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Short Communication

NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+) AND CELL AGING

Yutaka Takihara[1], Daisuke Sudo[1], Jun Arakawa[1], Mayu Takahashi[1], Akira Sato[2], Sei-ichi Tanuma[2] and Fumiaki Uchiumi[1,*]

[1]Department of Gene Regulation, [2]Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Yamazaki, Noda, Chiba, Japan

ABSTRACT

Nicotinamide adenine dinucleotide (NAD^+) is a co-enzyme that regulates reduction and oxidization in energy production. NAD^+ is also required for DNA repair in mammalian cells as a substrate for poly(ADP-ribose) polymerases (PARPs) to synthesize poly(ADP-ribose) (PAR) when DNA damage occurs. Excess DNA damage might cause exhaustion of the molecule that can hinder mitochondrial respiration. Therefore, the concentration of NAD^+ should be appropriately controlled. Recent studies have revealed that NAD^+-dependent deacetylase sirtuins play important

* Corresponding Author Email: uchiumi@rs.noda.tus.ac.jp.

roles in controlling the aging process. Moreover, a decrease in NAD^+ concentration has been suggested to correlate with aging or aging-related diseases.

In this study, we evaluated the effects of natural and chemical compounds on promoter activities of several human DNA repair-associated genes in HeLa S3 cells. The results indicated that naturally occurring compounds, for example, *trans*-resveratrol, upregulate *TP53* promoter activity. Sustaining an appropriate level of genes encoding DNA repair factors is thought to be necessary for cell survival by preventing the accumulation of DNA mismatches and epigenetic alterations. In this chapter, we discuss the possibility and eligibility of the NAD^+ molecule as a regulator of cell aging at the transcriptional level.

Keywords: DNA repair, ETS, GGAA, metabolism, mitochondria, nicotinamide adenine dinucleotide (NAD^+), transcription

ABBREVIATIONS

2DG	2-deoxy-D-glucose
3AB	3-aminobenzamide
Aβ	amyloid-β
ETC	electron transport chain
Luc	Luciferase
mtDNA	mitochondrial DNA
NAAD	nicotinic acid adenine dinucleotide
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NAMPT	nicotinamide phosphoribosyltransferase
NMN	nicotinamide mononucleotide
NMNAT	nicotinamide nucleotide adenylyltransferase
OLA	olaparib
PAR	poly(ADP-ribose)
PARP	poly(ADP-ribose) polymerase
PARPi	PARP inhibitors
PIC	piceatannol
Rsv	*trans*-resveratrol

TCA cycle tricarboxylic acid cycle
TEMPOL 4-hydroxy-TEMPO

INTRODUCTION

Several biosynthetic pathways for nicotinamide adenine dinucleotide (NAD^+) are known. Nicotinic acid and nicotinamide, which are also known as niacin or vitamin B_3, are generated from tryptophan, and they provide essential groups for the NAD^+ molecule [1]. NAD^+ plays important roles in energy metabolism, serving as a substrate for oxidation and antioxidation [2-4]. Reduction of NAD^+ to nicotinamide adenine dinucleotide (NADH) is coupled with the oxidization of a glyceraldehyde 3-phosphate to bisphosphoglycerate. Oxidization of NADH to NAD^+, by providing H^+ to pyruvate, gives rise to lactate. In the oxidization/reduction cycle of NAD^+/NADH in glycolysis, one glucose molecule can produce two ATP and two lactate molecules independent of mitochondrial function or the respiration process. It should be noted that the "Warburg effect" is frequently observed in cancer cells, which involve aberrant mitochondria in their structures and functions. In normal cells, pyruvate dehydrogenase oxidizes pyruvate [5] to produce acetyl CoA, which is completely oxidized to CO_2 and H_2O through the tricarboxylic acid cycle (TCA cycle). At least three enzymes, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase, which are encoded by the *IDH (IDH1*, *IDH2*, *IDH3A*, *IDH3B*, and *IDH3G)*, *OGDH*, and *MDH (MDH1*, *MDH2*, and *MDH1B)* genes, respectively, require NAD^+ as a coenzyme for oxidization of each substrate [6]. NADH will be immediately oxidized to NAD^+ by complex I of the mitochondrial electron transport chain (ETC). Moreover, NAD^+ can accept H^+ in the β-oxidation of fatty acids. β-hydroxybutyrate dehydrogenase, which is encoded on the *BDH1* and *BDH2* genes [1], catalyzes the conversion of β-hydroxybutyrate to acetoacetate. Furthermore, NAD^+ is required as a coenzyme for inosine 5'-phosphate dehydrogenase and dihydropyrimidine dehydrogenase [7], which are encoded by the

IMPDH1/*IMPDH2* and *DPYD* genes, respectively. Thus, NAD^+ plays key roles in the regulation of energy production and metabolism.

The other important function of NAD^+ is as the substrate for poly(ADP-ribose) polymerase (PARP) to synthesize poly(ADP-ribose) (PAR) in response to DNA damage-inducing signals [8]. Involvement of the NAD^+ molecule in the protection of chromosomal DNAs has been reviewed and discussed previously [4, 9]. ATP molecules can be supplied from nuclear PAR by NUDIX5, which affects chromatin remodeling [4, 9-11]. Moreover, NAD^+-dependent deacetylases or sirtuin proteins have histone deacetylase activity [12] to regulate both cancer generation and aging [13, 14].

In summary, NAD^+ is not only required for redox reactions, especially in the mitochondria, but also for DNA repair, chromatin remodeling, and epigenetic control in nuclei. Importantly, these biological events, both in mitochondria and nuclei, are tightly linked with cancer generation/development and aging. Before discussing the mechanism of aging, we should understand the biosynthesis or metabolism of NAD^+.

METABOLISM OF NAD^+

The biosynthesis of the NAD^+ molecule has been reviewed previously [4]. Nicotinamide phosphoribosyltransferase (NAMPT), which catalyzes the first rate-limiting step of NAD^+ synthesis from nicotinamide [15], can modulate glycolysis, the TCA (Citrate/Krebs) cycle, poly(ADP-ribosyl)ation, and sirtuin-mediated de-acetylation, which all depend on NAD^+. The reaction product, nicotinamide mononucleotide (NMN), can be bound with phosphoribosyl pyrophosphate to produce NAD^+ by nicotinamide nucleotide adenylyltransferases (NMNATs), which are encoded on the *NMNAT1*, *NMNAT2*, and *NMNAT3* genes. NMNAT2, which localizes in the cytosol-facing side of the Golgi apparatus, has been suggested to be involved in the NAD^+ transport system in mammalian cells [16]. Catalyzed by nicotinamide riboside kinases, NMN can be alternatively produced by phosphorylation of nicotinamide riboside. In addition, NAD synthetase synthesizes NAD^+ from nicotinic acid adenine dinucleotide

(NAAD), which is produced by de-phosphorylation of NAAD phosphate. It should be noted that PGC-1α also plays a pivotal role in mitochondrial biogenesis by upregulating the *de novo* synthesis of NAD^+ in mice [17].

NAMPT gene expression is induced in glioblastoma cells to upregulate the *E2F2*/*ID* pathway [18]. The NAMPT/E2F2/SIRT1 axis has been shown to promote proliferation of human melanoma cells [19]. Depletion of NAD^+ by NAMPT inhibitor FK866 effectively leads to Ewing sarcoma cell death [20]. In cancer cells, NAMPT inhibition suppresses both glycolysis and the TCA cycle [21]. These observations imply that NAMPT could be targeted in the treatment of some specific cancers, including gastric cancer and colorectal cancer, which overexpress the *NAMPT (PBEF)* gene [22-24].

NAD^+-Dependent Biological Reactions

TCA Cycle, ETC, and Mitochondrial Functions

A number of lines of evidence, including that of dysregulated TCA (Citrate/Krebs) cycle progression and the insufficient oxidative phosphorylation occurring in cancer cells, suggest that mitochondrial dysfunction might precede metabolic changes [25, 26]. Loss-of-function mutations on several TCA cycle/ETC enzyme-encoding genes, including *FH*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, and *SDHD*, are suggested to cause cancer [27]. We have confirmed that a common DNA sequence motif, or a duplication of the GGAA motif, is contained in the 5'-upstream regions of the *ACLY*, *ACO2*, *CS*, *FH*, *IDH1*, *IDH3A*, *IDH3B*, *SDHAF2*, *SDHB*, *SDHD*, and *SUCLG1* genes [28]. Therefore, a decrease in the NAD^+ level might impede TCA cycle progression and the dysregulation of respiration and oxidative phosphorylation, accompanied by a reduction in mitochondrial function-associated gene expression. Probably at this stage, the "Warburg effect" or low respiration dependency would be observed. As long as ETC is working well in accordance with TCA cycle progression, complex I can supply an adequate number of NAD^+ molecules in the mitochondria.

However, if TCA cycle progression would decline or be suppressed, ETC may produce a lower number of NAD^+ molecules. In this context, insufficient NAD^+ would lead to deficiencies in the mitochondria, which may cause various age-related diseases including cancer and neurodegenerative diseases. If an adequate number of NAD^+ molecules were produced, they could be used for the synthesis of PAR, which will act as a landmark for the DNA damage-induced sites requiring repair [8]. Thus, production of excess NAD^+ would cause no problem if it were utilized in the DNA repair system and controlled by reduction to NADH or by other degradation processes.

Poly(ADP-ribosyl)ation (PARylation)

NAD^+ not only plays essential roles in the regulation of DNA repair, mitochondrial functions, and cellular senescence [29, 30], but it also affects chromatin proteins [31] to modulate gene expression [32]. It should be noted that NAD^+ is a substrate for the PARP enzyme to synthesize PAR macromolecules, which modify both PARP itself and chromosomal proteins and DNA repair factors [4]. Histones and HMGB proteins can be poly(ADP-ribosyl)ated [33-36], suggesting that modifications by such macromolecules affect epigenetic gene regulation. Notably, tumor suppressor p53 protein can be poly(ADP-ribosyl)ated by PARP1 [37]. Epigenetic regulation can be affected by poly(ADP-ribosyl)ation of the chromosomal insulator protein CTCF [38, 39], which binds directly to PAR on the DNA lesion sites [40]. In addition, poly(ADP-ribosyl)ation affects methylation patterns in chromosomal DNAs [41, 42] and the acetylation of histone proteins [43]. A recent study showed that transcriptional regulation of the *EZH2* gene by PARP1 [44] affects the methylation of chromatin proteins [45]. Moreover, PARP1 activation upregulates transcription of the *TET1* gene, which belongs to the DNA methylation modulator Ten-Eleven translocation family enzymes [46]. Because the incidence of cancer increases with aging [47], a decline in cellular NAD^+ might lead to a decrease in PARP activity [48]. Taken together, NAD^+ and its polymerized form PAR can modulate the

aging process, altering the chromatin structure and the epigenetic regulator encoding gene transcription.

Sirtuin Proteins

Sirtuin proteins, or NAD^+-dependent de-acetylases, play important roles in the aging process [49]. Inhibition of the PARP1 enzyme ameliorates mitochondrial metabolism through the activation of SIRT1 [50]. If heavy DNA damage were induced, NAD^+ would be overused by the activated PARP enzyme, and that may hinder or reduce the functions of sirtuin proteins. Moreover, the NAD^+-binding pocket of PARP1 regulates interaction with DBC1 (deleted in breast cancer 1), which is a known SIRT1 inhibitor protein [51]. A decrease in NAD^+ will upregulate the interaction between DBC1 and PARP1, leading to the suppression of its activity. This might partly explain why DNA repair declines with aging [52]. It is hypothesized that a reduction in nutrient levels could induce the accumulation of NAD^+ to activate sirtuins. Histone de-acetylation is consistent with the finding that calorie restriction mimetics prolong the life span [53-55].

NAD^+ AND AGING/CANCER

NAD^+ and Aging

NAD^+ and its precursor nicotinamide ameliorate metabolism and mitochondrial functions [56-58]. Repletion of NAD^+ improves mitochondrial functions to prolong the life span of adult mouse stem cells [59]. Conversely, a decline in NAD^+ will cause aging or age-related diseases [60]. Very recently, it was reported that NAMPT delays the cellular senescence of mouse cells by activating SIRT1 and the expression of antioxidant-encoding genes *Sod2* and *Cat* [61]. These observations are

consistent with the concept that the NAD^+ level correlates with mitohormesis [62] and that nutrient sensing molecules can control aging [63].

NAD^+ and Cancer

The incidence of cancer arises in accordance with aging [64, 65]. Recent genetic studies suggest that the processes in aging and cancer generation overlap each other [66]. For example, mitochondrial deficiencies are observed both in senescent and cancer cells [67, 68]. Upregulation of the cellular NAD^+ level could improve mitochondrial integrity to suppress oncogenesis. In breast cancer cells, knockdown of the subunit NDUFV1 leads to an aberration in complex I that enhances aggressiveness or metastasis [69]. Mitochondrial biogenesis regulator protein PGC-1α drives NAD^+ biosynthesis to upregulate stress resistance [17]. PGC-1α suppresses the metastasis of melanoma, affecting the PGC-1α–ID2-TCF-integrin axis through transcriptional control [70]. A loss of mitochondrial localizing protein CSB [71] activates PARP1, which plays essential roles in DNA repair [72], to consume NAD^+ molecules. Therefore, dysregulation of the mitochondria or hyperactivation of the PARP enzyme will reduce the molecular ratio of NAD^+/NADH. Mutations on the *IDH1* and *IDH2* genes have been identified in human brain cancer cells [73-75]. In addition, mutation-introduced IDH2 can generate sarcomas [76]. Generally, it has been explained that mutant IDH product 2-hydroxyglutarate mainly affects epigenetic alterations to cause cancer [73, 76]. Alternatively, incomplete functioning of IDH might retard TCA cycle progression.

NAD^+ and Neurodegenerative Diseases

Recently, it was reported that mitochondrial proteostasis plays a role in reducing amyloid-β (Aβ) aggregation in a mouse model of Alzheimer's disease [77]. Metabolic dysfunction is thought to play a major role in the

development of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [78]. For example, high levels of deletions in mtDNA have been identified in Parkinson's disease [79]. Thus, mitochondrial dysfunction might cause neurodegenerative disease [80]. In addition, an NAD^+-dependent deacetylase SIRT3, which loalizes in mitochondria, may play a role in the development of neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's disease [81, 82]. Interestingly, SIRT2, which is a tubulin deacetylase, plays a role in accelerating Aβ aggregation [83]. In addition, SIRT6 can protect cells from Aβ42-induced DNA damage [84]. Therefore, NAD^+ might be involved in the pathogenesis of Alzheimer's disease and other neurodegenerative diseases by affecting sirtuin proteins.

TRANSCRIPTIONAL REGULATION OF MITOCHONDRIAL FUNCTION- AND DNA REPAIR-ASSOCIATED GENES

It should be noted that the 5'-upstream regions of the TCA cycle enzyme-encoding genes, including *IDH1*, *IDH3A*, and *IDH3B*, contain duplicated GGAA motifs, which are the target sequences of various transcription factors, including ETS (E26 transformation-specific) family proteins [85]. Surveillance of the human genomic DNA database (https://www.ncbi.nlm.nih.gov/gene/) indicated that the duplicated GGAA motifs are contained in the 5'-upstream regions of the mitochondrial functions [86] and the TCA cycle enzyme-encoding genes, including *PDH*, *MDH1*, and *MDH2*. Interestingly, *IDH3G*, *OGDH*, and *MDH1B* gene promoters have no such elements, but the encoded enzymes require NAD^+ as an acceptor of H^+. In this manner, TCA cycle progression would be dependent on both GGAA motifs-driven transcription and an NAD^+ molecule.

Depletion of NAD^+ by its metabolism-associated proteins/enzymes, including NAMPT, may effectively suppress proliferation of cancer cells by inhibiting glycolysis [21]. However, this might be a problem for healthy cells, which mainly produce energy from complete respiration via the TCA

cycle or oxidative phosphorylation. To avoid unfavorable side effects, it is essential to design and develop new concept-based anti-cancer drugs. The duplicated GGAA motifs are not only contained in the promoter regions of the mitochondrial function-associated genes but also those of the DNA repair factor-encoding genes [80]. That a certain compound upregulates both the cellular NAD^+ level and promoter activities of the DNA repair- and mitochondrial function-associated genes could be a compass guiding us to achieve currently undeveloped chemotherapeutics. To find such drugs that fulfill this goal, we have established an assay system to analyze multiple promoter activities of human DNA repair-associated genes in cultured cells [87, 88]. In this study, we performed pilot experiments to estimate the eligibility of several compounds as candidates for new concept-based anti-cancer drugs.

MATERIALS AND METHODS

Materials

trans-Resveratrol (Rsv) [89] was purchased from Cayman Chemical (Ann Arbor, MI), and 2-deoxy-D-glucose (2DG) [90] and 3-aminobenzamide (3AB) were obtained from WAKO Pure Chemical (Tokyo, Japan). Olaparib (OLA), piceatannol (PIC), and 4-hydroxy-TEMPO (TEMPOL) were purchased from ChemScene, LLC (Monmouth Junction, NJ), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and Sigma-Aldrich (St. Louis, MO), respectively.

Cells and Cell Culture

Human cervical carcinoma (HeLa S3) cells [91] were grown in Dulbecco's modified Eagle's medium (WAKO Pure Chemical) supplemented with 10% fetal bovine serum (Biosera, East Sussex, UK) and penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

NAD^+/NADH Assay

NAD/NADH-Glo™ Assay (Promega, Madison, WI) was performed according to the manufacturer's protocol. Briefly, cells (approximately 1 to 2×10^6) were suspended in PBS (50 μL), and 50 μL of base solution (+ DTAB) was added. The lysed cell sample was dispensed into aliquots to measure NAD^+ and NADH. To measure NAD^+, 25 μL of 0.4 N HCl was added to the lysed cell sample (50 μL), which was heated at 60°C for 15 min and equilibrated at room temperature for 10 min, and then Trizma base (25 μL) was added. To measure NADH, the lysed cell sample (50 μL) was heated at 60°C for 15 min, equilibrated at room temperature for 10 min, and HCl/Trizma solution (25 μL) was added. After incubating with NAD/NADH-Glo Detection Reagent (100 μL) at 25°C for 30 min, chemiluminescence was measured with an EnVision ARVO MX plate reader (Perkin Elmer Inc., Waltham, MA).

Construction of Luciferase Expression Reporter Plasmids

Luciferase (Luc) reporter plasmids containing 5'-upstream regions of the human *ATR*, *CDKN1A* (*p21*), *PARP1*, *PIF1*, *RB1*, *TP53*, *TERT*, and *WRN* genes have been constructed previously [87-92]. In this study, pGL4-ATM, pGL4-BRCA1, and pGL4-E2F4 were constructed by similar methods. Briefly, PCR was performed with sense and anti-sense primer pairs (Table 1) and template genomic DNAs that were extracted from HeLa S3 cells. Then, the PCR products were digested with *Kpn*I and *Xho*I, and they were ligated into the multicloning site of the pGL4.10[*luc*2] vector (Promega). Nucleotide sequences were confirmed by a DNA sequencing service (FASMAC, Greiner Japan Inc., Atsugi, Japan) with primers RV3 (5'-TAGCAAAATAGGCTGTCCCC-3') and GL2 (5'-CTTTATGTTTTTGGCGTCTTCC-3').

DNA Transfection and Luc Assay

Plasmid DNAs were transfected into cultured cells with the multiple DEAE-dextran method [87, 88]. DNA-transfected cells (approximately 1 to 2×10^6) were treated with various compounds for 24 h, and then cell lysates were prepared. They were subjected to the Luc assay (Promega). The cell lysate was centrifuged at 12,000 × *g* for 5 sec, and the supernatant was transferred to a new tube and stored at –80°C before use in the Luc assay, which was performed according to a protocol described previously [88]. Chemiluminescence was immediately measured for 7.5 sec with a Minilumat LB9506 luminometer (Berthold, Bad Wildbad, Germany). The light intensity measured in Relative Light Units (RLU) was referred to directly as Luc activity.

Table 1. Primer pairs used for amplifying the 5'-upstream regions of the human genes

Plasmid	Primer	Sequence (from 5' to 3')
pGL4-ATM	hATM-117122	TCGGTACCACAGCAGGAACCACAATAAG
	AhATM-117444	ATCTCGAGCGTTTGCGGCTCGCCCTTCG
pGL4-BRCA1	hBRCA1-1942	TCGGTACCGAAACTGGAGACCTCCATTAGG
	AhBRCA1-1533	ATCTCGAGCTCACGCCGCGCAGTCGCAG
pGL4-E2F4-466	hE2F4-9826	TCGGTACCTTTAGGAACAGGTTTGATGTGG
	AhE2F4-0291	ATCTCGAGGCCGGCGCCGCCGCCACTTCC

RESULTS

Upregulation of NAD^+/NADH Ratio by Chemical Compounds, Including Rsv

Because Rsv upregulates mitochondrial complex I, which oxidizes NADH to produce NAD^+ [93], we examined whether it affects the NAD^+/NADH ratio in HeLa S3 cells. The ratio increased about 20% by

treatment with Rsv (20 μM) for 24 h (Figure 1). Other compounds that have been reported to increase the cellular NAD^+/NADH ratio are 2DG [94] and TEMPOL [95]. We also suspected that PARP inhibitors (PARPi) could induce a change in the NAD^+/NADH ratio because they interfere with consumption of the NAD^+ molecule to synthesize poly(ADP-ribose). Therefore, the effects of 2DG (8 mM), TEMPOL (1 mM), 3AB (5 mM), and OLA (2.5 μM) on NAD^+ metabolism were tested in the same experimental setting. As shown in Figure 1, they upregulated the ratio by two- to three-fold. Moreover, PIC (20 μM), which resembles Rsv in its chemical structure and can extend the lifespan of *Caenorhabditis elegans* [96] with anti-cancer activity [97, 98], increased the ratio over three-fold.

Analysis of Promoter Activities of the Human DNA Repair-Associated Genes

To examine whether compounds upregulating the NAD^+/NADH ratio could affect transcription of the genes encoding factors controlling the DNA repair/cell cycle, Luc expression reporter plasmids containing approximately 500 bp of the 5'-upstream regions of 10 different human genes, including *TP53*, *RB1*, and *BRCA1*, were transfected into HeLa S3 cells, and Luc assay was carried out. As shown in Figure 2, all of the promoter activities, which were normalized to that of the *PIF1* promoter activity, increased in response to Rsv treatment for 24 h. Both 2DG and 3AB had activation of the *PARP1*, *WRN*, and *E2F4* promoters in common. Most of the promoter activities were induced by PIC, whereas OLA and TEMPOL treatment commonly induced *RB1* and *BRCA1* promoter activities. In this experimental condition, OLA specifically induced *TERT* and *PARP1* promoter activities and that TEMPOL activated *ATR* and *WRN* promoters.

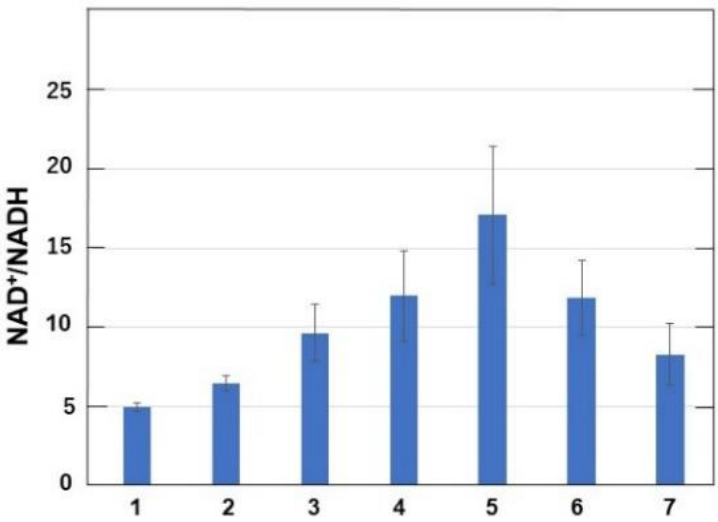

Figure 1. NAD^+/NADH ratio in HeLa S3 cells. HeLa S3 cells were treated with the following compounds: RSV (20 μM), 3AB (5 mM), 2DG (8 mM), PIC (20 μM), TEMPOL (1 mM), and OLA (2.5 μM) (Columns 2, 3, 4, 5, 6, and 7, respectively). After 8 h of treatment, cells were harvested, and cell extracts were subjected to NAD^+/NADH assay. Column 1 represents the NAD^+/NADH value of the DMSO (0.05%)-treated cell extract. Results are shown as means ± SD from four independent assays.

DISCUSSION

This study showed that NAD^+/NADH upregulating compounds could induce promoter activities of several essential genes encoding DNA repair factors, including *TP53* and *RB1*, in HeLa S3 cells. We previously discussed the beneficial effects of Rsv on health and longevity [55]. Moreover, we reported that the human *WRN* [89], *TERT* [89], *TP53* [91], and *HELB* [99] gene promoters are upregulated by Rsv treatment. In the present study, an apparent positive effect of Rsv on the *ATM*, *ATR*, *BRCA1*, *CDKN1A* (*p21*), *E2F4*, *PARP1*, and *RB1* gene promoters was also observed (Figure 2A). Although the magnitudes of promoter induction by the five other compounds tested were much lower than that by the Rsv treatment, several specific gene-drug combinations were remarkable, such as *ATR*, *BRCA1*, and *RB1*

promoter induction by TEMPOL, 2DG, and OLA, respectively (Figure 2B). It should be noted that the 5'-upstream regions of these genes that were examined in the present study commonly contain a duplicated GGAA motif [85], which is a target sequence of the ETS family and other transcription factors, that is frequently linked with the Sp1 family target sequence and GC-box elements. Furthermore, several of these genes have head-head links with partner genes, for example, the *ATM/NPAT*, *BRCA1/NRB2*, *RB1/LINC00441*, *TP53/WRAP53*, and *WRN/PURG* gene pairs [100], which could be classified as "bi-directional promoter"-driven gene sets [86]. A number of the genes associated with mitochondrial function are head-head oriented with other genes, and the GGAA duplications are contained within that region [86, 100]. These observations suggest that DNA-repair and mitochondrial functions would be diminished together in accordance with the decline in the cellular NAD^+ level that correlates with aging and the generation of aging-related diseases, including cancer.

To slow the aging process and prevent aging-related diseases, upregulation or control of the cellular NAD^+ level should be taken into account before the transcriptional state or the balance in the cells begins to collapse. The PARPi, which are especially effective in treating cancer with BRCA1 and BRCA2 mutations by disrupting specific types of DNA repair systems, are clinically approved drugs [101]. A part of the anti-cancer effect of the PARPi can be partially explained by inhibition of the over-consumption of NAD^+ molecules by the PARPs. The present study showed that *RB1* promoter activity was prominently upregulated by OLA (Figure 2B) and that 3AB could induce *TP53*, *WRN*, *E2F4*, and *CDKN1A* promoters. TEMPOL is an antioxidant with a suppressive effect on tumor cell proliferation [102], which increases the cellular NAD^+ level, that supports the DNA repair system [95, 103]. The compound PIC significantly induced activity of most of the promoters tested (Figure 2B). Based on the observations in the present study, chemicals and naturally resourced products could be expected to be anti-cancer medications if they induce a cellular NAD^+ level and ameliorate DNA repair systems.

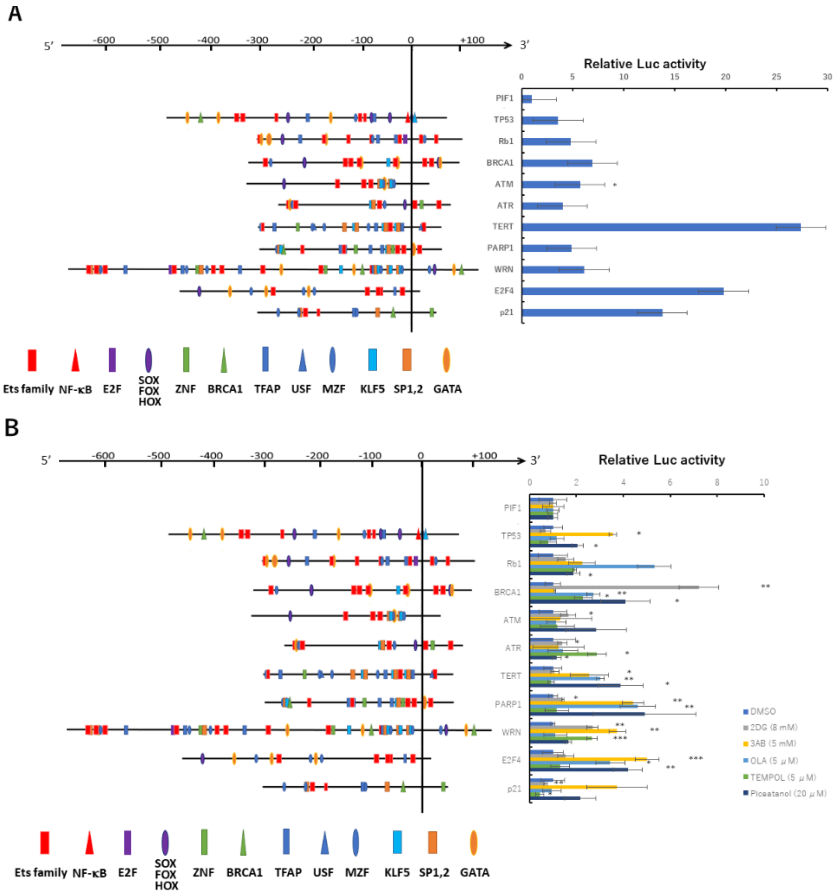

Figure 2. Promoter activities of the human DNA repair factor-encoding genes in HeLa S3 cells. (Left) PCR amplified 5'-flanking regions of various human DNA repair factor-encoding genes, which were inserted upstream of the Luciferase (Luc) gene of the pGL4.10[*luc*2] vector, are shown. Transcription start sites (or 5'-end of cDNAs) are designated +1. The JASPAR program (http://jaspar.genereg.net/) was performed, and putative transcription factor binding elements are shown schematically. (Right) HeLa S3 cells were treated with the following compounds: (A) Rsv (20 μM) and (B) 2DG (8 mM), 3AB (5 mM), OLA (5 μM), TEMPOL (5 μM), and PIC (20 μM). After 24 h of treatment, cells were harvested, and cell extracts were subjected to Luc assay. Luc activities were normalized to that of pGL4-PIF1 transfected cells. Relative Luc activity represents fold activation of the normalized Luc activity compared with that of control or 0.05% DMSO-treated cells. The results are shown as means ± SD from four independent assays. Significant differences between control and Rsv-treated cells were analyzed by Student *t*-test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$).

CONCLUSION

At present, it is not yet completely known how NAD^+ affects each DNA repair factor encoding gene promoter activity. However, observation of the human *BRCA1/NRB2* promoter, which depends on the metabolic state, suggests that an NAD^+-sensitive transcription control system is at work in cells. One of the candidate transcription factors is CtBP, which is a metabolic sensor transcriptional suppressor [104, 105]. In addition, the observation that poly(ADP-ribosyl)ation of transcription elongation factor NELF releases paused RNA pol II-dependent transcription [106] may support the idea that transcription is NAD^+ sensitive. Importantly, nuclear PAR can be utilized by NUDIX5 to supply ATP molecules, which are required for chromatin remodeling [11]. It should be remembered that damage on DNAs can activate the PARP enzyme, which consumes NAD^+ to synthesize PAR and also affects NAD^+-dependent transcription. Taken together, the findings suggest the importance of arranging nutrients in everyday foods because metabolites from niacin or vitamin B3 may affect DNA repair and mitochondrial function at the transcriptional level. The goal should not be just to obtain naturally resourced nutrients but also to utilize specific transcription factor expression vectors in the treatment of cancer and neurodegenerative diseases. We could thus expect that novel gene therapies developed on this basis would have many fewer side effects but with the advantage of anti-aging effects as well.

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BIOGRAPHICAL SKETCH

Fumiaki Uchiumi

Affiliation: Department of Gene Regulation, Faculty of Pharmaceutical Sciences, Tokyo University of Science

Education: Ph.D.

Business Address: Yamazaki 2641, Noda-shi, Chiba-ken 278-8510, JAPAN