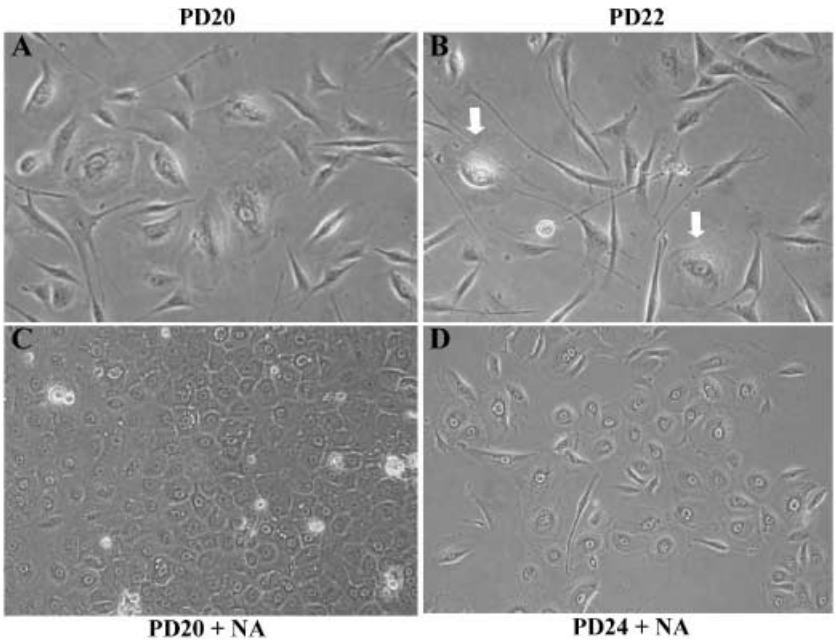

Fig. 9 Extension of the proliferative lifespan of normal human keratinocytes by nicotinamide treatment. Human foreskin keratinocytes were cultured in a keratinocyte SFM with (C and D) or without (A and B) a supplement of 5 mM nicotinamide from PD3. In the absence of nicotinamide, keratinocytes became enlarged and stopped dividing at PD20, and the culture became a mixture of keratinocytes and nonkeratinocytes (A). At PD22, keratinocytes were hardly noticeable (white arrows). Meanwhile, at PD24 in the culture maintained in the presence of nicotinamide, keratinocytes were still dividing as a majority.

culture was finally terminated at PD28. Therefore, the replicative lifespan of this keratinocyte line was also extended by the continuous treatment with 5 mM nicotinamide. In a separate experiment, the doubling capacity of a dermal fibroblast line obtained from a 75-year-old man also increased significantly by treatment with 5 mM nicotinamide (H. T. Kang & E. S. Hwang, unpublished result). These results suggest that continued treatment with 5 mM nicotinamide generally increases the cellular lifespan of normal cells of human origin.

Discussion

The most striking result of this study is a huge increase in lifespan of primary human fibroblasts (over a 1.5-fold increase) induced by the introduction of nicotinamide. Although antioxidants such as kinetin (Rattan & Clark, 1994) and carnosine (McFarland & Holliday, 1994) have been reported to cause a slight delay in the expression of senescence phenotypes, such a large increase of lifespan has been rarely achieved by chemical means. Treatment of IMR90 fibroblasts with the spin-trapping molecule *N*-*t*-butyl hydroxylamine has been reported to cause a large extension of the lifespan through its hydroxyl radical-scavenging activity (Atamna *et al.*, 2000). Although nicotinamide and *N*-*t*-butyl hydroxylamine caused a similar level of lifespan extension in human fibroblasts, their action mechanisms may not be the same. The magnitude of the effect of nicotinamide was dependent on treatment duration, and even after nicotinamide was removed, the population doublings continued for a further 20 or so PDs (Fig. 3). In the case of spin traps (Chen *et al.*, 1995), the magnitude of the effect was not dependent on treatment duration, nor is it cumulative. The lifespan gains were more prominent when they were applied to cells close to senescence. The effect of nicotinamide is also different from that of carnosine (McFarland *et al.*, 1994) and other antioxidant chemicals. Although carnosine had a pronounced effect in preventing senescent morphology, cells quickly entered senescence state upon its removal, neither does its effect appear to be cumulative. These differences suggest that nicotinamide's effect does not occur directly through antioxidation. Apparently, nicotinamide by itself is not an antioxidant at cellular level.

The antisenescence effect of nicotinamide has been reported previously by Matuoka *et al.* (2001), although their study noted only a small increase in replicative index and no increase in population-doubling capacity. This discrepancy may have occurred as a result of differences in the treatment dose and duration. In Matuoka's study, BJ fibroblasts were treated with 3 mM nicotinamide 2/3 into their lifespan (PD64 to PD88), while in this study, cells were treated with 5 mM nicotinamide from PD30 to PD83. Treatment for shorter periods indeed resulted in either a reduction in the extension of lifespan (at high-glucose condition) or no effect (at normal-glucose condition) (Fig. 3C,D).

In this study, two important characteristics of the nicotinamide-mediated lifespan extension are worth noting. First, the extension was not achieved by an acute modulation of activity of one or few cellular factors. Rather, certain cellular factors or program involved in controlling cell growth, telomere length, and/or ROS generation might be affected either by nicotinamide or NAD^+, and modified so that, even in its absence, their activity remains high. This is strongly supported by the variance in lifespan extension imposed by the treatments initiated at different PD points (Fig. 3). Second, even the nicotinamide-treated cells eventually reached a senescent state, indicating that senescence was delayed but not prevented. The cells, not being transformed, entered a delayed state of senescence with all its characteristic biochemical and cellular features, namely an increase in p53 and p21WAF1 levels, Rb hypophosphorylation, shortened telomeres, and a high ROS level. Therefore, the action mode of nicotinamide is noticeably different from that of carcinogens or viral oncoproteins.

We need to address the question of how ROS level was kept low and telomere shortening was decelerated in the nicotinamide-treated cells. Since nicotinamide itself is not an antioxidant, the decreased ROS level in late-passage cells is more likely due to reduced ROS generation. In the nicotinamide-treated cells, the ATP content decreased to 50%, while $\Delta\psi_m$ increased to 140% of those of untreated cells during a five-passage culture period. Although it requires further investigation, these changes may be attributed to the decreased cellular respiration (Fig. 6). The drop in cellular respiration rate occurred at the earliest time point (within 96 h) among the changes in the examined mitochondrial function, and unlike the other events, the decrease was quite rapid (being at 80% level in 72 h and at 20% level in 96 h). The 50% drop in the ATP level occurred steadily over 20 days and the 30% increase in $\Delta\psi_m$ occurred over 7 days. These results suggest that the decrease of cellular respiration might be precedent to the changes in ATP content and $\Delta\psi_m$, and therefore is likely to constitute an underlying reason for these changes overall. During this period, the superoxide anion content decreased to 30–40% of the untreated level. Although cellular hydroxyl radical content did not change during this short-term culture period, the lower production of superoxide anion would eventually lead to a decreased ROS level in cells cultured for a long period of time in the presence of nicotinamide. Previously, it has been reported that more ROS is produced under conditions where $\Delta\psi_m$ is high (Korshunov *et al.*, 1997). Closer examination of the superoxide anion level change in the nicotinamide-treated cells demonstrates that it increased (by nearly 20%) within 24 h (Fig. 6C); therefore the ROS production profile in the nicotinamide-treated cells does not contradict the earlier report. Interestingly, it kept fluctuating for the first 7 days, and steeply decreased later on.

Questions still remain about the action mechanism of nicotinamide. One would expect that an increase in $\Delta\psi_m$ leads to a higher ATP production as has been reported earlier by Kaim & Dimroth (1999). How does nicotinamide induce a decrease in ATP level and an increase in $\Delta\psi_m$ at the same time? It has been reported that NAD^+ at millimolar concentrations inhibits NADH:ubiquinone oxidoreductase, at the first stage in transferring electrons to the mitochondrial respiratory chain, and thereby down-regulates cellular respiration (Hatefi *et al.*, 1969).

In addition, treatment with millimolar concentrations of nicotinamide was shown to acutely decrease ATP synthesis in rat liver cells in a dose-dependent manner (Hoshino *et al.*, 1984). This suggests a possibility that nicotinamide treatment suppresses cellular respiration through an increase in free NAD^+. However, this is not likely the case as shown by the differing effects of nicotinamide and rotenone. Rotenone treatment caused a decrease in both $\Delta\psi_m$ and ATP level as expected for an inhibitor of Complex I, while nicotinamide treatment induced an increase in $\Delta\psi_m$ and a decrease in ATP level. Furthermore, cotreatment with nicotinamide resulted in a slight increase in $\Delta\psi_m$ as shown in Fig. 7B. One plausible explanation for the state of an increased $\Delta\psi_m$ and a decreased ATP content is that nicotinamide activates the mitochondrial ATP synthase in a reverse mode pumping protons into the mitochondrial intermembrane space by hydrolyzing ATP. This is partially supported by the effect of nicotinamide on the cells treated with oligomycin. It increased $\Delta\psi_m$ in the cells where the proton movement was frozen by oligomycin while decreasing ATP content (Fig. 7B). The proposed reversion of ATP synthase activity is not unprecedented. It has been reported that, in certain patho/physiological conditions, cells hydrolyze ATP to pump protons out of the mitochondrial matrix (Beltran *et al.*, 2000; Khaled *et al.*, 2001; Grover *et al.*, 2004; Takeda *et al.*, 2004).

In the long run, low respiration rate would be beneficial to cells in terms of the level of oxidative damage caused by ROS, once the level of ATP is high enough to support normal cell growth. Apparently, the ATP level maintained at 50% or above of that in the untreated cells is high enough to support normal proliferation of cells as demonstrated by the absence of any adverse effect on cell proliferation.

Telomere shortening is known to be affected by the levels of oxidative stress (von Zglinicki, 2002). Therefore, one plausible possibility is that the telomeres shortened at reduced rates in the nicotinamide-treated cells because of the decrease in the ROS levels. In these cells, cellular lifespan was extended possibly due to attenuated activation of the DNA-damage signal, which originates from shortened or damaged telomeres. Furthermore, the low ROS level would lead to reduced accumulation of other oxidative damage products, and, therefore, a delay in senescence marker expression. In addition, the activity of p53 itself might be suppressed, at least in part through the nicotinamide-mediated mechanism (McLure *et al.*, 2004). The reduced p21WAF1 induction would then lead to substantially lower Rb activation, and thereby, a delay in G1-growth arrest.

Finally, the result is somewhat contradictory to what has been expected from the inhibition of Sir2-like function in the human cells. In experiments not presented here, several independent fibroblast lines that either overexpress or underexpress SIRT-1 were generated and maintained in either the absence or presence of nicotinamide. There was no effect of the overexpression or knockdown of SIRT-1 on the extension of lifespan upon nicotinamide treatment (H. T. Kang & E. S. Hwang, unpublished results). Therefore, the effect of nicotinamide is not likely mediated through SIRT-1. Furthermore, it has previously been reported that the presence of SIRT-1 is not a determinant of cellular longevity in human cells (Michishita *et al.*, 2005).

Whatever the underlying mechanism(s) of nicotinamide, it may not be the same as that of calorie restriction (CR) in animals or *in vitro* cell culture models. CR resulted in a decreased ROS production possibly due to a decrease in $\Delta\psi_m$ in mitochondria from the CR rat (Lambert & Merry, 2004). Furthermore, in an *in vitro* model in which cells were incubated in the presence of serum from CR animals, both $\Delta\psi_m$ and oxygen consumption were reduced but the ATP level was not significantly altered (Lopez-Lluch *et al.*, 2006). These different effects on $\Delta\psi_m$ in the nicotinamide-treated and CR mitochondria may suggest that there is a regimen other than CR for healthy longevity. However, since the effect of CR on mitochondrial function has not been unanimously decided, conclusive distinction should wait for more understanding on the effects of both treatments. However, it is of a great interest whether continued intake of nicotinamide at high doses could have any impact on the lifespan and health of the human body, especially since high doses of nicotinamide are currently being examined in clinical trials for a number of therapeutic purposes.

Experimental procedures

Cell culture

Normal human fibroblasts (NHF) from a newborn foreskin were provided by Dr Sang Chul Park (Seoul National University, Korea) at population doubling 7, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing glucose either at 25 mM (high-glucose medium) or 5.5 mM (normal-glucose medium) plus 10% fetal bovine serum (Bio-Whitacker, Walkersville, MD, USA). Cultures were maintained in a 100-mm dish, and when confluent, were split in 1 : 4 ratio during early passages and 1 : 2 during late passages. The number of PD (n) was calculated using the equation, $n = \log_2 F/I$, where F and I are the numbers of cells at the end and those seeded at the beginning of one passage, respectively. Nicotinamide (Sigma, St. Louis, MO, USA) was added to the medium at 5 mM concentration from PD30 and kept in the medium throughout the culture unless otherwise indicated. NCI-H460, a human lung carcinoma line, and SKOV3, a human ovarian carcinoma line, were obtained from American Type Culture Collection. A pooled line of primary human foreskin keratinocytes was purchased from Invitrogen (12332–011, Carlsbad, CA, USA) and passaged in 1 : 2 ratio in Defined Keratinocyte-SFM (Invitrogen) in the presence or absence of 5 mM nicotinamide.

Western blot analysis

Cells were lysed with RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with NaF, $NaVO_4$, and a protease inhibitor mixture (Sigma). Typically, 20 μg of proteins were separated by SDS-PAGE, and transferred to nitrocellulose membrane (Hybond ECL; Amersham, NJ, USA). The following

primary antibodies were used: antip53 (Calbiochem, La Jolla, CA, USA), antip21WAF1, anti-ERK 1/2 (Santa Cruz, Santa Cruz, CA, USA), anti-Rb (Pharmingen, San Diego, CA, USA), antiacetylated K382 p53 (Cell Signaling, Beverly, MA, USA). Protein bands were visualized by using horseradish peroxidase-conjugated secondary antibodies and Lumi-Light^{PLUS} Western Blotting substrate (Roche, Indianapolis, IN, USA).

Measurement of telomere length by TRF assay

Genomic DNA was extracted by DNeasy tissue kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol. Approximately 2 μg genomic DNA was digested with *Rsa*I and *Hinf*I, electrophoresed on a 0.7% agarose gel, and transferred to nylon membrane. Membrane was probed with radio-labeled $(TTAGGG)_4$ oligonucleotide and exposed to X-ray film. The mean TRF length was determined using Telorun program (http://www.swmed.edu/home_pages/cellbio/shay_wright/research/sw_lab_methods.htm).

TRAP assay

Telomerase activity was determined by the protocol of Kim & Wu (1997). The extract of MCF-7 cells was used for positive reaction. Extracts of NHF (at PD50) that were passaged with or without 5 mM nicotinamide were used for telomerase activity determination. Extension reaction was carried out at 23 °C for 30 min, and stopped by heating at 94 °C for 3 min. PCR was carried out in the following conditions: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min for 30 cycles.

Measurement of ROS

To determine intracellular content of H_2O_2 and superoxide anion, cells were incubated with 5 μg mL^{-1} of 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA) (Sigma) or 2 μM dihydroethidium (Invitrogen) for 30 min at 37 °C, washed with PBS, trypsinized and collected in 1 μL PBS, and applied to FACS (Beckton Dickson FACScan, San Jose, CA, USA). The result was analyzed by using CellQuest 3.2 software (Beckton Dickson). Values of mean fluorescence intensity were plotted by using SigmaPlot 9.01 software (Systat Software Inc., Richmond, CA, USA).

RT–PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen) following manufacturer's protocol. Total 5 μg of RNA was converted to cDNA by using MMLV reverse transcriptase (Promega, Madison, WI, USA) and oligo d(T) primer (Promega), and 1/40 volume of synthesized cDNA was applied to PCR.

Measurement of cellular ATP level

Cells 2×10^4 at the indicated PD were seeded per well in 24-well plates. Cells were lysed with 150 μL lysis buffer and ATP contents were measured using ViaLight Plus Kit (Cambrex, Walkersvile, MD, USA) according to the manufacturer's instruction. To determine the dose–response, cells at PD30 were incubated in the medium containing various concentrations of nicotinamide for 24 h before being assayed.

Measurement of cellular [NAD$^+$] and [NADH]

To determine cellular NAD$^+$ content, 1×10^6 cells were washed in 0.9% NaCl solution and lysed by adding 200 μL cold $HClO_4$ solution (0.5 M) and incubating in ice for 15 min. The extracts were neutralized by adding 61 μL 2 M KOH/0.2 M K_3PO_4 (pH 7.5) and spun at 13 000 ***g*** for 3 min 10 μL of supernatant or NAD$^+$ standard diluents were mixed with 150 μL reaction solution, and after incubation for 30 min at 37 °C, absorbance at 450 nM was measured. Reaction solution was composed of 16 μL WST-1 (Takara, Otsu, Japan), 70 μM 1-methoxy-5methylphenazinium methyl sulfate, 20 IU alcohol dehydrogenase (Sigma), 64 mM nicotinamide, 0.32 M ethanol (Sigma) in 64 mM Gly-Gly buffer (pH 7.4). To determine cellular NADH content, cells were lysed in 200 μL 0.02 N NaOH solution containing 0.5 mM L-cysteine for 10 min at 60 °C. Thirty microliter (30 μL) of the neutralized extract or NADH standard diluents was incubated with 130 μL reaction solution 60 min at 37 °C, and absorbance at 450 nM was measured.

Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

Cells at PD30 were incubated in the medium containing 20 mM nicotinamide only or together with 20 μM rotenone or 8 μg mL^{-1} oligomycin for 24 h before being assayed. To determine the effect of nicotinamide on $\Delta\psi_m$, 0.3 μg mL^{-1} JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidalohylcarbocyanine iodide; Invitrogen) was added to cells. After 30 min at 37 °C, cells were washed with PBS, trypsinized and collected in 1 mL PBS, and applied to FACS. The fluorescence at 585 nm (FL2) and 525 nm (FL1) was measured for the JC-1-treated cells as well as the mock-treated cells. To correct for autofluorescence, the FL2 and FL1 values from the JC-1-treated cells were subtracted with those from the mock treated cells. $\Delta\psi_m$ was determined by dividing the corrected FL2 value by the corrected FL1 value.

Respirometric assays

Cellular oxygen consumption was measured as previously described (Hynes *et al.*, 2005). Briefly, 2×10^5 cells were placed in a well of black-walled 96-well plate containing A65N oxygen probe (0.15 μM; Luxcel, Cork, Ireland) in a total volume of 150 μL. Each well was then covered with 100 μL of prewarmed (37 °C) heavy mineral oil (Sigma) and the plate was placed on a fluorescence plate reader (BIO-TEK FLx800; BIO-TEK Instruments, Winooski, VT, USA), and read kinetically for 3.5 h at 37 °C at 2 min intervals using excitation at 380 nm and emission at 645 nm. Oxygen consumption rates were assessed

as previously described (Hynes *et al.*, 2005). Cells cultured in normal medium were used as control, and the relative value compared to that of the control cells at each time point was plotted (expressed as relative respiration).

Statistical analysis

All quantitative measurements were done at least in triplicate, and mean ± SEM was presented. Comparison of the mean values between different groups was performed by one-way analysis of variance using InStat 3.06 (GraphPad Software Inc., San Diego, CA, USA). A *P*-value of 0.05 was considered to be statistically significant.

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Supplementary Material

The following supplementary material is available for this article:

Fig. S1 To determine passage-dependent response in cellular ROS generation (hydroxyl radical (DCFH-DA) and superoxide anion (DHE)) and $\Delta\psi_m$ (FL2/FL1 of JC-1), fibroblasts at PD 40 or 83 were incubated in medium containing 0 or 5 mM nicotinamide for 24 h. After incubation, cells were stained with DCFH-DA (5 μg mL^{-1}), dihydroethidium (2 μM) or JC-1 (0.3 μg mL^{-1}) for 30 min at 37 °C, and applied to FACS analysis. Cells at higher passage have higher ROS and lower $\Delta\psi_m$ levels (subtracted with autofluorescence from the mock treated cells). But, there was no significant difference by the treatment of nicotinamide at any PD number.

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