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Downregulation of transcription factor, Sp1, during cellular senescence

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Abstract

We found that the protein level of Sp1 transcription factor decreases as normal human fibroblasts undergo replicative aging. Sp1 also undergoes a rapid decrease in the protein level and activity in MCF-7 cells that are induced to a state of cellular senescence. In the cells treated with other DNA damaging chemicals such as actinomycin D and H_2O_2, the Sp1 level decreased progressively as well. Inhibition of ATM/ATR kinases prevented this downregulation, suggesting that DNA damage signaling is involved in the regulation of the Sp1. This decrease in Sp1 protein level is due to the accelerated proteasomal degradation since a proteasome inhibitor, ALLN, blocked this downregulation. Therefore, the global decrease in gene transcription frequently reported in aging cells and tissues could be attributed at least in part to the decrease in Sp1 level.

Keywords: Sp1; DNA damage; Cellular senescence; Aging; Adriamycin

In normal cells, a state of senescence is reached after a finite number of cell division [1]. Cellular senescence is marked by irreversible growth arrest and expression of certain cellular phenotype such as high level autofluorescence and flat and enlarged cell morphology. As cell proliferation continues, levels of p53, p21WAF1, and/or p16INK4a proteins increase gradually, and eventually cell growth is arrested irreversibly [2,3]. During cellular lifespan, cells undergo alterations in various activities including DNA damage signaling [4] and cell stress response [5,6] as well as in their energy and glucose metabolism [7]. In addition, a number of recent studies on tissues from aged animals and cells undergoing *in vitro* senescence showed that expression of a panel of genes involved in such cellular activities are either up- or downregulated during the aging progression [8–10]. In general, genes involved in energy production, cell growth, and DNA transcription/replication have a pattern of decrease, while those involved in inflammation and oxidative stress response have a pattern of increase during aging [9,11]. These findings suggest that the changes in cellular function and the senescence phenotype are attributed at least in part to the alterations in transcriptional activity of certain genes. Therefore, a significant change in the activities of certain transcription factors during aging progression is expected.

Sp1 is a key transcription factor for basal level transcription of diverse genes that lack a TATA-box [12]. Cellular Sp1 activity and protein level are subject to change according to multiple factors associated with various cellular signaling pathways and cellular metabolic status, possibly through either phosphorylation by various kinases [13] or *O*-glycosylation (*O*-GlcNAcylation) which, in turn, is regulated by the flux of glucose and metabolism [14]. Indeed, a substantial variation in Sp1 level was noted during animal development, suggesting that Sp1 has a regulatory function in addition to its general role in the transcription of housekeeping genes [15]. Recently, a number of studies reported a decreased activity of Sp1 in aged animal tissues as well as in cells undergoing replicative senescence [16–20]. However, the status of Sp1 protein itself has never been closely examined during cellular aging. In the present study, we

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determined how Sp1 and its activity are affected during *in vitro* aging of cells. The protein level of Sp1 decreased in both replicative and induced senescence due to a decrease in its stability. DNA damage could trigger Sp1 downregulation via proteasome-dependent degradation. Furthermore, activation of ATM/ATR signaling pathway in response to DNA damage appears to be involved in Sp1 downregulation.

Materials and methods

Cell culture and chemicals. NIH-H460 and MCF7 cell lines were purchased from American Type Cell Culture, and a primary line of normal human fibroblasts isolated from newborn foreskin was provided by Dr. Sang Chul Park, Seoul Nat'l University (Seoul, Korea). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Bio-Whitacker, Walkersville, MD, USA).

Western analysis. Cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Sigma). Proteins were applied to Western blotting analysis using antibodies to either p53 (DO-1; Calbiochem, La Jolla, CA, USA), p21WAF1 (C-19, HRP; Santa Cruz, CA, USA), Sp1 (07-645; Upstate, Lake Placid, NY, USA) or ERK 1 and 2 (K-23; Santa Cruz).

In situ staining of SA β-Gal activity. Cultured cells were washed in PBS (pH 7.4), fixed with 3.7% formaldehyde, and incubated over night at 37 °C in freshly prepared staining buffer (1 mg/ml X-gal, 5 mM $K_3Fe[CN]_6$, 5 mM $K_4Fe[CN]_6$, and 2 mM $MgCl_2$ in PBS, pH 6.0). At the end of the incubation, cells were washed with H_2O and examined at 200× magnification.

Sp1 reporter assay. H460 cells in 24-well plates were transfected with 0.3 μg/well of pGL2-0.3 plasmid (provided by Dr. J.Y. Lee, Hallym University, Korea) by using MegaFectin™ Opti-Kit (QBiogene, Irvine, CA, USA). At 36 h post-transfection, adriamycin was added to the medium, and the cells were further incubated before being lysed and applied to luciferase assay using a luciferase assay kit (Promega, Madison, WI, USA).

RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. A total of 5 μg RNA was converted to cDNA and 1/40 vol of the cDNA reaction was applied to PCR. PCR amplification was carried out using the following sets of primers: 5′-ACAGGTGAGCTTGACCTCAC-3′ and 5′-GTTGGTTTGCACCTGGTATG-3′ for Sp1; 5′-CCAAAACTGAA GCCGAGAAG-3′ and 5′-ATCGATGCGGTACAATGTGA-3′ for IGF-1 receptor; 5′-ACATTGACCCTGTCCCTGAGG-3′ and 5'-CGAGGGG TTCCTTCCACTTCT-3′ for PDGF-α receptor; 5′-GGCTCCCTCCTT CCGGCTG-3′ and 5′-TCCCGCAGCACGCCGAT-3′ for eNOS; 5′- GTCGCCCTGGACTTCGAGCAAGAG-3′ and 5′-CTAGAAGCAT TTGCGGTGGACG-3′ for β-actin.

Results

Decrease in Sp1 protein level during cellular senescence

First, we determined whether Sp1 protein level changes as normal fibroblasts continue population doublings (PD). Proteins were extracted from fibroblasts at different PD points starting from the middle of the lifespan (PD44) and ending at a point close to termination of cell proliferation, which is PD75 as determined by a lack of cell number increase during a 20-day period. As shown in Fig. 1A, cellular Sp1 protein level decreased progressively as cells continued proliferation. In the cells at PD64 and PD75, the Sp1 protein level normalized by that of ERK protein bands was reduced to 53% and 37%, respectively, of that in the cells at PD44 (Fig. 1B). A senescent state is also induced in MCF-7 by a 4-h pulse of 0.25 μM adriamycin and chase over 3 days as shown in Fig. 1C [21]. As previously demonstrated [21], in the cells pulsed with adriamy-

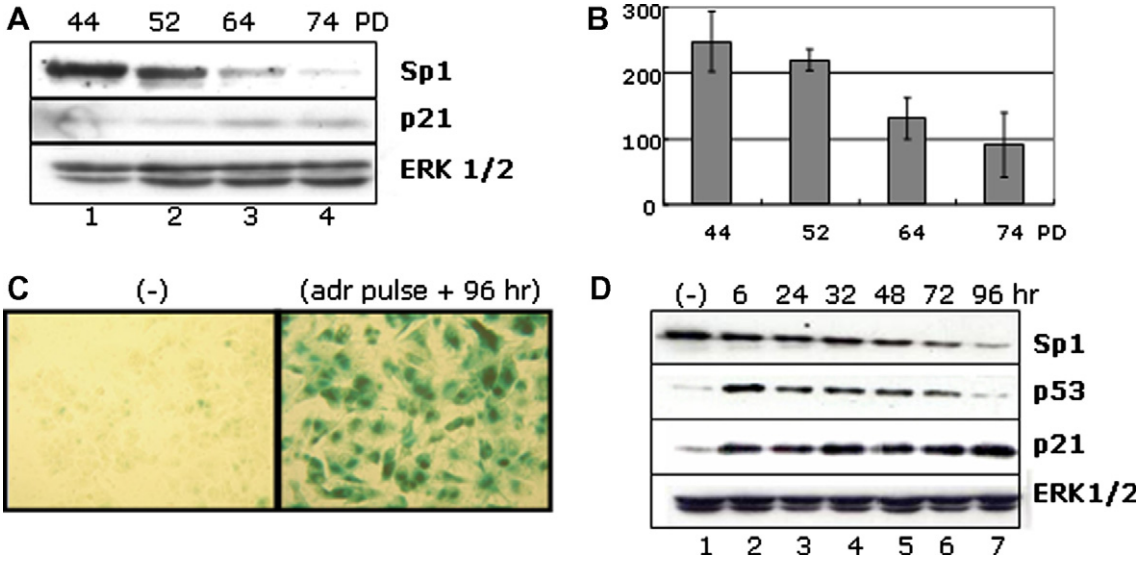

Fig. 1. Decrease in Sp1 protein level during cellular senescence. (A) Extract of cells collected at indicated PD points were applied to Western blot analysis for Sp1, p21WAF1, and ERK1 and 2. (B) Western blottings were carried out with cells cultured in two different culture dishes at each PD number. The intensity of protein band was quantified, the numbers were normalized against those of ERK protein bands, and the average was plotted on a bar graph. (C) Near 100% MCF-7 cells expressed senescence phenotype in 96 h after a 4 h pulse of 0.25 μM adriamycin (adr pulse + 96 h). (D) MCF-7 cells were collected at the indicated time points after the 4 h pulse of adriamycin. Cell extracts were applied to Western blotting for Sp1, p53, p21WAF1, and ERK1/2.

cin, p53 protein level increased immediately but slowly decreased thereafter, while p21WAF1 expression was induced along with an increase in p53 level and sustained highly. Sp1 protein level decreased gradually reaching close to 1/3 of the untreated level in 96 h. A similar but expedite decrease in Sp1 level was found in H460 cells undergoing senescence induced by the same adriamycin treatment (data not shown). These results together suggest that Sp1 level decreases as cells progress to a state of senescence either induced or replicative.

Interestingly, in both fibroblasts and MCF-7 cells, p21WAF1 expression level did not change in accordance with the level of Sp1 which is known to be essential for p21WAF1 transcription [22] (Fig. 1B and D).

Downregulation of Sp1 activity in cells undergoing cellular senescence

Whether Sp1 activity changes in accordance to the decrease of the Sp1 protein level in the cells undergoing cellular senescence was determined. First, MCF-7 cells were transiently transfected with a plasmid, pGL2-0.3, which contains luciferase cDNA downstream of a promoter that was derived from p21WAF1 gene but has only the proximal region containing six putative Sp1-binding sites (Fig. 2A, top). At 36 h post-transfection, the cells were divided into five dishes and treated with 0.25 μM adriamycin for 4 h, collected either at 0, 12, 24, 48, and 72 h post-adriamycin treatment, and applied to a luciferase activity assay. As shown in Fig. 2A, the reporter activity decreased as cells were chased reaching a 54% level at 72 h post-treatment. A similar downregulation in the promoter activity was observed in H460 cells treated in the same manner (data not shown). Therefore, the Sp1 transcriptional activity decreased as did its protein level in cells undergoing the induced senescence.

Next, it was determined whether the change in the Sp1 protein level has impact on the expression of the genes known to be dependent on Sp1 during the cellular progression toward replicative senescence. RNA from cells at PD 30, 46, 62, 66, and 70 were collected and applied to RT-PCR to examine the changes in mRNA levels of the genes encoding either IGF-1 receptor, PDGF-β receptor, or eNOS, all of which are known to be regulated by Sp1 [23–25, respectively]. As shown in Fig. 2B, while the level of β-actin mRNA was stably maintained, mRNA levels of all the Sp1-dependent genes showed a general pattern of decrease reaching approximately 20–40% of that in the cells at PD30 (Fig. 2B). These results suggest that the Sp1 activity is indeed downregulated in cells undergoing cellular senescence.

Decrease in Sp1 level in cells at non-senescent condition

The Sp1 downregulation may be induced as a part of senescence program or simply induced by DNA damages accumulated in cells undergoing either replicative senescence or senescence induced by the adriamycin. A continuous treatment (instead of the 4 h pulse) of 0.25 μM adriamycin causes apoptotic death instead of senescence in a large portion of MCF-7 cells with an apparent increase in BAX1, a DNA damage-dependent proapoptotic protein [21]. We reasoned that these different cellular responses could be used to answer the question raised above. When cells were continuously treated with adriamycin, Sp1 protein level decreased as well (Fig. 3A), and a comparison to that in Fig. 1D indicates a much rapid and severe decline in the continuously treated cells (a reduction was apparent in 12 h post-treatment). A rapid decrease in Sp1 activity in

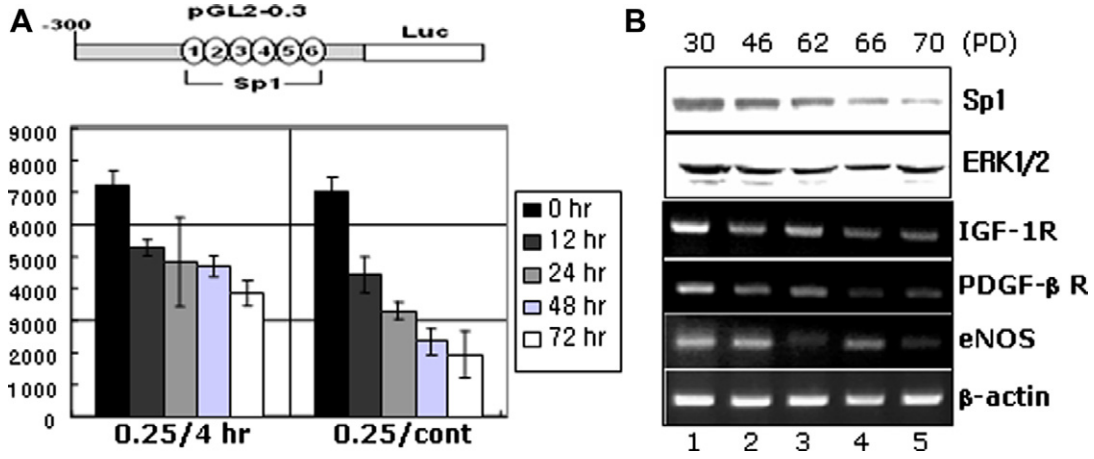

Fig. 2. Downregulation of Sp1 activity during cellular senescence. (A, top) Map of pGL2-0.3 plasmid showing 6-putative Sp1 binding motifs in the 300 bp proximal upstream of p21WAF1 gene. (A, bottom) MCF-7 cells were transfected with pGL2-0.3. After 36 h, cells were pulsed with 0.25 μM adriamycin for 4 h, splitted into five plates, and incubated either in its absence (left) or presence (right) for indicated period of time. The same amount of protein was applied to luciferase assay. (B) Total RNAs were extracted from fibroblasts at indicated PD points, and applied to RT-PCR with primers for IGF-1 receptor, PDGF-β receptor, eNOS, and β-actin. Protein levels of Sp1 and ERK1/2 at each PD were shown in parallel (upper two strips). RT-PCR was carried out twice with RNA prepared from two separately cultured cells, and a representative result is shown.

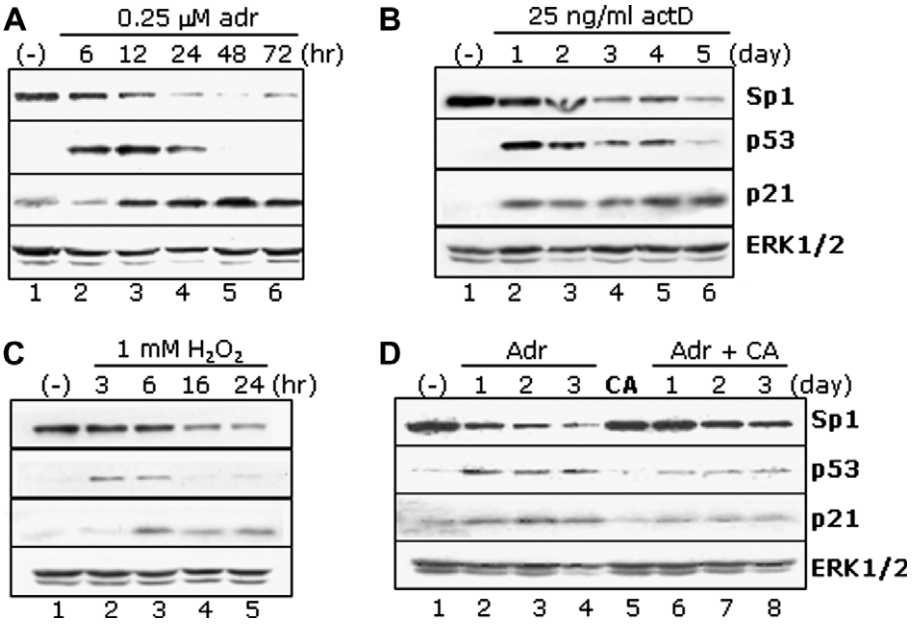

Fig. 3. Decrease in Sp1 protein level in cells induced to apoptosis by DNA damaging treatment. MCF-7 cells were either continuously treated with 0.25 μM adriamycin (A), pulsed for 4 h with 25 ng/ml actinomycin D (B), or pulsed for 1 h with 1 mM H_2O_2 (C). Extract of cells collected at the indicated time points were applied to Western analysis for Sp1, p53 , p21 , and ERK 1/2. (D) MCF-7 cells were pulsed with adriamycin and chased either in the presence (Adr + CA; lanes 6–8) or absence of 8 mM caffeine (Adr; lanes 2–4). Cells were collected at 1, 2, or 3 days of chase. In lane 5, cells were spiked with caffeine without the adriamycin pulse.

the continuously treated cells was also demonstrated by the luciferase-reporter assay in Fig. 2A (right). These results suggest that the decrease in Sp1 level is not due to the cellular state of senescence per se but caused by an event induced by the adriamycin treatment such as DNA damage. This proposal is supported by the finding that treatments of actinomycin D, another DNA damaging chemical [26], and H_2O_2, a chemical inducing oxidative stress including DNA damages [27], also caused a progressive decline in Sp1 level (Fig. 3B and C, respectively). Actinomycin D at the dose of 25 ng/ml and H_2O_2 at 1 mM did not cause a state of senescence in MDF-7 cells even after a prolonged incubation, but apparently induced DNA damage as judged by an activated p53-checkpoint response. Overall, these results indicate that the decrease in the Sp1 protein level during cellular senescence is likely caused by DNA damage and/or ROS stress.

Effect of ATM/ATR inhibition on Sp1 stability

Next, whether a cellular signaling from DNA damage indeed is involved in Sp1 downregulation was investigated. Cells were treated with adriamycin along with 8 mM caffeine, a potent inhibitor of ATM/ATR [28], and its effect on the Sp1 level was checked. In the cells treated with both adrimaycin and caffeine, the increase in the protein levels of p53 and p21WAF1 was minimal demonstrating that the ATM/ATR-mediated DNA damage signaling was effectively blocked by the treatment of caffeine in these cells (Fig. 3D, lanes 2–4 vs. 6–8). In these cells, the decrease in the Sp1 level was much less than in the cells treated with adriamycin alone. These results strongly suggest that DNA-damage signaling is quite likely involved in the downregulation of Sp1 protein.

Proteasomal degradation of Sp1

The decrease in the Sp1 level can be caused either by a reduction in Sp1 transcription or by an increase in degradation rate. The underlying reason for Sp1 downregulation was determined. First, total RNA was isolated from MCF-7 cells being chased after the adriamycin pulse, converted to cDNA and applied to RT-PCR. As shown by the pattern of the amplified DNA bands in Fig. 4A, there was no significant change in the level of Sp1 mRNA during the 72 h time course suggesting that Sp1 was not modulated at the mRNA level. Sp1 protein is known to undergo proteasome-dependent degradation [29]. To check the accelerated proteasomal degradation of Sp1, cells were treated with a proteasome inhibitor *N*-acetyl-L-leucinal-L-leucinal-L-norleucinal (ALLN) for 24 h prior to cell harvest. In the cells pulsed with adriamycin for 4 h, the decrease in Sp1 protein level was substantially reduced when they were chased in the presence of ALLN (Fig. 4B, lanes 2–5 vs. 6–9). An earlier increase in the levels of p53 and p21WAF1 indicates that the proteasome function was effectively blocked in the ALLN-treated cells based on previous reports that p53 and p21WAF1 are targets of proteasome activity [30,31]. An identical result was obtained with the cells continuously treated with adriamycin (Fig. 4C). As previously shown in Fig. 3A, continuous treatment of adriamycin caused an earlier decline in the Sp1 level (Fig. 4C, lanes

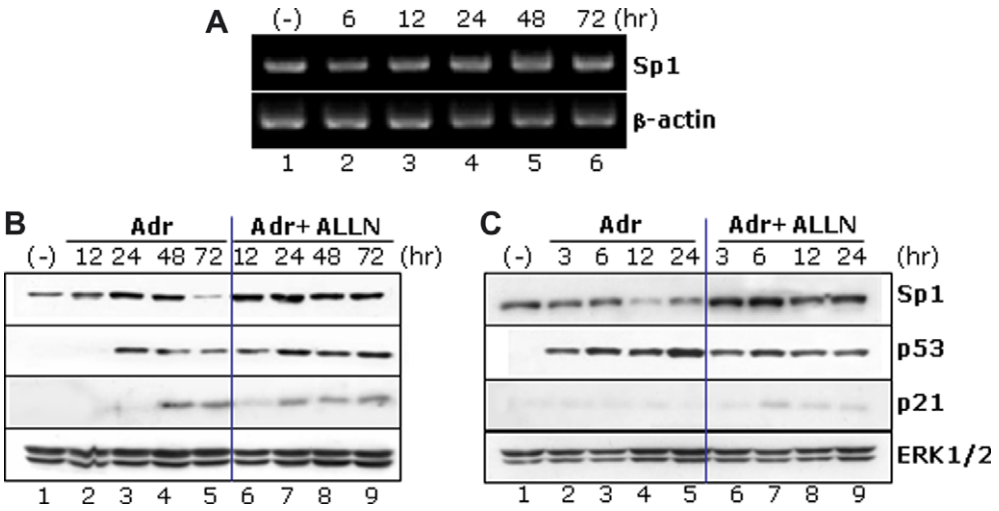

Fig. 4. Inhibition of Sp1 downregulation by the treatment of ALLN, a proteasome inhibitor, in MCF-7 cells undergoing induced senescence. (A) MCF-7 cells were pulsed by 0.25 μM adriamycin for 4 h, and collected at the indicated time points during the chase. Total RNAs were isolated and applied to RT-PCR using sets of primers for Sp1 and β-actin. (B) The adrimycin-pulsed MCF-7 cells were chased for the indicated time points. Twenty-five micromolar ALLN was added for 12 h before collection (Adr + ALLN; lanes 6–9). Cells were lysed and cell extracts were applied in Western blot analysis for Sp1, p53, p21WAF1, and ERK1/2. (C) MCF-7 cells were continuously treated with 0.25 μM adriamycin alone (Adr; lanes 2–5) or together with 25 μM ALLN (Adr + ALLN; lanes 6–10).

2–5), but, the presence of ALLN lead to maintenance of Sp1 at high levels (Fig. 4C, lanes 6–9). These results together indicate that the adriamycin-induced decrease of the Sp1 level is caused by an accelerated proteasomal degradation at least in part, and suggest that Sp1 downregulation during cellular senescence is due to an increase in the Sp1 protein degradation rate.

Discussion

Aging is apparently accompanied by progressive changes in the expression of certain subset of genes. Results from recent DNA array studies indicate that a small but a significant number of genes are either up- or downregulated in aged organs or cells of rat, rhesus monkey as well as human [8–10]. For example, in 6912 genes compared between fibroblasts from young and aged donors, 6.3% displayed significant differences [11]. These alterations in transcriptional activity indicate that activities of certain transcription factors change during aging and cellular senescence. Our results indicate that Sp1 is a plausible candidate as such, since both Sp1 activity and protein level decreased during senescence of normal fibroblast (replicative senescence) and MCF-7 and NIH-460 cells (induced senescence). This possibility is strongly supported by a number of recent reports. Sp1 DNA binding activity has been found to be reduced in aged T cells [32] and in the nuclear extracts of aged rats [17]. An age-related decrease in Sp1 binding activity on the 5′ regulatory element of human transferrin gene was reported as well [18]. Aging of both mice and rats induced a decrease in AP-1 and Sp-1 activities in liver [19]. Replicative senescence of human WI-38 fibroblasts induced a strong decrease in the DNA binding activity of Sp1 and certain others [19]. In addition, the insulin receptor gene, *INSR*, which has been reported to be regulated by Sp1 [20], showed reduced expression in the fibroblasts from old donor and Werner patients [11]. Our results provide a strong mechanistic support for the decrease in Sp1 activity during aging and cellular senescence. It would be of a great interest to check whether the downregulated genes in the above-mentioned DNA array studies are those that are regulated by Sp1. However, the finding in this study that p21WAF1, a representative gene known to be dependent on the activity of Sp1 [22], did not respond accordingly to the level of Sp1 (as well as to that of p53) (Figs. 1D and 3) suggest that not all the genes containing the Sp1-binding motif would decrease in accordance to the Sp1 level.

Our results also provide a plausible explanation on how Sp1 is downregulated during cellular aging. The decrease in Sp1 level not only in cells undergoing senescence but also in those under DNA damaging stresses not accompanying senescence strongly indicates that DNA damage accumulating during replicative senescence could be a causative event. The facts that Sp1 downregulation was more severe in cells under a harsher stress (Fig. 3A–C) and that an inhibition of the ATM/ATR pathway caused Sp1 to be maintained at high levels strongly support this possibility. Meanwhile, a previous report showed that the DNA-binding efficiency of Sp1 is substantially reduced by high ROS stress [33]. These findings together suggest a possibility that Sp1 may play a role as a sensor for cellular stress. Sp1 already has been proposed a sensor for nutrient status due to its susceptibility to modulation by *O*-GlcNAcylation through the influx of glucose [34] (see below). *O*-GlcNAcylation has been suggested a main mechanism that determines the stability of Sp1 [29]. Reduced GlcNAcylation of Sp1 is associated with an increased susceptibility to

proteasome-dependent degradation, while hyper-GlcNAcylation attenuates the Sp1-specific proteasome activity [29]. Therefore, it is likely that, during cellular progression toward senescence, Sp1 stability may be regulated by *O*-GlcNAcylation. Further study is required to clarify the possibility of the altered post-transcriptional modification of Sp1 during cellular proliferative lifespan.

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