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p21$^{Waf1/Cip1}$ Plays a Critical Role in Modulating Senescence Through Changes of DNA Methylation

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Abstract It has been reported that genomic DNA methylation decreases gradually during cell culture and an organism's aging. However, less is known about the methylation changes of age-related specific genes in aging. p21$^{Waf1/Cip1}$ and p16^{INK4a} are cyclin-dependent kinase (Cdk) inhibitors that are critical for the replicative senescence of normal cells. In this study, we show that p21$^{Waf1/Cip1}$ and p16^{INK4a} have different methylation patterns during the aging process of normal human 2BS and WI-38 fibroblasts. p21$^{Waf1/Cip1}$ promoter is gradually methylated up into middle-aged fibroblasts but not with senescent fibroblasts, whereas p16^{INK4a} is always unmethylated in the aging process. Correspondently, the protein levels of DNA methyltransferase 1 (DNMT1) and DNMT3a increase from young to middle-aged fibroblasts but decrease in the senescent fibroblasts, while DNMT3b decreases stably from young to senescent fibroblasts. p21$^{Waf1/Cip1}$ promoter methylation directly represses its expression and blocks the radiation-induced DNA damage-signaling pathway by p53 in middle-aged fibroblasts. More importantly, demethylation by 5-aza-CdR or DNMT1 RNA interference (RNAi) resulted in an increased p21$^{Waf1/Cip1}$ level and premature senescence of middle-aged fibroblasts demonstrated by cell growth arrest and high β-Galactosidase expression. Our results suggest that p21$^{Waf1/Cip1}$ but not p16^{INK4a} is involved in the DNA methylation mediated aging process. p21$^{Waf1/Cip1}$ promoter methylation may be a critical biological barrier to postpone the aging process. J. Cell. Biochem. 98: 1230–1248, 2006.

Key words: DNA methylation; p21$^{Waf1/Cip1}$; p16^{INK4a}; senescence; DNA methyltransferas

DNA methylation patterns change with aging in a complex fashion [Wilson and Jones, 1983; Mays-Hoopes, 1989; Richardson, 2003]. In general, total genomic deoxymethylcytosine (dmC) has been found to decrease during aging in various organisms and cultured normal cells [Liu et al., 2003]; nevertheless, CpG island-methylation progressively increases at multiple gene loci [Issa et al., 1994, 1996; Bornman et al., 2001]. Age-dependent CpG island hypermethylation typically results in suppression of the associated genes, and may contribute to carcinogenesis by inhibiting the expression of genes with various growth-suppressing functions [Issa, 1999]. On the other hand, the gradual loss of genomic dmC content leads to cellular senescence and has been proposed as a counting mechanism of cell aging [Wilson and Jones, 1983; Neumeister et al., 2002]. However, it is not known whether the aging-related loss of methylation involves specific regions of the genome or it is a stochastic process.

It is now well accepted that two inhibitors of cyclin-dependent kinases (Cdks), p21$^{Waf1/Cip1}$,

Abbreviations Used: Cdk, cyclin-dependent kinase; PD, population doubling; FBS, fetal bovine serum; PBS, phosphate-buffered saline; pRb, retinoblastoma protein; MSP, methylation-specific polymerase chain reaction; DNMT, DNA (cytosine5)-methyltransferase; 5-aza-CdR, 5-aza-2′-deoxycytidine; MTT, 3-[4,5-Dimethylthiazolzyl]-2,5-Diphenyl Tetrazolium Bromide; DMSO, dimethyl sulfoxide; X-gal, 5-Bromo-4-chloro-3-indolyl-β-D-galactoside; SA-β-Gal, senescence-associated-β-galactosidase; siRNA, small interfering RNA; RNAi, RNA interference; nt, nucleotide.

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and p16^{INK4a}, whose amounts increase with age, have an essential role in inactivating Cdks in senescent fibroblasts [Noda et al., 1994; Brown et al., 1997; Duan et al., 2001; Wang et al., 2001]. p21$^{Waf1/Cip1}$ binds to and inactivates most cyclin–Cdk complexes, whereas p16^{INK4a} blocks cyclin D-Cdk activation by binding specifically to Cdk4 and Cdk6, thus prevents their association with cyclin D [Sherr and Roberts, 1999]. Cdk inactivation, in turn, allows the accumulation of unphosphorylated retinoblastoma protein (pRb), which exerts a negative regulation of cell-cycle progression by forming complexes with members of the E2F transcription factor family [Grana et al., 1998]. This event finally inhibits the expression of late G1 genes whose products are required for S-phase initiation and progression, thus resulting in cell-cycle G_1/S growth arrest [Weinberg, 1995]. Senescent cells exhibit phenotypic changes including altered morphology, increased cell volume, and expression of a neural senescence-associated β-galactosidase (SA-β-Gal) activity [Dimri et al., 1995].

Aberrant methylation of p16^{INK4a} promoter has been intensively studied in various cancer cells and tumors [Baylin and Herman, 2000; Esteller, 2002]. Although, p21$^{Waf1/Cip1}$ methylation is not frequent in tumors compared to p16^{INK4a}, it occurs in acute lymphoblastic leukemia and rhabdomyosarcoma [Chen et al., 2000; Roman-Gomez et al., 2002]. Hypermethylation of p21$^{Waf1/Cip1}$ promoter around the consensus Sp1-binding sites may directly reduce Sp1/Sp3 binding, therefore lead to a reduced p21$^{Waf1/Cip1}$ expression in response to histone deacetylase inhibitors [Zhu et al., 2003]. In addition, p21$^{Waf1/Cip1}$ methylation is also considered as a critical mechanism to abrogate p53-dependent signal transduction caused by DNA damage [Allan et al., 2000].

It has been demonstrated that inhibition of DNA methyltransferase induces the expression of p21$^{Waf1/Cip1}$and an irreversible growth arrest in normal human fibroblasts [Young and Smith, 2001], while in contrast, normal human fibroblasts lacking a functional p21$^{Waf1/Cip1}$ fail to undergo cell-cycle arrest following DNA methyltransferase inhibition, indicating that p21$^{Waf1/Cip1}$ is an essential component of this arrest. Milutinovic et al. [2000] reported that the induction of p21$^{Waf1/Cip1}$ expression points to a mechanism that is not associated with p21$^{Waf1/Cip1}$ promoter demethylation in lung carcinoma cell line A549 cells. However, recent studies suggest that the silencing of p21$^{Waf1/Cip1}$ involves DNA methylation through recruitment of DNA methyltransferase to its promoter [Brenner et al., 2005]. Therefore, it remains to be determined whether DNA methyltransferase participation in the determination of cellular life span is mediated by its methyltransferase activity and/or its capacity for methylation-independent transcriptional regulation.

Here, we tested the promoter methylation patterns of p21$^{Waf1/Cip1}$ and p16^{INK4a} in the life span of normal human fibroblasts. We found that p16^{INK4a} has no promoter methylation in the process of cell aging, whereas p21$^{Waf1/Cip1}$ promoter is gradually methylated up into middle-aged fibroblasts but loses methylation in the senescent fibroblasts, which may be associated with the expression changes of DNMT1 and DNMT3a. p21$^{Waf1/Cip1}$ promoter methylation represses its expression and its role in cell-cycle control. Demethylation by DNA methyltransferase inhibitors or RNA-interference enhanced p21$^{Waf1/Cip1}$ levels, which in turn contributes to the onset of premature senescence.

MATERIALS AND METHODS

Cell Culture and DNA Extraction

Human embryonic lung diploid fibroblast 2BS cells (obtained from the National Institute of Biological Products, Beijing, China) were previously isolated from female fetal lung fibroblast tissue and have been fully characterized [Li et al., 1995; Duan et al., 2001; Wang et al., 2001; Huang et al., 2004; Zheng et al., 2004]. The current expected life span is approximately 70 population doublings (PD). 2BS cells are considered to be young at PD30 or below, middle-aged at PD30-50, and fully senescent at PD55 or above. WI-38 human fetal lung fibroblasts were obtained from American-type culture collection (ATCC). Cells at PD less than 30 were considered young, cells at PD 30–50 were considered middle-aged, and cells at PD 50 or above were considered senescent [Sitte et al., 2000]. Cells were maintained in Dulbacco's modified Eagle's medium (GIBCO.BRL), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. Cell cultures were expanded through sequential subculturing using trypsin-EDTA (GIBCO) to achieve a higher PD level. Cells grown to about 80%–90% confluence were used for all the

experiments. The human lung cancer cell line A549 in which $p21^{Waf1/Cip1}$ promoter is not methylated was obtained from ATCC.

For genome DNA extraction, cells were washed with phosphate-buffered saline (PBS) and detached with 0.25% trypsin; then cells were resuspended in extraction buffer (10 mmol/L Tris-HCl pH 8.0, 0.1 mol/L EDTA pH 8.0, 0.5%SDS, 2 μg/ml Ribonuclease A) and incubated at 37°C for 1 h. Proteinase K was added at the final concentration of 100 μg/ml and incubated at 55°C for 3 h. Genome DNA was extracted with phenol/chloroform three times and the DNA concentration was measured.

Methylation-Specific PCR (MSP)

DNA from young, middle-aged, and senescent 2BS, and WI-38 cells were treated with bisulfite as previously described [Zhu et al., 2003]. Briefly, genomic DNA (1 μg) in a volume of 50 μl was denatured by NaOH (final concentration, 0.275 M) for 10 min at 42°C. The denatured DNA was then treated with 10 μl of 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite at 50°C overnight. The bisulfite-modified DNA was purified with a Qiaquick gel extraction kit (Qiagen) according to the manufacturer's instructions and then precipitated with sodium acetate (final concentration, 0.45 M) and isopropanol. DNA was eluted with distilled H_2O and used for PCR. The primers for methylation specific polymerase chain reaction (MSP) of $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ promoter were designed as follows: methylation-specific primers for $p21^{Waf1/Cip1}$, forward primer, 5′-ttgtagtacgcgaggtttcg-3′ and reverse primer, 5′-caactcaacgcgaccctaat-3′; for $p16^{INK4a}$, forward primer, 5′-ttattagagggtggggcggatcgc-3′ and reverse primer, 5′-gaccccgaaccgcgaccgtaa-3′, unmethylation-specific primers: for $p21^{Waf1/Cip1}$, forward primer, 5′-ttttgggattggttggtttg-3′ and reverse primer, 5′-acacccaactccaactccac-3′; for $p16^{INK4a}$, forward primer, 5′-ttattagagggtggggtggattgt-3′ and reverse primer, 5′-caaccccaaaccacaaccataa-3′.

Bisulfite Sequencing

DNA was treated with bisulfite as described above and purified for PCR. The primers for sequencing of $p21^{Waf1/Cip1}$ promoter were designed as follows: forward primer, 5′-gggaggagggaagtgtttt-3′ and reverse primer, 5′-acaactactcacacctcaact-3′. The PCR products were gel extracted (Qiagen) and ligated into a pGEM-T® Easy vector by using the TA cloning system (Promega). At least 10 separate clones were chosen for sequencing analysis.

Extraction of Nuclear Proteins

Nuclear extracts for Western blotting were prepared using the NE-PER Nuclear and cytoplasm Extraction Regents kit (Pierce) according to the manufacture's protocol. A total of 80 μg of each extract was resolved by 7% SDS-polyacrylamide electrophoresis.

Western Blotting

Cells were lysed in lysis buffer (50 mM Tris-HCL, pH 7.6, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.15% IgepalCA-630, 1% Protease Inhibitor Cocktail [Sigma]). The protein concentration of each sample was determined by Bio-Rad protein assay regent and equal amounts of protein (100–150 μg) were size fractionated by electrophoresis on 9%–15% SDS–polyacrylamide gel. Proteins were then transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Nonspecific protein binding to the membrane was blocked with blocking buffer (5% nonfat milk, 200 mM NaCl, 50 mM Tris-HCL pH 7.5, 0.05% Tween 20). The blocked membrane was then incubated with primary antibodies at 4°C overnight with rocking. After the membrane was washed six times with TBS-T (20 mM Tris-HCL, pH 7.5, 500 mM NaCl, 0.1% Tween 20) for 5 min each time, the membrane was incubated with secondary antibody conjugated to horseradish peroxidase at 4°C for 1 h. Proteins were visualized using a chemiluminescence kit (Pierce) according to the manufacture's protocol. The antibodies against DNMT1 (sc-10219), DNMT3a (sc-10231), DNMT3b (sc-10235), $p21^{Waf1/Cip1}$ (sc-6264), p53 (sc-126), β-actin (sc-1616) were purchased from Santa Cruz Biotechnology and used at 1 μg /ml.

In Vitro Methylation and Luciferase Activity Analysis

Wild-type $p21^{Waf1/Cip1}$ promoter-luciferase fusion plasmid pWWP-Luc in this study has been described previously [Zhu et al., 2003]. pWWP-Luc (20 μg) was treated with 20 units of *Sss*I methylase (New England Biolabs) for 20 h with 160μM S-adenosylmethionine (New England Biolabs). Methylation status was verified by digestion with *Hpa*II and *Msp*I. Methylated and unmethylated $p21^{Waf1/Cip1}$ promoter

plasmids were transiently transfected into A549 cells and assayed for luciferase activity. Methylated and mock-methylated pGL3-Basic plasmids without a promoter were served as background control.

RT-PCR

Cells were washed twice with PBS and harvested. RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacture's protocol. Reverse transcription reactions were performed using ThermoScript^{TM} RT-PCR System (Invitrogen) and using an oligo(dT)$_{20}$ primer. PCR amplification to detect first strand cDNA for p21$^{Waf1/Cip1}$ mRNA used primers: forward primer, 5′-gaggaagaccatgtgg-ac-3′ and reverse primer, 5′-cagcactcttaggaa-cctc-3′. Glyceraldehyde-3-phosphate dehydrogenase was amplified as loading control and used primers: forward, 5′-cgagtcaacggatttggtg-gtat-3′ and reverse, 5′-agccttctccatggtgaagac-3′.

γ-Irradiation

Fresh medium was added to cells 1 h prior to irradiation. Young (PD 28), middle-aged (PD 42), and senescent (PD 58) 2BS cells were then gamma irradiated at 1.02 Gy/min. The irradiated cells were then incubated at 37°C in 5% CO_2 until harvest. Experiment was performed at least two times.

Cell-Cycle Analysis

Young (PD 28), middle-aged (PD 42), and senescent (PD 58) 2BS cells were treated with γ-irradiation as described above; 24 h later, cells were trypsinized and washed with cold PBS once, then fixed with 70% ethanol overnight. After being treated with 100 μg/ml of RNase A (Sigma), Propidium iodide (10 μg/ml; Sigma) was added to the cells for staining. The DNA contents were measured by fluorescence-activated cell sorting on a FACScan flow cytometry system (Becton-Dickinson, San Jose, CA). Cell-cycle distributions were analyzed using Cell Fit software.

5-aza-CdR Treatment

Middle-aged (PD 39) 2BS cells were plated at a density of 10^4cells/cm^2 and 24 h later treated with 1 μM 5-aza-2′-deoxycytidine (5-aza-CdR) (Sigma). The medium was changed every 24 h with medium containing fresh 5-aza-2′-deoxycytidine by the time the control cells achieved three PDs.

Growth Curve Analysis

Middle-aged, 5-aza-CdR treated middle-aged, and senescent 2BS cells were detached and seeded into 96-well plates, 2,000 cells per well. At the indicated times, cells were stained with MTT (10 μg/ml in PBS; Sigma) for 4 h; then the cells were dissolved with 200 μl DMSO. Their optical density at 570 nm was determined. Each point was determined in triplicate.

Senescence-Associated-β-Galactosidase (SA-β-Gal) Staining

Middle-aged (PD 42), 5-aza-2′-deoxycytidine treated middle-aged, and senescent 2BS cells were washed twice with PBS, fixed to plates using 3% formaldehyde for 3–5 min and washed with PBS again. Then the cells were incubated overnight at 37°C without CO_2 in a freshly prepared staining buffer (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$).

Small Interfering RNA (siRNA) Preparation and Transfection

Small interfering RNAs (siRNAs) corresponding to DNMT1 were designed according to the pSilencer neo instruction manual (Ambion). In brief, the 21 nt potential sequences in the target mRNAs that begin with an AA dinucleotide were found and compared to the human genome database using BLAST (www.ncbi.nlm.nih.gov/BLAST), any target sequences with more than 16–17 contiguous base pairs of homology to other coding sequences were eliminated from consideration. The hairpin siRNA template oligonucleotides were designed by a web-based insert design tool (www.ambion.com/techlib/misc/psilencer converter.html) and chemically synthesized with 5′phosphate, 3′hydroxyl, and two bases overhangs on each strand. The following gene-specific sequence for DNMT1 was used successfully: 5′-AATCTGTCCGTTCA-CATGTGT-3′, then the siRNAs were inserted into the BamH I and Hind III sites of pSilencer 2.1-U6 neo vector (Ambion), and referred to as pSilencer-DNMT1 (siDNMT1). They were transfected by Lipofectamine 2000 reagent (Life Technologies, Inc.) into 4×10^5 middle-aged 2BS cells (PD 39) in 80%–90% confluence. pSilencer NC vector (negative control of pSilencer 2.1-U6 neo vector that expresses a hairpin siRNA with limited homology to any known

sequence in the human, mouse, and rat genomes) was also transfected as a control. To improve the tranfection efficiency, 2BS cells were selected with G418 (Life Technologies, Inc.) for 6 days before harvest.

RESULTS

$p21^{Waf1/Cip1}$, but not $p16^{INK4a}$, is Aberrantly Methylated in 2BS and WI-38 Cells, Which may be Associated With Expression Changes of DNMT1 and DNMT3a

$p21^{Waf1/Cip1}$ and $p16^{INK4a}$ are essential mediators of cellular senescence by inactivation of the G1 cyclin–Cdk complexes responsible for phosphorylation of the pRb [Sherr and Roberts, 1995; Alcorta et al., 1996; McConnell et al., 1998; Stein and Dulic, 1998]. Promoter methlylation of $p16^{INK4a}$ but rarely $p21^{Waf1/Cip1}$ frequently occurs in cancer cells [Rocco and Sidransky, 2001; Esteller, 2002; Zhu et al., 2003]. To investigate the methylation status of $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ in the aging process of human fibroblasts cultured in vitro, we chose a fragment within the CpG island of the $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ promoter which was confirmed by computer program (http://www.itba. mi.cnr.it/webgene/) (Fig. 1A). First, MSP was used to determine the methylation status of $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ promoter in the young (PD 28), middle-aged (PD 42), and senescent (PD 58) 2BS cells, respectively. In this assay, bisulfite modification results in a change from C to T when the C is unmethylated in the context of a CG dinucleotide. However, when the cytosine is methylated, bisulfite modification leaves the methylated cytosine intact. Therefore, after bisulfite modifications, methylation specific and unmethylation-specific primers were designed and promoter methylation status was detected

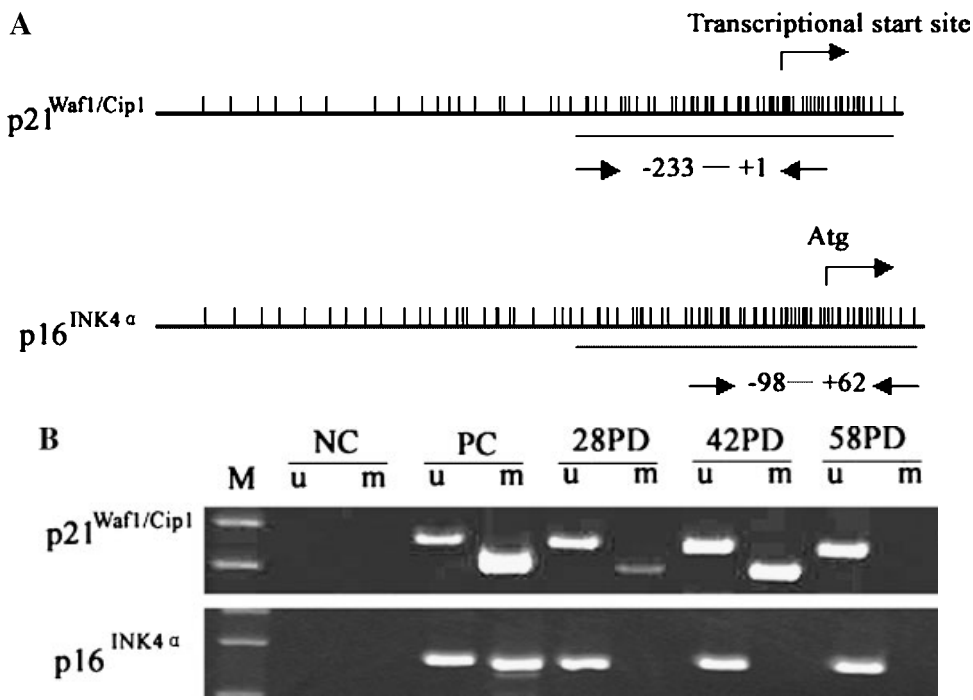

Fig. 1. $p21^{Waf1/Cip1}$ promoter but not $p16^{INK4a}$ is gradually methylated from young to middle-aged normal human fibroblasts. **A**: Physical maps of the $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ promoter showing the CpG island (straight line) analyzed in this study. The primers used to amplify the bisulfite DNA are shown as arrows at the bottom of their promoter. **B**: MSP analysis of $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ promoter methylation status in the young (PD 28), middle-aged (PD 42), and senescent (PD 58) 2BS cells. **C**: MSP analysis of $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ promoter methylation status in the young (PD 22), middle-aged (PD 38), and senescent (PD 50) WI-38 cells. M, marker; NC, negative control; PC, positive control; u, unmethylation-specific PCR products; m, methylation-specific PCR products. **D**: Bisulfite sequencing of CpG island in the $p21^{Waf1/Cip1}$ promoter (NCBI no. U24170). This sequence spans 234 bp between positions −233 to +1 relative to the transcription start site, including 24 CGs upstream of the transcriptional start site, DNA from different-aged 2BS and WI-38 cells was treated with bisulfite, and the $p21^{Waf1/Cip1}$ promoter was PCR amplified. The PCR product was ligated into pGEM-T® Easy vector by using the TA cloning system. At least 10 separate clones were chosen for sequencing analysis. Symbols: ○, unmethylated cytosine, ●, methylated cytosine.

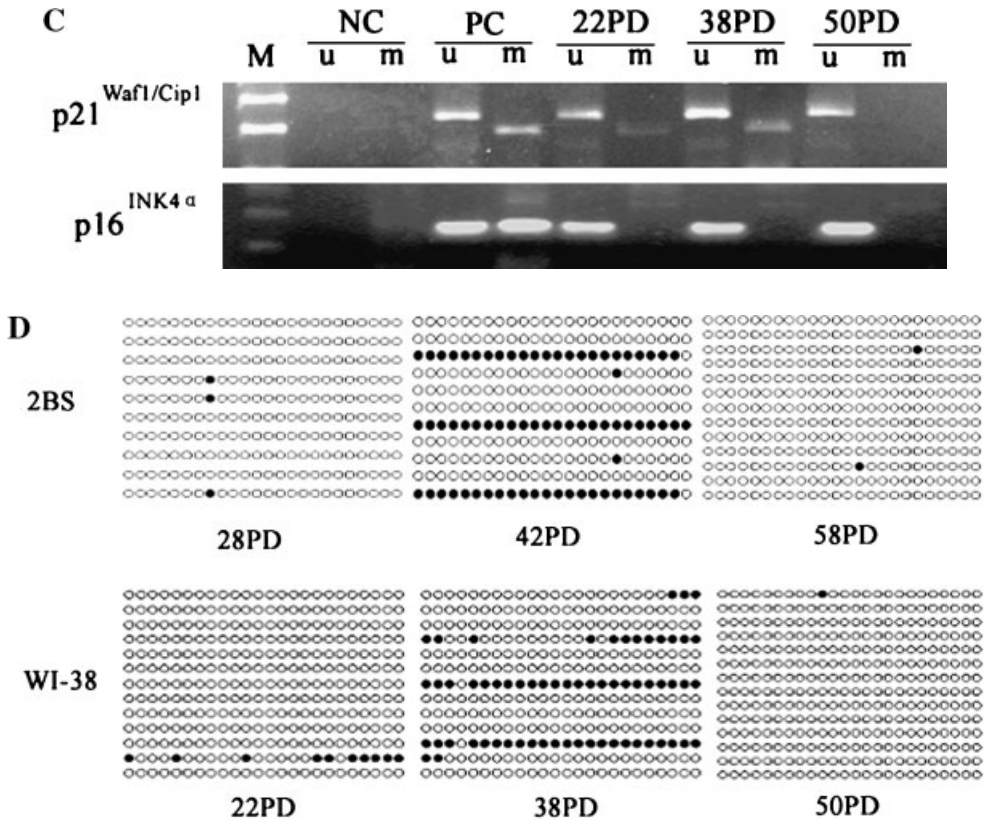

Fig. 1. (*Continued*)

by PCR. As shown in Figure 1B, there was a low level of p21$^{Waf1/Cip1}$ promoter methylation in the young 2BS cells illustrated by a weak band of methylation-specific PCR products. It was increased in the middle-aged 2BS cells demonstrated by a clear band of methylation-specific PCR products. However, p21$^{Waf1/Cip1}$ promoter was demethylated when 2BS cells became senescent because no methylation-specific PCR products appeared in the senescent 2BS cells. In contrast to the methylation changes of p21$^{Waf1/Cip1}$, p16^{INK4a} promoter was always unmethylated in the young, middle-aged, and senescent 2BS cells (Fig. 1B).

To further identify the methylation patterns of p21$^{Waf1/Cip1}$ promoter in different aged 2BS cells, primers for bisufite sequencing were designed and PCR products were subcloned into the pGEM-T® Easy vector (Promega), and at least 10 separate subclones were sequenced. Table I and Figure 1D showed the methylation status for all CGs in the tested CpG island (24 CGs) between the −233 to +1 positions relative to the transcriptional start site of p21$^{Waf1/Cip1}$ promoter in the young, middle-aged, and senescent 2BS cells. Overall, 1.25% of CGs were methylated in this fragment (10 clones, 3 of 240 of CGs were methylated) of young 2BS cells. However, there was an elevated p21$^{Waf1/Cip1}$ promoter methylatin in the middle-aged 2BS cells, in which about 27.27% of CpGs were methylated in this fragment (11 clones, 72 of 264 CpGs were methylated), whereas only 0.64% of CpGs were methylated in this fragment (13 clones, 2 of 312 CpGs were methylated) of senescent 2BS cells. In addition, sequencing analysis revealed that methyled CGs were not

TABLE I. Bisulfite Sequencing Analysis of the CpG Island in the p21$^{Waf1/Cip1}$ Promoter of Different-Aged 2BS and WI-38 Cells

Cell	PD	Clones	Total CpGs	mCpGs	(mCpGs/total CpGs)%
2BS	28	10	240	3	1.25
	42	11	264	72	27.27
	58	13	312	2	0.64
WI-38	22	13	312	10	3.21
	38	13	312	63	20.19
	50	14	336	1	0.29

uniformly distributed among the clones of middle-aged 2BS cells. However, some clones were densely methylated, while other clones were rarely or no methylated.

To test whether these wavy changes of $p21^{Waf1/Cip1}$ promoter methylation are cell specific, we examined $p21^{Waf1/Cip1}$ promoter methylation patterns in the young (PD 22), middle-aged (PD 38), and senescent (PD 50) WI-38 cells. Both MSP (Fig. 1C) and bisulfite sequencing (Table I and Fig. 1D) results of $p21^{Waf1/Cip1}$ promoter are consistent with that of 2BS cells, and $p16^{INK4a}$ promoter is totally unmethylated in the aging process of WI-38 cells (Fig. 1C). Furthermore, the heterogeneity of $p21^{Waf1/Cip1}$ promoter methylation was also observed in the young and middle-aged WI-38 cells (Fig. 1D).

To elucidate the basis for the aberrant DNA methylation changes in aging, nuclear protein was extracted and the expression of DNMT1, DNMT3a and DNMT3b was analyzed in the young, middle-aged, and senescent 2BS cells, respectively. Changes in these DNMTs were observed in Figure 2, with the protein levels of DNMT1 and DNMT3a increased from young to middle-aged 2BS cells, but decreased in the senescent cells, whereas DNMT3b decreased from young to senescent cells. These results indicate that $p21^{Waf1/Cip1}$ promoter methylation status may be associated with the expression changes of DNMT1 and/or DNMT3a.

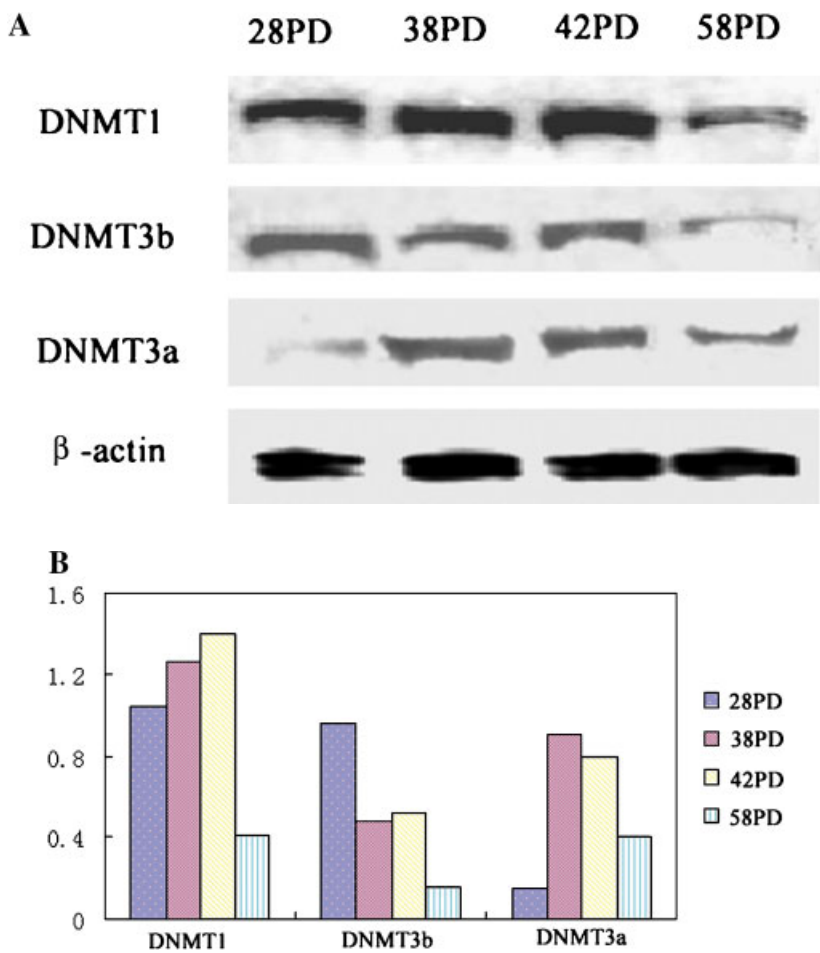

Fig. 2. Protein levels of DNMT1 and DNMT3a increase from young to middle-aged fibroblasts but decrease in senescent fibroblasts. **A**: Western-blot analysis of DNMT1, DNMT3b, and DNMT3a expression in different-aged 2BS cells. Nuclear proteins were extracted from young (PD 28), middle-aged (PD 38 and PD 42), and senescent (PD 58) 2BS cells, Western-blot was performed using specific antibodies against DNMT1,DNMT3b, and DNMT3a as indicated, β-actin lane serves as loading control. **B**: Ratios of DNMT1, DNMT3b, and DNMT3a message to β-actin message. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

p21$^{Waf1/Cip1}$ Promoter Methylation Represses p21$^{Waf1/Cip1}$ Expression and its Function

To test the effect of p21$^{Waf1/Cip1}$ promoter methylation on its expression, we chose the p21$^{Waf1/Cip1}$ promoter-luciferase fusion plasmid pWWP-Luc, and modified in vitro with *Sss*I methylase, an enzyme that methylates every CpG dinucleotide. To ensure that the plasmids were methylated, they were digested with either *Hpa*II or *Msp*I. Methylation prevented digestion of the pGL3 basic and pWWP-Luc by *Hpa*II (Fig. 3A, lane 4 and 9), whereas unmethylated plasmids were digested by both *Hpa*II and *Msp*I (Fig. 3A, lane2, 3, 7, and 8). The results presented in Figure 3B showed that after transfection, the expression of the methylated pWWP-Luc was reduced about 30-fold compared to its unmethylated counterpart, while the methylated pGL3 control vector was only 1.5-fold lower than its unmethylated counterpart (Fig. 3B). These results demonstrate that p21$^{Waf1/Cip1}$ promoter methylation exerts a strong negative effect on its expression.

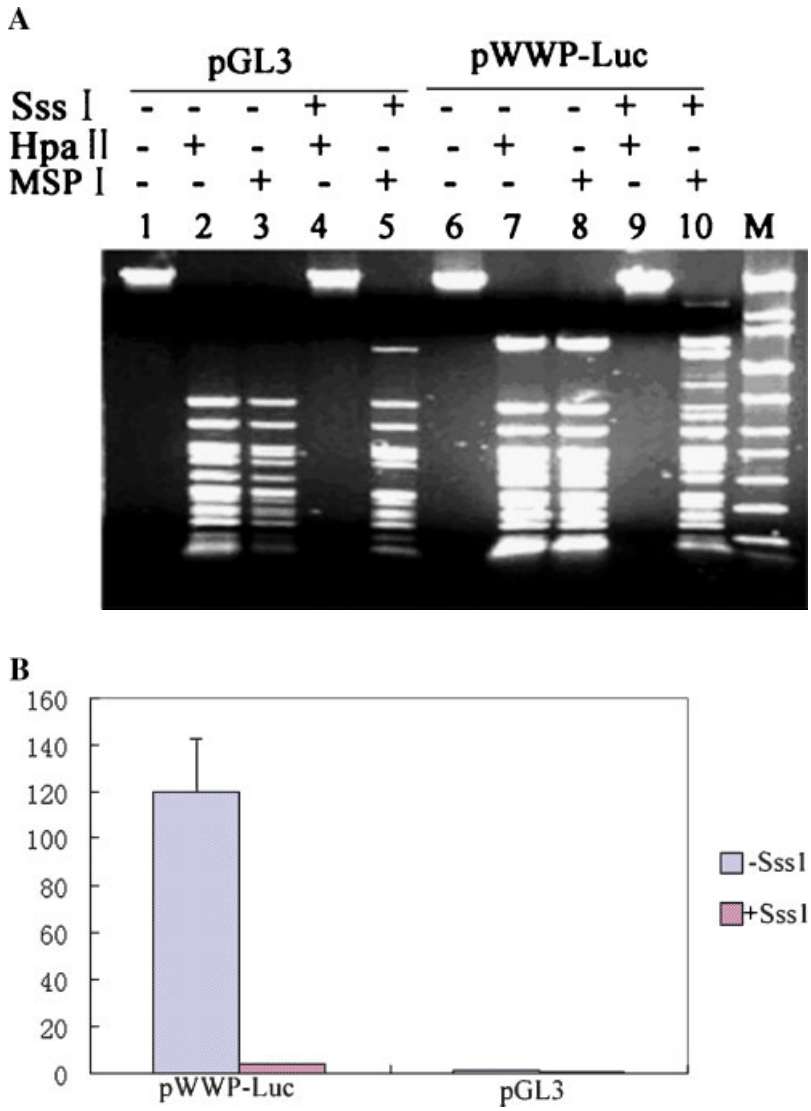

Fig. 3. p21$^{Waf1/Cip1}$ promoter methylation directly represses its expression. **A:** pGL3 control and p21$^{Waf1/Cip1}$ promoter-luciferase fusion plasmid pWWP-Luc were methylated in vitro with *Sss*I methylase, the extent of methylation was assessed by comparing digestion patterns of unmethylated (lanes 2, 3 and 7, 8) and methylated (lanes 4, 5 and 9, 10) pGL3 and pWWP-Luc with *Hpa*II and *Msp*I. **B**: Unmethylated and methylated pGL3 control and pWWP-Luc plasmids were transfected into A549 cells and assayed for promoter activity. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

To obtain evidence that this modification affects expression in vivo, RT-PCR and Western blot were performed to analyze the changes of $p21^{Waf1/Cip1}$ expression in the aging process of 2BS cells. The results from Figure 4A showed that expression of $p21^{Waf1/Cip1}$ decreased in the middle-aged (38PD, 42PD, 46PD) 2BS cells at the transcriptional level except that the mRNA of $p21^{Waf1/Cip1}$ first changed slightly from 28PD to 33PD, and it decreased again from 55PD to 60PD. Furthermore, the changes of $p21^{Waf1/Cip1}$ expression were also confirmed at the protein level (Fig. 4B,C).

It is well established that DNA damage upregulates $p21^{Waf1/Cip1}$ expression by the p53 tumor suppressor gene, and through its inhibition of cyclin-dependent kinases (Cdks), $p21^{Waf1/Cip1}$ blocks entry into S and S phase progression [Xiong et al., 1993; Luo et al., 1995]. Next, whether $p21^{Waf1/Cip1}$ promoter methylation affects this function of cell-cycle control was detected in the middle-aged 2BS cells. Young (PD 28), middle-aged (PD 42), and senescent (PD 58) 2BS cells were treated with γ-irradiation, respectively, and flow cytometry was used for measuring cell-cycle changes. After γ-irradiation, the percentage of cells in the S phase decreased sharply in the young (23.82%/1.12%, 21.2-fold) and senescent (5.51%/1.23%, 4.5-fold) 2BS cells (Fig. 5A), while in the middle-aged 2BS cells, the percentage of cells in the S phase decreased slightly (8.70%/5.21%, 1.6 fold) compared to young and senescent 2BS cells (Fig. 5A). This difference of cell-cycle

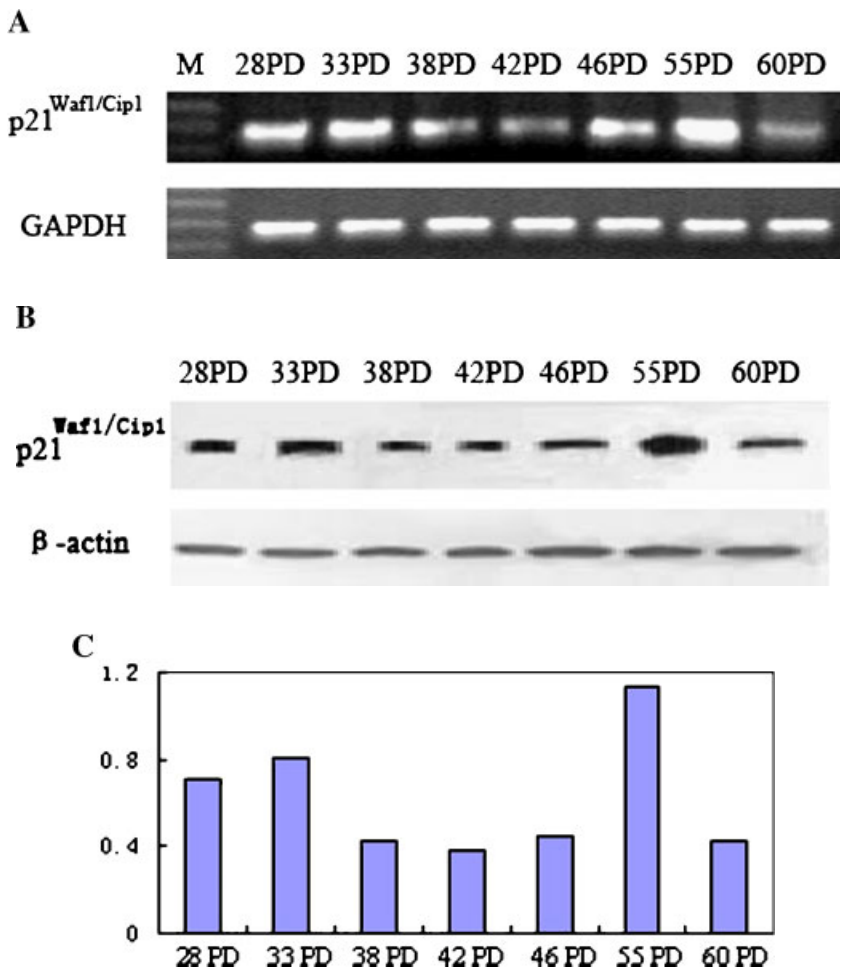

Fig. 4. $p21^{Waf1/Cip1}$ expression decreased in the middle-aged 2BS cells at both mRNA and protein level. **A**: mRNA was extracted from the young (PD 28), middle-aged (PD 33, PD 38, PD 42, PD 46), and senescent (PD 55, PD 60) 2BS cells and RT-PCR was performed to analyze the $p21^{Waf1/Cip1}$ expression changes in the aging process of 2BS cells. **B**: Western blot analysis of $p21^{Waf1/Cip1}$ expression in the young (PD 28), middle-aged (PD 33, PD 38, PD 42, PD 46), and senescent (PD 55, PD 60) 2BS cells. **C**: Ratios of $p21^{Waf1/Cip1}$ message to β-actin message.

changes in responding to γ-irradiation is a reflection of p21$^{Waf1/Cip1}$ protein changes in these cells. As shown in Figure 5B, after γ-irradiation at 2Gy and 8Gy, the p53 protein level was increased in young, middle-aged, and senescent 2BS cells. In contrast, although the p21$^{Waf1/Cip1}$ protein level was increased in the young and senescent 2BS cells after γ-irradiation, there was little increase of p21$^{Waf1/Cip1}$ protein level in the middle-aged 2BS cells (Fig. 5B,C). These data are consistent with the cell-cycle changes in middle-aged 2BS cells after γ-irradiation.

5-aza-CdR is a potent DNA methyltransferase inhibitor, which is widely used to demonstrate the correlation between loss of

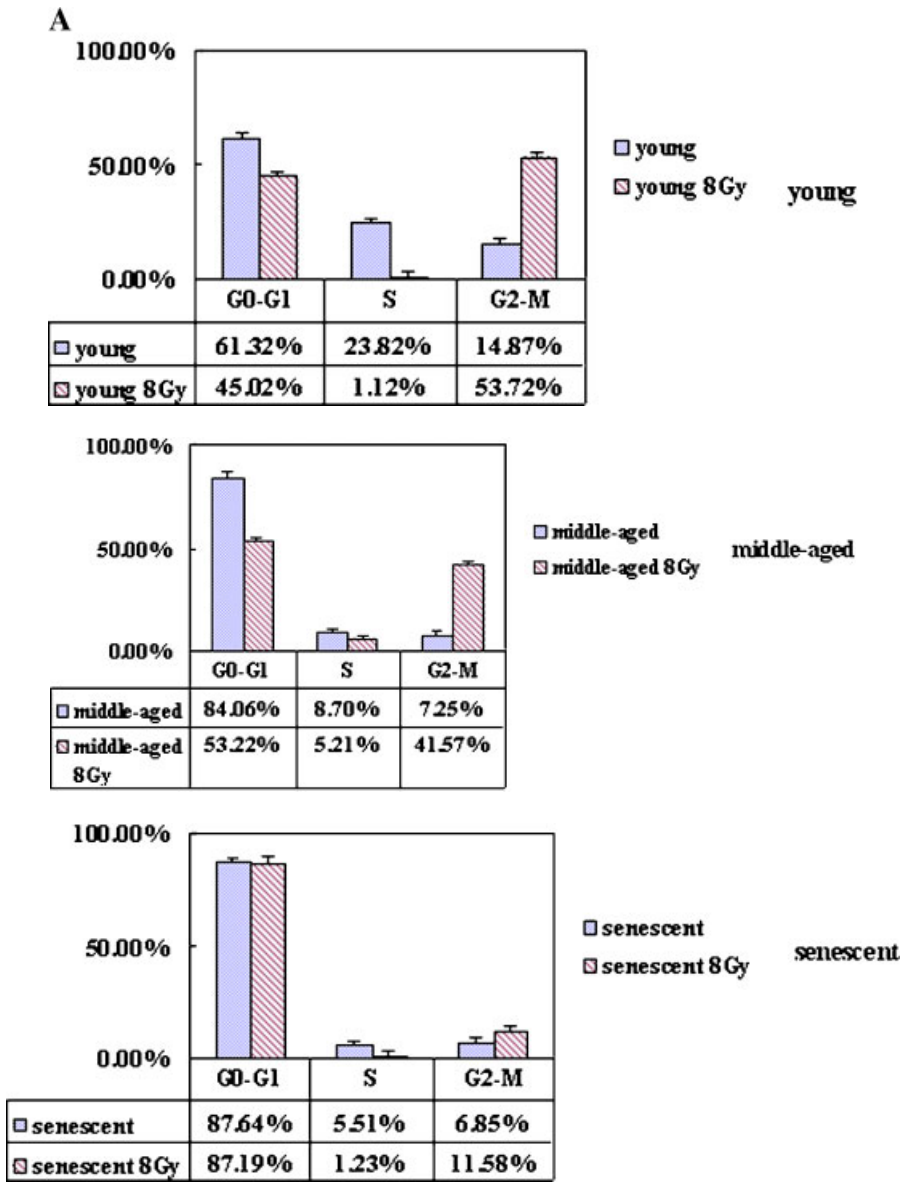

Fig. 5. p21$^{Waf1/Cip1}$ promoter methylation inhibits its function in γ-irradiation induced DNA damaged signal pathway. **A**: The graph depicted cell-cycle distribution of different-aged 2BS cells after γ-irradiation. Young (PD 28), middle-aged (PD 42), and senescent (PD 58) 2BS cells were treated with γ-ray of 8Gy, cells were trypsinized and the DNA contents were measured by flow cytometry system. **B**: Western-blot analysis of the effect of γ-irradiation on p53 and p21$^{Waf1/Cip1}$ expression in different-aged 2BS cells. Young (PD 28), middle-aged (PD 42), and senescent (PD 58) 2BS cells were treated with γ-ray of 2Gy and 8Gy, total proteins were extracted, respectively, and Western-blot was performed using specific antibodies against p53 and p21$^{Waf1/Cip1}$ as indicated, protein from untreated young, middle-aged, and senescent 2BS cells serves as normal control and β-actin lane serves as loading control. CTR, normal control. **C**: Ratios of p53 and p21$^{Waf1/Cip1}$ message to β-actin message. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

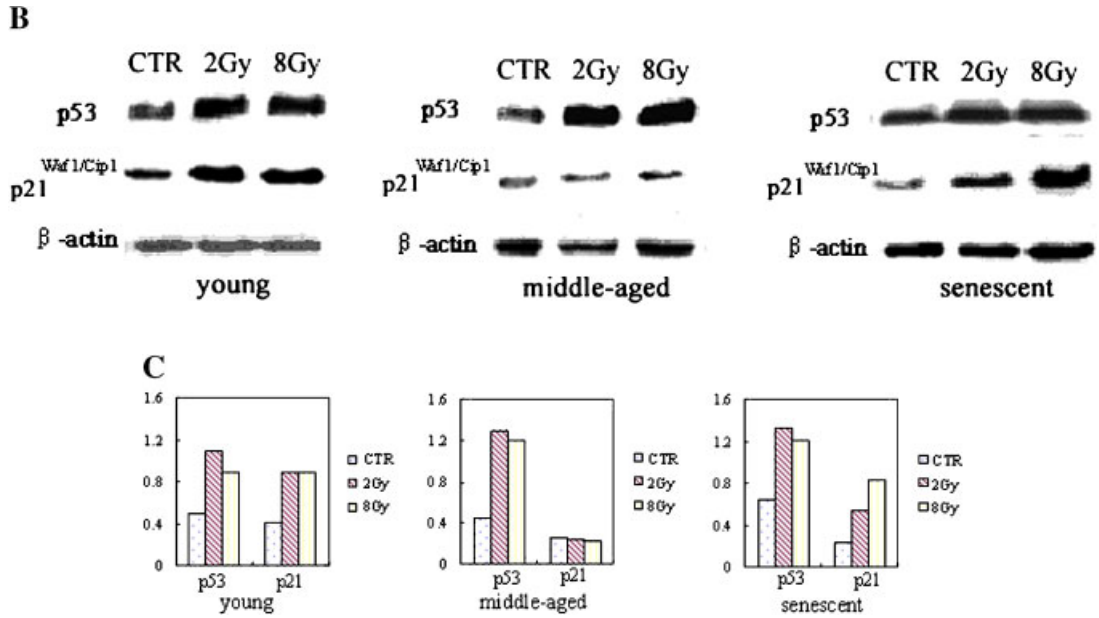

Fig. 5. (*Continued*)

methylation in specific gene regions and activation of the associated genes [Christman, 2002]. To further investigate the effects of $p21^{Waf1/Cip1}$ promoter methylation on its expression, we treated middle-aged 2BS (PD 39) cells with 5-aza-CdR for 9 days (three doublings). As observed in Figure 6A, $p21^{Waf1/Cip1}$ promoter was demethylated after 5-aza-CdR treatment, which in turn led to an elevated $p21^{Waf1/Cip1}$ protein level (Fig. 6B). Moreover, $p21^{Waf1/Cip1}$ protein increased after exposing the 5-aza-CdR treated middle-aged 2BS cells to γ-irradiation (Fig. 6B), and the percentage of cells in the S phase decreased sharply from 5.39% to 1.05% (Fig. 6C). These data demonstrate that $p21^{Waf1/Cip1}$ promoter methylation blocks the p53-induced $p21^{Waf1/Cip1}$ expression in middle-aged 2BS cells.

5-aza-CdR Treatment and DNMT1 RNA Interference Induced Cell Growth Arrest and Premature Senescence of Middle-Aged 2BS

To analyze the phenotypes of the 5-aza-CdR treated middle-aged 2BS cells, the growth curves of 5-aza-CdR treated and untreated normal middle-aged 2BS cells were compared. The growth curve of 5-aza-CdR treated cells was similar to that of senescent cells, showing nearly complete growth arrest, (Fig. 7A). Furthermore, middle-aged 2BS cells treated with 5-aza-CdR exhibited a senescent-like morphology and expressed the senescence-associated-β-galactosidase activity (Fig. 7B). These data suggest that DNA methyltransferase inhibition in middle-aged 2BS cells induces a cellular senescence response.

DNMT1 is the predominant methyltransferase required to maintain DNA methylation patterns in mammalian cells [Robert et al., 2003]. To clarify the specificity of DNA methyltransferase inhibition on the response of cellular senescence, we used the pSilencer vector system to transiently suppress the DNMT1 gene. Middle-aged 2BS (PD 39) were transfected with the pSilencer-DNMT1 (siDNMT1) or pSilencer NC vector (NC). To improve the transfection efficiency, transformants were selected with G418 at a concentration of 50 μM for 6 days before harvest. The siRNA transfected cells were analyzed for the relative senescence markers compared with pSilencer NC vector (NC) transfected and untransfected normal middle-aged 2BS cells (N).

To determine the efficiency of siDNMT1, we detected the expression of related proteins in siDNMT1 transfected cells by Western blotting and $p21^{Waf1/Cip1}$ promoter methylation status by MSP. We found that in siDNMT1 transfected cells, the DNMT1 protein level was reduced nearly 90% compared with pSilencer NC vector (NC) transfected and normal middle-aged 2BS cells (N) (Fig. 8A), which established the efficiency of the DNMT1 siRNA. At the same

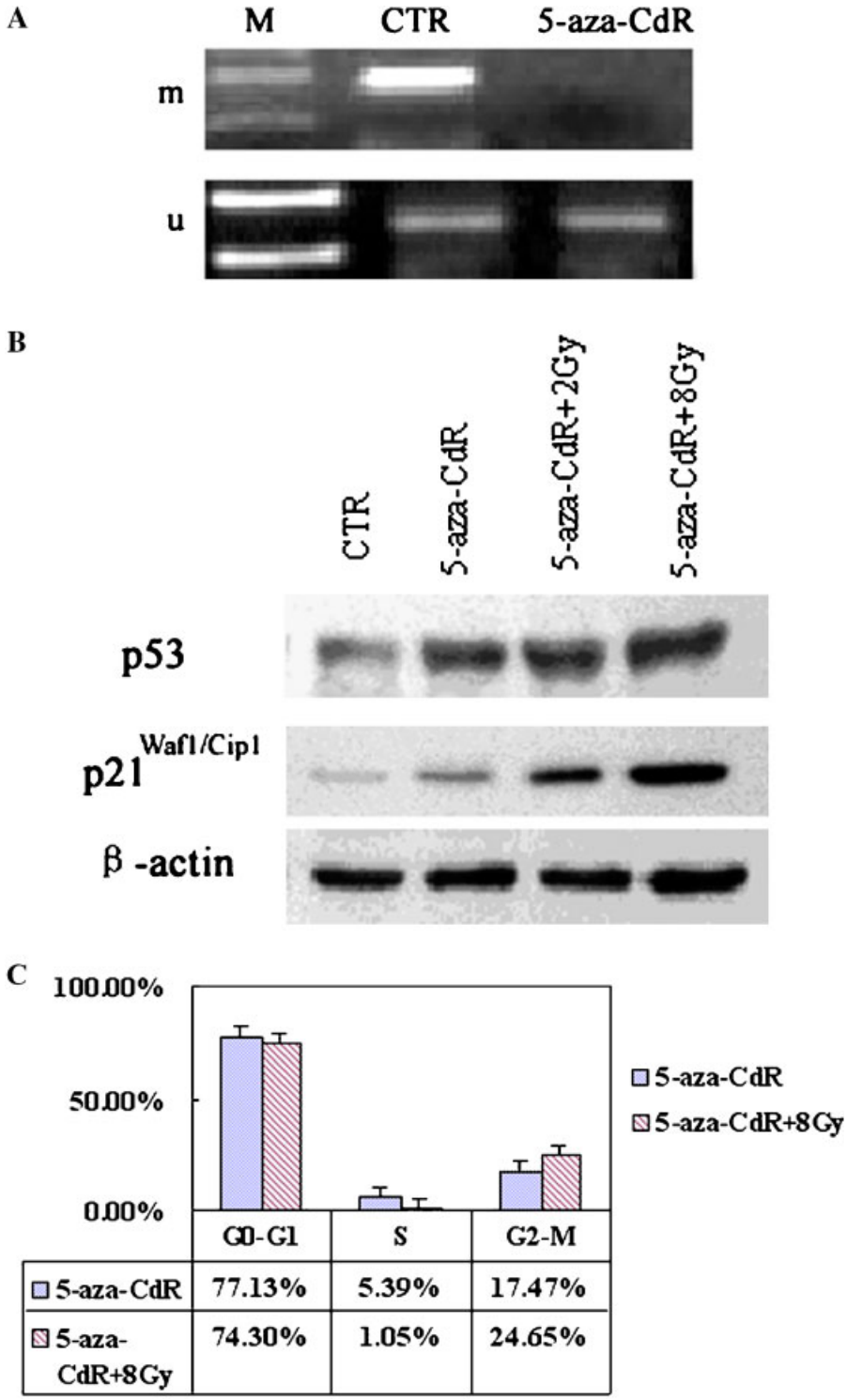

Fig. 6. 5-aza-CdR treatment increased p21$^{Waf1/Cip1}$ expression in normal and γ-irradiation treated middle-aged 2BS cells. **A**: MSP analysis of p21$^{Waf1/Cip1}$ promoter methylation status in 5-aza-CdR treated middle-aged 2BS cells. **B**: Western-blot analysis of p53 and p21$^{Waf1/Cip1}$ expression in 5-aza-CdR and 5-aza-CdR + γ-irradiation treated middle-aged 2BS cells. **C:** The graph depicted cell-cycle distribution of 5-aza-CdR and 5-aza-CdR + γ-irradiation treated middle-aged 2BS cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

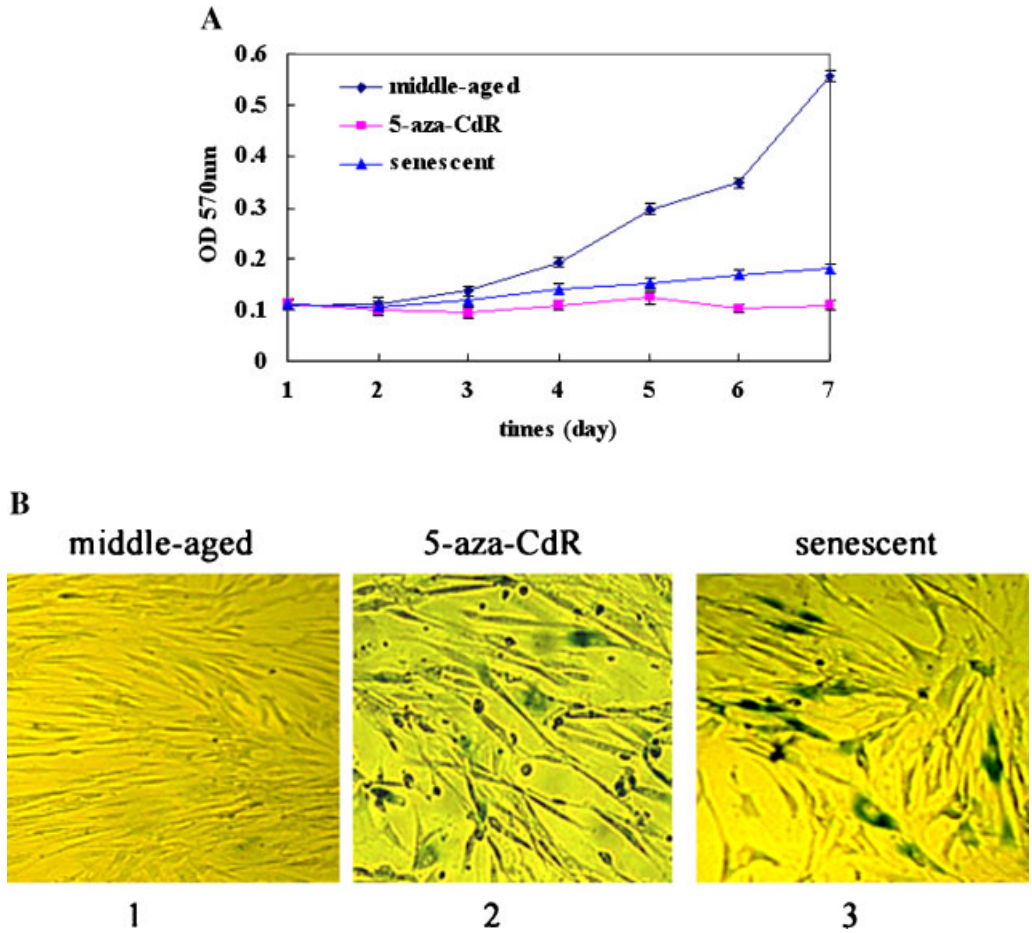

Fig. 7. 5-aza-CdR treatment induces cell growth arrest and premature senescence of middle-aged 2BS cells. **A**: Growth curves of normal middle-aged (PD 42), 5-aza-CdR treated, and senescent (PD 55) 2BS cells. Cells (2000) per well were plated into 96-well plates, at the indicated time points, cells were stained with MTT and cell number was obtained from OD at 570 nm. **B**: Morphology and SA-β-gal staining for 5-aza-CdR treated middle-aged 2BS cells. 1, normal middle-aged 2BS cells; 2, middle-aged 2BS cells with 5-aza-CdR treatment; 3, senescent 2BS cells. Photographs were 400× magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

time, the genome DNA of siDNMT1 and NC vector transfected cells was extracted for MSP analysis. We did not find any methylation in the $p21^{Waf1/Cip1}$ promoter of siDNMT1 transfected cells; however, NC vector transfected cells have the same promoter methylation pattern as normal middle-aged 2BS cells (Fig. 8B). Introduction of siDNMT1 efficiently increased the expression of $p21^{Waf1/Cip1}$, since the level of $p21^{Waf1/Cip1}$ protein significantly increased (about 60%) in siDNMT1 transfected cells (Fig. 8A). More importantly, transfection of siDNMT1 into middle-aged (PD 39) 2BS cells resulted in an irreversible cell-cycle G_1/S arrest (Fig. 8C), they stopped proliferation at about 42-45PD and displayed several other cell senescence features, such as enlarged cell volume and expression of the senescence-associated-β-galactosidase activity (Fig. 8D). These results provide direct evidence for $p21^{Waf1/Cip1}$ demethylation mediated cellular senescence in middle-aged 2BS cells.

DISCUSSION

It has been reported that DNA methyltransferase inhibition in normal human fibroblasts induces a $p21^{Waf1/Cip1}$-dependent cell growth arrest and senescent phenotypes [Young and Smith, 2001]. However, it was unknown whether this response was involved in the methylation changes of age-related specific genes. Here, we have detected the methylation status of $p16^{INK4a}$ and $p21^{Waf1/Cip1}$ in the life span of normal human fibroblasts cultured in vitro. We found that $p16^{INK4a}$ has no promoter

methylation in the aging process of human fibroblasts; but interestingly, p21$^{Waf1/Cip1}$ promoter is gradually methylated from young to middle-aged fibroblasts, while losing methylation in senescent fibroblasts (Fig. 1). Therefore, the expression of p16^{INK4a} is not regulated by promoter methylation in the aging process of human fibroblasts, in spite of its frequent promoter methylation in cancer cells. Moreover, Herman et al. [1995] [1997] were unable to find p16^{INK4a} promoter methylation in normal tissues. Mice fibroblasts derived from PASG mutant embryos cause global hypomethylation and show a replicative senescence phenotype. Although a markedly increased expression of p16^{INK4a} is induced, it is independent of promoter methylation; instead, it is associated with downregulation of bmi-1, a negative regulator of p16^{INK4a} [Sun et al., 2004]. Thus, p16^{INK4a} promoter methylation is probably cancer related, as an integral feature of the neoplastic process [Issa, 1999]. However, p21$^{Waf1/Cip1}$ is

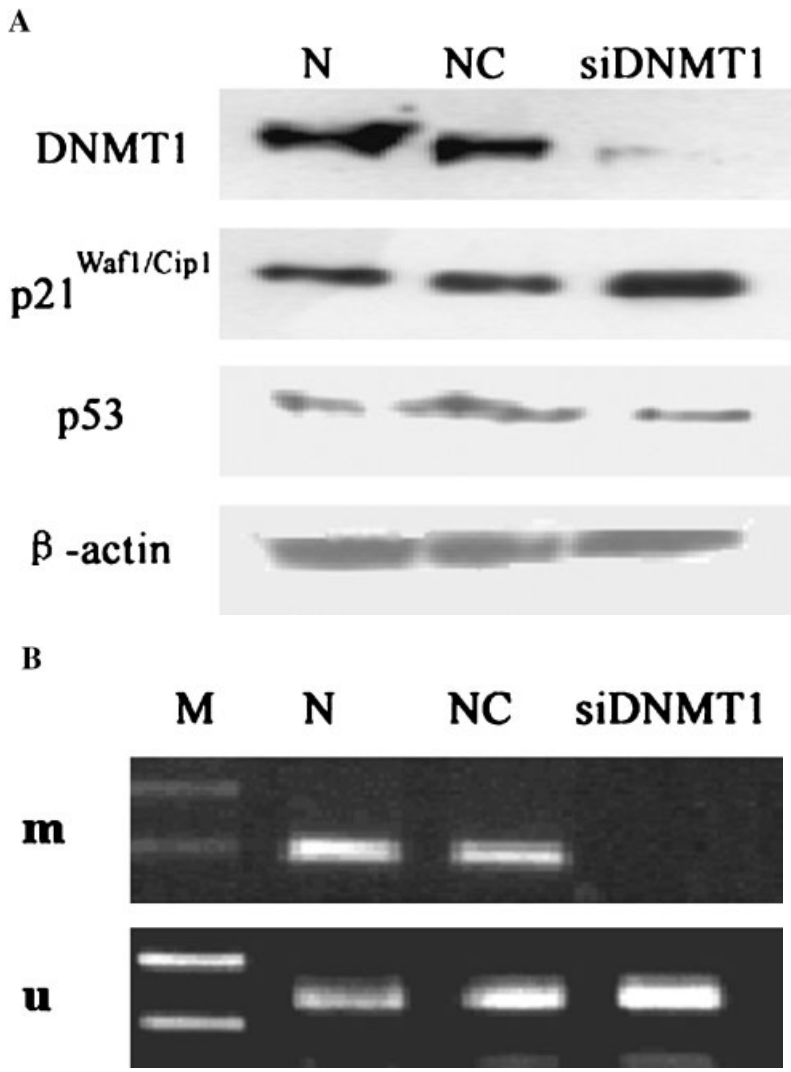

Fig. 8. DNMT1 RNA interference induces premature senescence of middle-aged 2BS cells. **A**: Western-blot analysis of DNMT1, p21$^{Waf1/Cip1}$ and p53 expression in siDNMT1 transfected (siDNMT1) compared with normal (N) and NC vector transfected middle-aged 2BS cells (NC). **B**: MSP analysis of p21$^{Waf1/Cip1}$ promoter methylation patterns in normal (N), NC vector transfected (NC), and siDNMT1 transfected (siDNMT1) middle-aged 2BS cells. M, marker; m, methylation-specific PCR products; u, unmethylation-specific PCR products. **C**: The graph depicted cell-cycle distribution of siDNMT1 transfected middle-aged 2BS cells compared with NC vector transfected, and normal young, middle-aged, and senescent 2BS cells. **D**: Morphology and SA-β-gal staining for siDNMT1 transfected middle-aged 2BS cells. 1, NC vector transfected middle-aged 2BS cells; 2, siDNMT1 transfected middle-aged 2BS cells. Photographs were 400× magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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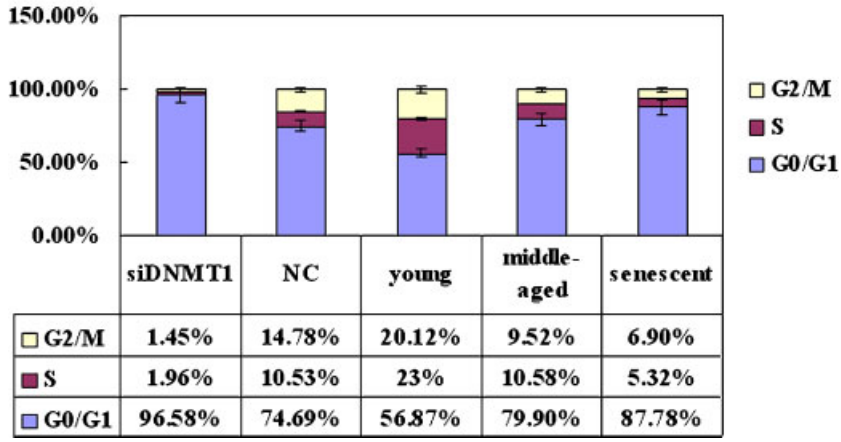

	siDNMT1	NC	young	middle-aged	senescent
G2/M	1.45%	14.78%	20.12%	9.52%	6.90%
S	1.96%	10.53%	23%	10.58%	5.32%
G0/G1	96.58%	74.69%	56.87%	79.90%	87.78%

D

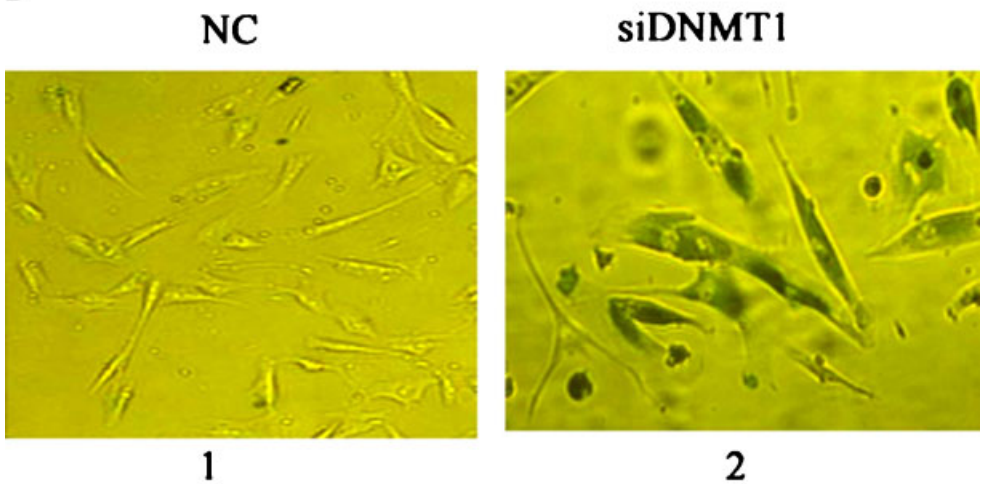

Fig. 8. (*Continued*)

maintained in a partially methylated state in normal tissues in a tissue-specific pattern, such as blood, muscle, and lung tissue [Chen et al., 2000].

Although the mechanism causing clonal heterogeneity of p21 promoter methylation is uncertain, several studies have addressed the issue of clonal variation in the methylation status of specific genes or DNA sequences. Fitzpatrick et al. [1998] analyzed IFN-gamma and *IL-3* gene methylation in primary CD8 + T cells. They observed substantial heterogeneity in the methylation patterns of the two genes between clones. In the studies by Shmookler Reis and Goldstein [1982], a mass culture of human diploid fibroblasts and eight clones isolated from the mass culture were examined for the methylation patterns of several genes. They found a striking degree of interclonal heterogeneity, with each gene exhibiting a clone-specific pattern of DNA methylation.

It is not clear how $p21^{Waf1/Cip1}$ promoter methylation patterns are established and maintained in the process of cell aging. Changes in the expression levels of DNMT1 and DNMT3a may be responsible for it, since their expression patterns are parallel to that of $p21^{Waf1/Cip1}$ methylation changes in the cell aging process (Fig. 2). Recent studies suggest that DNMT1 cooperates with other transcriptional factors displaying a role of de novo methylation [Di Croce et al., 2002; Esteve et al., 2005; Zhang et al., 2005], and DNMT1 and DNMT3a cooperates with PML-RAR to induce hypermethylation of *RAR* β gene (Di Croce et al., 2002). Brenner et al. [2005] found that *myc*-mediated

repression of p21$^{Waf1/Cip1}$ is required for the recruitment of DNMT3a and aberrant methylation of its promoter. On the other hand, DNMT1 can be stimulated for methylation of unmodified DNA if the DNA already carries some methyl groups [Fatemi et al., 2002]. DNMT3b is unlikely to be involved in p21$^{Waf1/Cip1}$ promoter methylation because it has the highest expression level in the young cells in which p21$^{Waf1/Cip1}$ has a low level of methylation. Moreover, it has been reported that DNMT3b appears to be specialized for the methylation of a particular compartment of the genome; loss of DNMT3b activity in ICF syndrome causes demethylation of only specific families of repeated sequences around pericentric regions and CpG islands on the inactive X chromosome. [Bestor, 2000; Jiang et al., 2005].

Although there is still debate on whether methylation controls developmental and tissue-specific patterns of gene expression, our studies about the p21$^{Waf1/Cip1}$ promoter favor the idea that methylation is involved in gene silencing. This is directly supported by the inactivation of p21$^{Waf1/Cip1}$ promoter through in vitro methylation (Fig. 3B). In addition, there was a lower p21$^{Waf1/Cip1}$ expression in the middle-aged (38PD, 42PD, 46PD) 2BS cells than in the young (28PD) and senescent (55PD) cells at both mRNA and protein level. (Fig. 4). Because p21$^{Waf1/Cip1}$ promoter is partly methylated in vivo, the decrease of its expression is not very remarkable compared with that of in vitro methylation analysis in which p21$^{Waf1/Cip1}$ promoter was completely methylated. However, it is evident that p21$^{Waf1/Cip1}$ promoter methylation repressed its expression and prevented its rapid increase in the middle-aged 2BS cells. It is possible that p21$^{Waf1/Cip1}$ promoter methylation was formed slowly in the middle-aged 2BS cells since its expression did not decrease at 33PD. We did not see elevated p21$^{Waf1/Cip1}$ expression in the fully senescent (PD 60) 2BS cells in which p21$^{Waf1/Cip1}$promoter is not methylated; this is because the role of p21$^{Waf1/Cip1}$ is to initiate cell senescence and its expression declines quickly after the cells achieve senescence, whereas the amount of p16^{INK4a} increases and maintains the state of senescence [Alcorta et al., 1996; Stein et al., 1999].

Since the functions of p21$^{Waf1/Cip1}$ are predominantly limited to cell-cycle control at the G_1/S phase transition in the presence of functional pRb [Niculescu et al., 1998], cultured p21$^{Waf1/Cip1}$ deficient fibroblasts were compromised in their ability to undergo G_1 arrest in response to DNA damage [Deng et al., 1995], while p53 prevents G_2/M transition by a p21$^{Waf1/Cip1}$-independent reduction of intracellular levels of cyclin B1 protein [Innocente and Lee, 2005]. Therefore, the lack of p21$^{Waf1/Cip1}$ protein increase resulting from promoter methylation offers a potential explanation for the obscure decrease of cell-cycle S phase cells in the middle-aged 2BS cells after γ-irradiation, which is consistent with the observations of Allan et al. [2000] that p21$^{Waf1/Cip1}$ promoter methylation is a main mechanism to abrogate p53-dependent p21$^{Waf1/Cip1}$ expression caused by DNA damage.

Treating middle-aged 2BS cells with 5-aza-2-CdR, we observed that p21$^{Waf1/Cip1}$ promoter was demethylated and p21$^{Waf1/Cip1}$ protein level increased. At the same time, the 5-aza-2-CdR treated cells displayed an apparent growth arrest and some senescent-like features, such as increased size and expressed senescence-associated-β-galactosidase activity (Fig. 7B). Unexpectedly, p53 protein increased after 5-aza-CdR treatment (Fig. 6B), but by MSP detection, we did not find p53 methylation in 2BS cells (data not shown). It is reported that in addition to its demethylation function, 5-aza-2′-deoxycytidine also plays a role in the induction and activation of wild type, but not mutant p53 protein in DNA damage [Karpf et al., 2001; Zhu et al., 2004]. Thus, it is possible that p53 may be involved in the premature senescence of 2BS cells induced by 5-aza CdR treatment [Bond et al., 1996; Chen et al., 2004]. However, it is p21$^{Waf1/Cip1}$ dependent [Brown et al., 1997]. Furthermore, p21$^{Waf1/Cip1}$ upregulation and cell-cycle arrest after DNA methyltransferase inhibition were also observed in cell lines lacking a functional p53 [Young and Smith, 2001].

DNMT1 RNA interference (RNAi) eliminates the pleiotropic effects of 5-aza-CdR. In contrast to normal and NC vector transfected middle-aged 2BS cells, the siDNMT1 transfected cells exhibited demethylated p21$^{Waf1/Cip1}$ promoter and elevated p21$^{Waf1/Cip1}$ expression, while p53 protein level was not increased in the siDNMT1 transfected cells. Moreover, siDNMT1 transfected cells show an almost absolutely cell-cycle G_1/S arrest, accompanied with irreversible growth arrest and a shortened life span as well as several other cell senescent features. These

data supplied direct evidence for the role of $p21^{Waf1/Cip1}$ promoter demethylation on aging. Several other genes may be activated by DNA demethylation in normal human fibroblasts, but their role in cell aging is obscure [Liang et al., 2002]. Up to now, no genes have been found in the major pathways of cell aging to be regulated by DNA methylation. Previous studies have demonstrated that progressive losses of 5-mc levels is a characteristic phenomenon of cell aging and the DNA methyltransferase inhibitor 5-azacytine or 5-aza-2′-deoxycytidine has been reported to shorten the life span of cultured fibroblasts [Wilson and Jones, 1983; Holliday, 1986; Fairweather et al., 1987]. However, little is known about the methylation changes of specific genes involved in the regulation of cell aging. Our results proved that loss of DNA methylation in the process of cell aging is not a stochastic process, but involves the demethylayion of the age-related $p21^{Waf1/Cip1}$ gene.

This aberrant change in $p21^{Waf1/Cip1}$ promoter methylation status is likely to be associated with the cell aging process and has a function of anti-senescence, since recent experiments in our lab have shown that introduction of antisense $p21^{Waf1/Cip1}$ can extend the lifespan of 2BS cells, as evidenced at the cellular level by an increase in the maximum number of PDs and at the molecular level by a reduction in the senescence-associated markers, while high expression of $p21^{Waf1/Cip1}$ promotes transcriptional activation of p16INK4 and accelerates senescence [Huang et al., 2004; Xue et al., 2004]. $p21^{Waf1/Cip1}$ promoter methylation represses its expression in the middle-aged 2BS cells, thus delaying the onset of cell aging. $p21^{Waf1/Cip1}$ demethylation either by DNA methyltransferase inhibitors or by siDNMT1, results in elevated $p21^{Waf1/Cip1}$ expression and accelerated cellular senescence. On the other hand, $p21^{Waf1/Cip1}$ promoter methylation blocks p53 induced $p21^{Waf1/Cip1}$ expression in the condition of DNA damage (Fig. 5), which in turn may result in apoptosis [Huang et al., 2004] or transformation of middle-aged fibroblasts [Jackson et al., 2003]. It is not clear how $p21^{Waf1/Cip1}$ promoter methylation is established and maintained in the life span of normal human fibroblasts. Thus, further studies should be pursued to explore the mechanism of $p21^{Waf1/Cip1}$ methylation and its effects on the normal cells apart from cell senescence.

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