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[Original Article]

# Suppression of Senescent Phenotypes in Normal Human Diploid Cells by Nicotinamide and phosphoinositide 3-kinase inhibitors

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Normal human diploid cells undergo physiological aging by serial subculture in vitro as defined by the loss of doubling potential, the appearance of senescence-associated β-galactosidase activity, a decrease of cellular motility and a change of cell morphology. Alternatively, these phenotypes can be induced by exposing young cells to a brief oxidative stress such as hydrogen peroxide-treatment. We have previously reported that nicotinamide (NAM), a vitamin and energy metabolism-related molecule, reversibly suppresses the senescent phenotypes associated with physiological aging as well as the stress-induced aging. The aim of the present study is to investigate whether other small molecules that are known to be involved in modulating key signaling pathways may act like NAM in affecting the senescent phenotypes. Specifically, we found the activators for sirtuin, AMP kinase and autophagy signaling all suppress the senescent phenotypes, whereas their inhibitors induce senescent phenotypes. On the other hand, the inhibitors of phosphoinositide 3-kinase (PI3K) suppressed senescent phenotypes. To further analyze the role of NAM and PI3K in the senescence modulating process, we examined the expression of various PI3K catalytic units and the phosphorylation of Akt, a PI3K downstream target. Our study suggested that the expression of senescent phenotypes in human diploid cells is intimately connected to signaling networks involved in energy metabolism and autophagy.

## 1. INTRODUCTION

Living organisms exhibit gradual and continual changes during their life process and among those changes, the most fundamental one is "aging" at cellular level[1~2)]. However, the mechanism of cellular aging is yet to be fully elucidated. A number of biological pathways have been suggested to play some role in the aging process. Chief among them is the protein family of sirtuins[3)]. Sirtuins are a class of histone deacetylases which use nicotinamide adenine dinucleotide ($NAD^+$) as co-factor for their activities in regulating a whole array of gene expressions. In this regard, our earlier finding that nicotinamide (NAM) can reverse the expression of the senescence-related phenotypes in aging cells[4)] is noteworthy, as NAM is an end product of the sirtuin enzyme reaction[5)], hence a putative sirtuin inhibitor. This work provides an indirect evidence that signaling pathway utilizing $NAD^+$ may be involved in the aging process. Human diploid cells maintained under serial subculture undergo physiological aging process with gradual loss of dividing potential, accompanied by the appearance of senescent phenotypes such as enlarged cellular and nuclear sizes[2)] and expression of senescent cell-associated β-galactosidase (SA-gal) [6)]. These senescent phenotypes in normal cells can also be induced by a low dose of hydrogen peroxide ($H_2O_2$), probably due to accumulation of cell damages[7)].

In the present study we have examined effects of NAM and a variety of related signaling molecules on the cellular aging and have found that, as with NAM, phosphoinositide 3-kinase (PI3K) inhibitors and autophagy activators are suppressive on

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the expression of the senescent cell phenotypes.

## 2. MATERIALS AND METHODS

### 2.1 Cell culture

Normal human fibroblast BJ cells (ATCC CRL-2522) was cultured with HEPES-buffered D-MEM medium (D1152, Sigma) supplemented with 10% fetal bovine serum, 40 μg/mL kanamycin sulfate, and GlutaMax I (Gibco). By serial subculture with 0.25% trypsin + EDTA (Gibco), cells at various aging stages from population doubling level (PDL) 20 (early passage, young cells) through PDL 80 (late passage, old cells) were prepared and used in the present study.

### 2.2 Chemicals and reagents

The following chemicals or reagents were used. Unless otherwise indicated, chemicals were diluted in the culture medium from aqueous or DMSO stock solutions. Nicotinamide (NAM, [$NAD^+$ precursor], Wako): 4-20 mM; Dorsomorphin (AMP kinase inhibitor; Sigma): 30–300 nM; AICAR (AMP kinase stimulator; Cell Signaling Technology): 0.3–3 mM; Resveratrol (3,5,4'-trihydroxy *trans*-stilbene [sirtuin activator], Sigma): 5-50 μM; Splitomicin (sirtuin inhibitor; Sigma): 80 μM; LY294002 (inhibitor of phosphoinositide 3-kinase (PI3K); Selleck): 5-50 μM; Wortmannin (PI3K inhibitor; Selleck): 10-100 nM; ZSTK474 (PI3K inhibitor; Selleck): 30-300 nM; BEZ235 (PI3K inhibitor; Cellagen Technology): 30-300 nM; Thymulin (FTS, thymic factor; potential anti-senescent peptide), a generous gift from Dr. A. Awaya[8]): 0.1-1.1 μM; Rapamycin (Rap, autophagy inducer; LC Laboratories): 10 nM-1 μM; Chloroquine (Cqn, autophagy inhibitor; Enzo Life Sciences): 0.3-50 μM.

### 2.3 Cell culture experiments

Cells were treated with the above reagents and analyzed by fluorescent or enzyme staining and time-lapse microscopy with a microscope Nikon TE2000-E and an image analysis software NIS-Elements AR. Cells were inoculated, treated with the reagents on the next day, and cultured for additional 2-8 d, unless otherwise specified.

For cell staining Giemsa staining (Giemsa's azur eosin methylene blue solution, 1.09204.0503, Merck) was used. SA-gal staining was carried out as reported[6]. For an autophagy marker MAP-LC3[9], cells were fixed and stained with anti-LC3 antibody (PM036, MBL), according to the manufacturer's instructions. Oxidative stress analysis was carried out by using Total ROS/Superoxide Detection Kit (Enzo life sciences). The Akt proteins were detected by Western blotting as previously described[10] with antibodies, anti-phosphoThr308-AKT1 (SAB4504332, Sigma) and anti-β-actin (G043, abm) and analyzed by using ImageJ software[11].

### 2.4 qPCR

qPCR was carried out by using CellAmp Direct TB Green RT-qPCR Kit (TaKaRa), according to the manufacturer's instructions. Primers for the catalytic subunits of PI3K enzymes were designed for qPCR by using the web data resources Ensembl and Primer3Plus[12,13]. The following oligonucleotides were purchased as custom DNA primers from ThermoFisher Scientific Life Technologies:

Table 1 Primer sequences for RT-PCR

| Subtype (gene) | Sence/antisense strand | Sequence (5' - 3') |
|---|---|---|
| Class I-α (p110α) | Forward | TAG CTG TGG AAA TGC GTC TG |
| | Reverse | AGC TTA AAA AAG AAT GAT AGT GA |
| Class I-β (p110β) | Forward | GCA AGT CAG CGG GAG AGT AG |
| | Reverse | CTT GAT CTT GCA GCA TTC CA |
| Class I-δ (p110δ) | Forward | GAG CAG CCG GAA GAC TAC AC |
| | Reverse | AGG ATG GAG GAG GAA TGG AC |
| Class II-α (PI3K-C2α) | Forward | TGG AAA ATC CCT TTC TGT GG |
| | Reverse | TTT GCA AGC TGA GTT GTT CG |
| Class II-β (PI3K-C2β) | Forward | GGA CCG ACC TCA AGC TGT TA |
| | Reverse | TTG GGA GGT AGA GTG GTT GG |
| ClassⅢ (Vps34) | Forward | AAA GTT CCT GAC CCC CAG AT |
| | Reverse | ATC GTG GTC AGA AGG TCC AC |
| β-Actin | Forward | TCC TCC CTG GAG AAG AGC TAC |
| | Reverse | TGA AGG TAG TTT CGT GGA TGC |

## 3. RESULTS AND DISCUSSION

Fig. 1 extends our previous observation that NAM treatment can reverse the senescent phenotypes associated with physiological or stress-induced aging by showing that the effect of NAM is reversible. The old cells at PDL 71 exhibited typical senescent morphology with enlarged cellular and nuclear sizes (Fig. 1C: Control). After 3 days of exposure to NAM (Fig. 1C: 6 mM NAM), the morphology of the treated old cells resembled that of young cells (Fig. 1A: Control). However, once NAM was removed, the old cells exhibited senescent morphology again (Fig. 1C: NAM -NAM). The same observation of the effect of NAM on morphology was made for the oxidative stress-induced cells. Thus, cells at PDL 24 exhibited slender morphology typical for young cells (Fig. 1A: Control). But after a 2-h exposure to 100 μM $H_2O_2$, cell

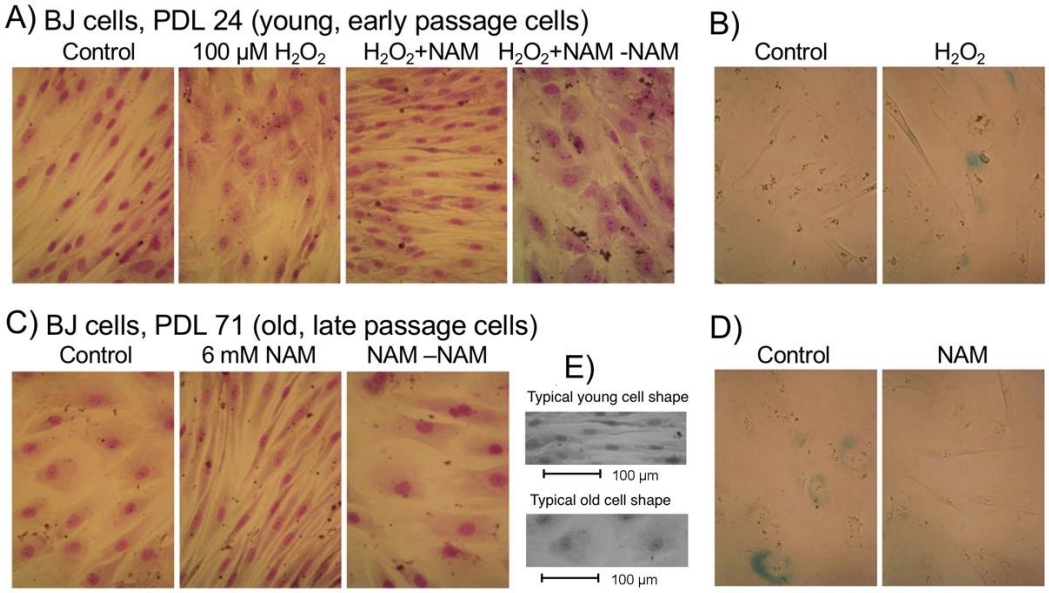


Fig. 1 Effects of aging, oxidative stress and NAM treatment on normal human fibroblasts (A, B: young cells at PDL 24; C, D: old cells at PDL 71; A, C: Giemsa staining; B, D: SA-gal staining)

BJ cells were treated with 100 μM $H_2O_2$ for 2 h, with 6 mM NAM for 3 – 6 d, or with $H_2O_2$ and thereafter with NAM. "-NAM" indicates "NAM-treated cells were subjected to additional culture without NAM for 3 d". After the treatments cells were examined by Giemsa or SA-gal staining. Typical shapes of young and old cells at the same magnification (for all the cells in Fig. 1 as well) are shown as an inset (E). Old cells and $H_2O_2$-treated cells exhibited typical senescent phenotypes (*i.e.* enlarged, flattened cell and nuclear shapes and strong SA-gal activity), which were suppressed upon NAM treatment. Removal of NAM returned the cells back to the senescence again.

morphology drastically changed to that resembled old cells (Fig. 1A: 100 μM $H_2O_2$). Such stress-induced change can be reversed by NAM (Fig. 1A: $H_2O_2$+NAM) and the effect of NAM was reversible (Fig. 1A: $H_2O_2$+NAM -NAM). Similarly, the expression of the aging marker SA-gal activity associated with old cells (Fig. 1D: Control) or $H_2O_2$-treated young cells (Fig. 1B: $H_2O_2$) was able to be suppressed by NAM(Fig. 1D: NAM). We also examined whether NAM treatment may affect the level of reactive oxygen species (ROS) in cells, we did not find any significant correlation, indicating that the effect of NAM is not directly channeled via ROS (data not shown).

NAM, a precursor/metabolite of $NAD^+$, plays important roles in cellular energy metabolism and a whole array of signaling pathways including sirtuins, PI3K, AMPK and autophagy[5,14]. The finding that NAM could reversibly modulate senescent phenotypes in human diploid cells prompted us to investigate whether other small molecules that inhibit or activate these pathways may produce similar effects on senescent phenotypes. Such study could shed additional light on the mechanism of the action of NAM on cellular senescence. For PI3K pathway, we tested 4 inhibitors: LY294002, wortmannin, ZSTK474, and BEZ235. For sirtuins, we tested resveratrol as the activator and splitomicin as the inhibitor. For AMP kinase, we tested AICAR as the activator and dorsomorphin as the inhibitor. Fig. 2 shows the effects of these small molecules on the morphology of young and old BJ cells. Table 2 summarizes the results. We found that the inhibitors of PI3K suppressed senescent phenotypes, with BEZ235 being more effective (Fig. 2, Table 2). In contrast, inhibitors for sirtuins and AMPK, *i.e.* splitomicin and dorsomorphin, respectively, slightly enhanced the senescent phenotypes, whereas AICAR, the AMPK activator suppressed the senescent phenotypes (Fig. 2). PI3K and autophagy signals are both critical in the regulation of cellular aging, survival and longevity[3,15]. MAP-LC3 is a component of the autophagy machinery "autophagosome" and induced under starvation or stress, hence used as an autophagy marker[9]. The LC3 signal was stronger in old cells and $H_2O_2$-pretreated cells, while it was weaker in young cells and NAM-treated cells (Fig. 3). It could be interpreted that under the senescent or stressed conditions the autophagic system is activated for cell protection.

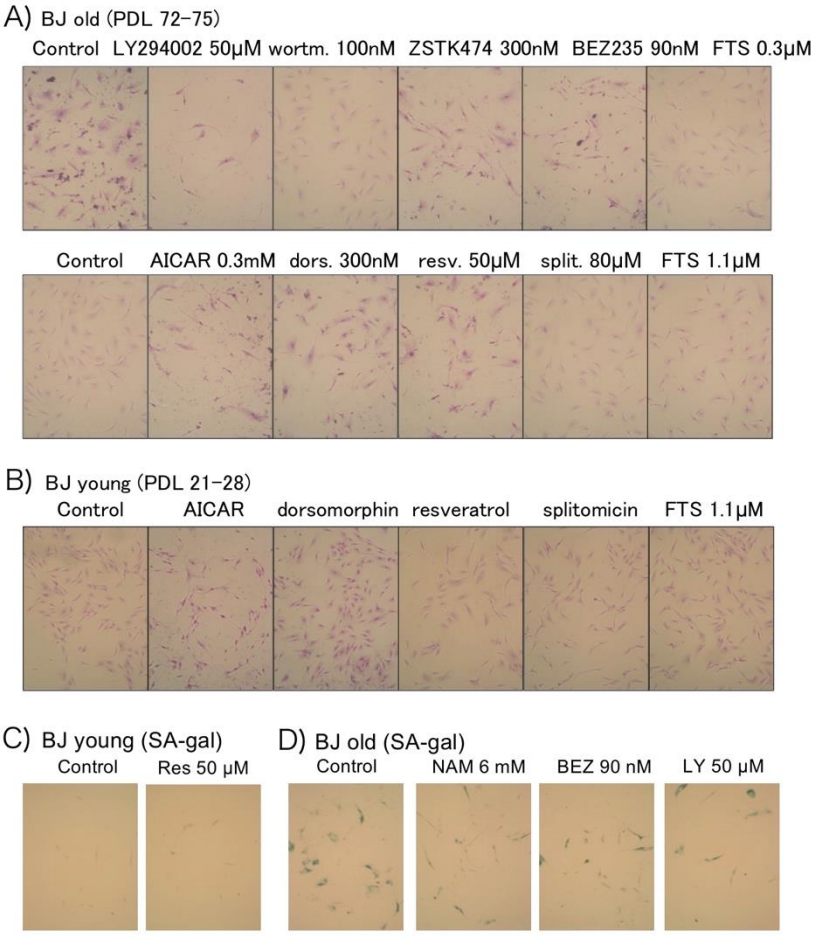


Fig. 2 Effects of bioactive compounds on normal human fibroblasts (A, B: Giemsa staining; C, D: SA-gal staining; B, C: young cells at PDL 21-28; A, D: old cells at PDL 72-75)

BJ cells were treated as described in the legend to Fig. 1. wortm.: wortmannin, dors.: dorsomorphin, split.: splitomicin, Res: resveratrol, BEZ: BEZ235, LY: LY294002

NAM, PI3K inhibitors, AMP kinase activator and FTS (thymic factor) suppressed senescent cell phenotypes (enlarged cellular/nuclear sizes and SA-gal activity), whereas AMP kinase inhibitor, sirtuin inhibitor and resveratrol appeared to enhance them to some extents.

Table 2 Effects of signaling compounds on the cell size distribution of normal human fibroblasts

| Cell (age) | Treatment | Cell size ($\mu m^2$) Average ± Std Dev | Median |
|---|---|---|---|
| Young (PDL 26 - 37) | None (control) | 1,160 ± 920 | 830 |
| | Dorsomorphin 300 nM | 1,560 ± 1,120 *a* | 1,250 |
| | Resveratrol 50 μM | 2,140 ± 1,780 *a* | 1,510 |
| Old (PDL 66 - 80) | None (control) | 2,700 ± 1,880 *a* | 2,170 |
| | NAM 4 mM | 1,320 ± 810 *b* | 1,050 |
| | NAM 6 mM | 1,560 ± 1,460 *b* | 1,070 |
| | NAM 10 mM | 1,480 ± 1,530 *b* | 930 |
| | Resveratrol 50 μM | 2,540 ± 2,180 *b* | 1,660 |
| | LY294002 50 μM | 1,750 ± 1,310 *b* | 1,190 |
| | BEZ235 90 nM | 1,290 ± 650 *b* | 880 |
| | AICAR 0.3 mM | 1,700 ± 1,690 *b* | 940 |
| | FTS 1.1 μM | 1,640 ± 1,190 *b* | 1,190 |

*a*: $p<0.01$ as compared with young control; *b*: $p<0.01$ as compared with old control

BJ cells were treated as described in the legend to Fig. 1. NAM, PI3K inhibitors, AICAR (AMP kinase stimulator) and FTS (thymic factor) changed old cells smaller, whereas Dorsomorphin (AMP kinase inhibitor) and resveratrol enlarged them

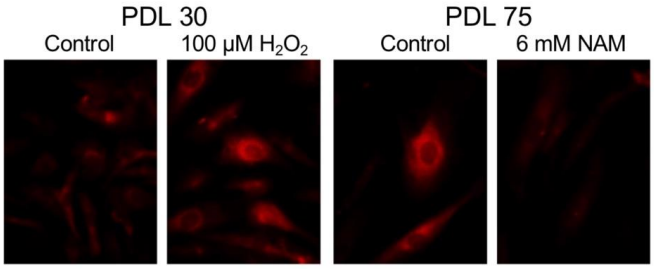


Fig. 3 Effects of aging, oxidative stress and NAM treatment on the expression of autophagy marker MAP-LC3

BJ cells were treated as described in the legend to Fig. 1. LC3 was detected by immunofluorescence with anti-LC3 antibody. The signal of LC3 was detected to be strong in old or H2O2-treated cells and weak under NAM treatment.

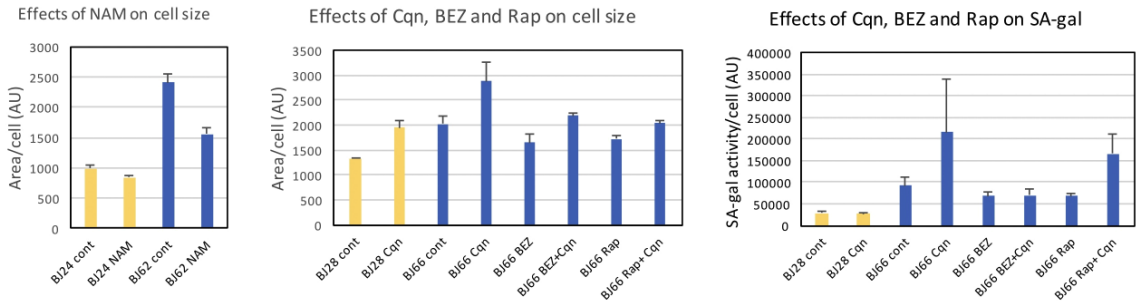


Fig. 4 Effects of autophagy-modulating compounds on normal human fibroblasts

Young cells: BJ24 (at PDL 24) or BJ28 (at PDL 28); old cells: BJ62 (at PDL 62) or BJ66 (at PDL 66)

The cells were treated as described in the legend to Fig. 1: 3 days with 4 mM NAM, 90 nM BEZ235 (BEZ, PI3K inhibitor), 10 μM Rapamycin (Rap, autophagy inducer), 10 μM Chloroquine (Cqn, autophagy inhibitor) or normal medium (Cont). Cell size and SA-gal activity are shown in arbitrary unit (AU). NAM, BEZ and Rap reduced senescent cell phenotypes (enlarged cellular size and high SA-gal activity), which Cqn enhanced.

Table 3 Effects of signaling compounds on cell motility

| BJ cells | Treatment | Motility (μm/h) |
|---|---|---|
| PDL28 | None (control) | 29.9 ± 6.8 |
| PDL21 | 300 nM Dorsomorphin | 18.7 ± 7.8 [a] |
| PDL27 | 50 μM Resveratrol | 25.4 ± 11.2 [a] |
| PDL74 | None (control) | 11.2 ± 6.9 [a] |
| PDL73 | 10 mM NAM | 28.0 ± 15.4 [b] |
| PDL72 | 90 nM BEZ235 | 31.1 ± 12.1 [b] |

Mean ± Std Dev; a: $p<0.01$ as compared with PDL28 control; b: $p<0.01$ as compared with PDL74 control

BJ cells were inoculated and, on the next day, treated with the indicated compounds. Movement of the cells were monitored the indicated compounds. Movement of the cells were monitored by time-lapse photography at every min for a period of 72 h and the results after 30 h from the start of chemical treatments were used to calculate cell motilities. Old cells at PDL 72-74 crawled on the culture vessel slowly, but their motility increased by treatment with NAM and BEZ.

Table 4 Effects of aging and NAM treatment on the expression of PI3K subspecies

| Cell age | PI3K subtypes | NAM treatment | PI3K - β-ACT (ΔCt) Average | SD | | PDL61 - PDL22 (ΔΔCt) | 【% change】 | NAM - control (ΔΔCt) | 【% change】 |
|---|---|---|---|---|---|---|---|---|---|
| PDL 22 | I α | None (control) | 7.218 | 1.871 | | | | | |
| | | 5 mM NAM | 8.115 | 0.459 | | | | 0.897 | 【 -46.3 】 |
| PDL 61 | | None (control) | 7.108 | 1.986 | | -0.110 | 【 7.9 】 | | |
| | | 5 mM NAM | 7.358 | 2.082 | | | | 0.250 | 【 -15.9 】 |
| PDL 22 | I β | None (control) | 10.510 | 0.554 | | | | | |
| | | 5 mM NAM | 10.065 | 0.264 | | | | -0.445 | 【 36.1 】 |
| PDL 61 | | None (control) | 10.335 | 0.759 | | -0.175 | 【 12.9 】 | | |
| | | 5 mM NAM | 10.533 | 1.344 | | | | 0.198 | 【 -12.7 】 |
| PDL 22 | I δ | None (control) | 8.663 | 1.195 | | | | | |
| | | 5 mM NAM | 9.283 | 0.279 | | | | 0.620 | 【 -34.9 】 |
| PDL 61 | | None (control) | 8.098 | 1.084 | | -0.565 | 【 47.9 】 | | |
| | | 5 mM NAM | 8.123 | 1.131 | | | | 0.025 | 【 -1,7 】 |
| PDL 22 | II α | None (control) | 7.918 | 0.399 | | | | | |
| | | 5 mM NAM | 7.758 | 0.255 | | | | -0.160 | 【 11.7 】 |
| PDL 61 | | None (control) | 6.880 | 1.442 | *a* | -1.038 | 【105.2】 | | |
| | | 5 mM NAM | 6.463 | 0.813 | | | | -0.418 | 【 33.5 】 |
| PDL 22 | II β | None (control) | 12.825 | 0.320 | | | | | |
| | | 5 mM NAM | 11.780 | 1.219 | *b* | | | -1.045 | 【 106.3 】 |
| PDL 61 | | None (control) | 12.275 | 0.120 | *c* | -0.550 | 【 46.4 】 | | |
| | | 5 mM NAM | 11.900 | 0.825 | | | | -0.375 | 【 29.6 】 |
| PDL 22 | III | None (control) | 9.435 | 0.339 | | | | | |
| | | 5 mM NAM | 9.178 | 0.318 | | | | -0.258 | 【 19.5 】 |
| PDL 61 | | None (control) | 9.100 | 0.515 | | -0.335 | 【 26.1 】 | | |
| | | 5 mM NAM | 8.410 | 0.348 | | | | -0.690 | 【 61.3 】 |

*a*: p<0.05 as compared with PDL22 IIα control; *b*: p<0.05 as compared with PDL22 IIβ control; *c*: p<0.05 as compared with PDL22 IIβ control BJ cells were treated as described in the legend to Fig. 1. Expression of PI3K catalytic subunits was analyzed by qPCR (ΔΔCt method). The results are shown in ΔCt and ΔΔCt for the difference PI3K vs. β-ACT, PDL61 vs. PDL22, and NAM vs. untreated, respectively. The PI3K expression seems to be more or less high in old cells for all the subtypes. Upon NAM treatment there may be possible tendencies that classes II and III expression was elevated, with most class I expression being lowered. For the most results here, however, statistically significant differences were not observed.

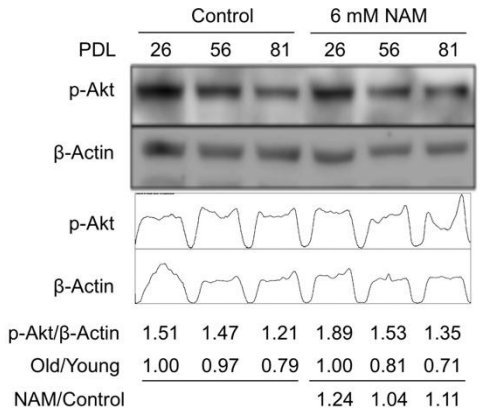


Fig. 5 Effects of aging and NAM treatment on Akt phosphorylation BJ cells were treated as described in the legend to Fig. 1 and cellular proteins were analyzed by Western blotting. Akt(Thr308) phosphorylation was, although slightly, low in old cells and high on NAM treatment.

Thus, we compared the effects of NAM and BEZ235 with that of Rap and Cqn, chemicals that modulate autophagy signaling (Fig.4). In general, the inhibitor of autophagy signaling enhanced senescent phenotypes (cell size and SA-gal), whereas Rap, the activator of autophagy signaling, acted similar to NAM and BEZ235 in suppressing the senescence. Rap induced no apparent change on young cells (not shown).

In addition to cell size and SA-gal, cell motility correlated well with the PDL level of diploid cells. Using time-lapse

microscopy we were able to show that NAM and BEZ235 significantly enhanced the motility of old cells to the degree comparable to that of young cells (Table 3). This result indicated that the effects of NAM and BEZ235 are not limited at morphological level. Another observation here is that resveratrol appears to be somewhat "pro-aging" (Table 2, Table 3). Although resveratrol is well known as a sirtuin activator, conflicting biological effects of resveratrol have been reported, thus further work is needed to elucidate its action.

PI3K comprises many subclasses and subtypes[15]. In light of the effect of PI3K inhibitors on suppressing the senescent phenotypes, we examined the effect of NAM on the expression of each of the subtypes in young and old cells by qPCR. When expression of the messages of PI3K components was analyzed, the catalytic subunits of class I γ (expressed mainly in immune system) and class II γ (mainly in liver) were not detected (data not shown), most likely due to their low expression in the skin fibroblasts employed here[15]. Thus, in the present study, those of class I α, β and δ, class II α and β, and class III were examined (Table 4). Taking the level of β-actin as a reference, the most abundant species were class I α and class II α (approximately 1/70~1/240 of β-actin), and the least one was class II β (1/4900~1/7200 of β-actin). The expression level of all these subtypes in old cells was higher than in young cells to varying degrees (Table 4). Upon treatment with NAM, there were tendencies that, with both young and old cells, the expression of the PI3K class I subtypes, with the exception of 1b, was reduced, whereas those of class II and class III subtypes were elevated substantially (Table 4). In this experiment, however, statistically significant difference was detected at $p<0.05$ only with class II α and II β for PDL 63 vs. PDL 22, and with class II β for 5 mM NAM vs. control and therefore, those observations need further analysis to draw a distinct conclusion.

As to the PI3K pathway, we further investigated the status of phosphorylation at Thr308 of Akt, the downstream target PI3K signaling (Fig. 5). Akt phosphorylation in senescent cells was about 20% less than that in young cells. This ratio did not change with the treatment with NAM, even though NAM enhanced phosphorylation in both young and senescent cells. It has been reported that PI3K class I activates Akt (protein kinase B) and mTOR signaling, and then suppress autophagy[18], whereas the functions of PI3K class III (and possibly, class II as well) are considered to be prerequisite for induction of autophagy[15]. Clearly the complex PI3K signaling pathways emphasize the importance of fine tuning of each components in achieving global effect on physiological processes such as aging. Our initial finding of fluctuations of PI3K subspecies in response to NAM is encouraging. Future work will explore the causal relationship of such fluctuation and aging.

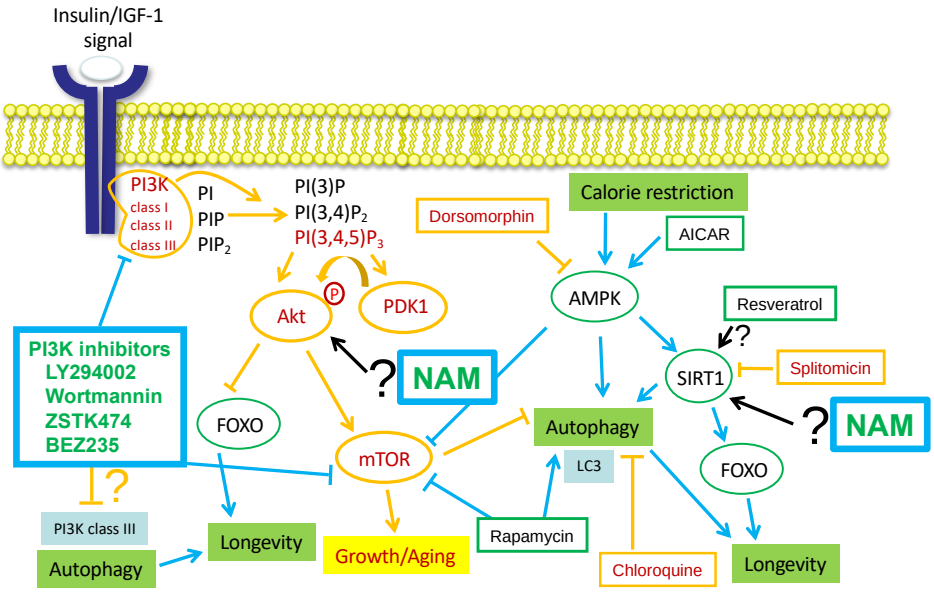


Fig. 6 Possible network of NAM and PI3K-related molecules in modulation of the aging signaling (scheme)

In general, PI3Ks function pro-aging, while sirtuins are anti-aging, inducing autophagy. Complication is that PI3K class III works for autophagy and that NAM, a precursor of $NAD^+$ and a putative sirtuin inhibitor, is implied to function as a sirtuin activator. Their roles and mechanisms for the aging phenomena are yet to be revealed.

In summary, we have found that senescent phenotypes, whether from physiological aging or stress-induced aging, can be suppressed by NAM, and the effect was reversible. In addition to NAM, small molecules such as BEZ235, the inhibitor of PI3K signaling, and rapamycin, the activator of autophagy, are also effective in suppressing the senescent phenotypes. Fig. 6 summarizes our finding and current thinking about the potential central role of NAM in aging process. It has been reported that sirtuins work for maintenance of genome stability, cell longevity[19], and activating autophagy[20]. Autophagy is considered to work for protection of the cell from such damages as starvation, stress and inflammation[3,21]. Taken together, the modulation of senescent phenotypes, including cell morphology, SA-gal, and cell motility by small molecules associated with key signaling pathways such as sirtuin, PI3K and autophagy provides us the opportunity to further delineate the mechanism of aging at single cell level.

**5. ACKNOWLEDGMENT**

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