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Reactive oxygen species promotes cellular senescence in normal human epidermal keratinocytes through epigenetic regulation of $p16^{INK4a}$

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ABSTRACT

Reactive oxygen species (ROS) can cause severe damage to DNA, proteins and lipids in normal cells, contributing to carcinogenesis and various pathological conditions. While cellular senescence arrests the early phase of cell cycle without any detectable telomere loss or dysfunction. ROS is reported to contribute to induction of cellular senescence, as evidence by its premature onset upon treatment with antioxidants or inhibitors of cellular oxidant scavengers. Although cellular senescence is known to be implicated in tumor suppression, it remains unknown whether ROS initially contributed to be cellular senescence in normal human epidermal keratinocytes (NHEK) and their malignant counterparts. To clarify whether ROS induce cellular senescence in NHEKs, we examined the effect of hydrogen peroxide (H_2O_2) on the expression of cellular senescence-associated molecules in NHEKs, compared to in squamous carcinoma cells (SCCs). Hydrogen peroxide increased the number of cells positive in senescence associated-β-galactosidase (SA-β-Gal) activity in NHEKs, but not SCCs. The expression of cyclin-dependent kinase (CDK) inhibitors, especially $p16^{INK4a}$ was upregulated in NHEKs treated with H_2O_2. Interestingly, H_2O_2 suppressed the methylation of $p16^{INK4a}$, promoter region in NHEKs, but not in SCCs. Hydrogen peroxide also suppressed the expression of phosphorylated Rb and CDK4, resulting in arrest in G0/G1 phase in NHEKs, but not SCCs.

Our results indicate that the ROS-induced cellular senescence in NHEKs was caused by the upregulation $p16^{INK4a}$ through demethylation in its promoter region, which is not detected in SCCs, suggesting that ROS-induced cellular senescence contributes to tumor suppression of NHEKs.

1. Introduction

Cellular senescence has been triggered by telomere erosion or prematurely by various stresses, and arrests a phase G1 in cell-cycle process in a variety of conditions [1]. The senescent cells fail to proliferate without loss of viability and metabolic capacity, which are not quiescent and terminally differentiated cells. Cellular senescence is thought to be involved in aging, which is characterized by the inability of tissues to maintain homeostasis, and aging-related diseases [2]. No specific markers or hallmarks of senescent cells have not been identified, but Rodier and Campisi [3] raise the features of senescent cells as follows: (a) the senescence growth arrest is permanent. (b) Senescent cells increase in size. (c) Senescent cells express a SA-β-Gal, which reflects the increase in lysosomal mass. (d) Most senescent cells express $p16^{INK4a}$, a tumor suppressor. (e) Senescent cells with persistent DNA damage response (DDR) contain nuclear foci, termed DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS). (f) Senescent cells with persistent DDR signaling secrete growth factors, proteases, cytokines and other factors, which are referred to as the senescence-associated secretory phenotype (SASP), which exerts effects on neighboring cells to result in age-related disorders.

Cellular senescence is considered to be an anti-cancer mechanism that prevents the uncontrolled growth of DNA-damaged cells. In light of the steadily increasing evidence that senescence protects humans and other organisms against cancer, ideas are being launched to restore this process in tumor cells in which it has become deficient. Tumor suppressor genes, whose inhibition contributes to the tumor initiation and maintenance, have been reported to be related to cellular senescence. Thus, cellular senescence may act as a barrier to cancer and play an important role in tumor suppression [4].

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ROS, such as superoxide anions, hydroxyl radicals, and H_2O_2 can cause severe damage to DNA, protein, and lipids. Increased levels of ROS produced during normal cellular metabolism such as mitochondrial electron transport, and from environmental stimuli such as cytokines, UV radiation, perturb the normal ROS balance and shift cells into a state of oxidative stress [5]. Oxidative stress is believed to contribute to the etiology of various degenerative diseases such as diabetes, atherosclerosis, arthritis, cancer, and the process of aging. There have been several reports indicating that elevated ROS has been found in many types of tumor cells, and contributes to carcinogenesis and metastasis [6]. We recently have reported that ROS induces the epithelial mesenchymal transition (EMT) in tumor progression in NHEKs through TGF-β1 secretion [7].

It is widely assumed that ROS produced by mitochondria are involved in replicative senescence [8,9]. ROS also is reported to be contributed to induction of cellular senescence, as evidence by delayed and premature onset upon treatment with antioxidants and inhibitors of cellular oxidant scavengers. Although cellular senescence is known to be implicated in tumor suppression, it remains unknown whether ROS initially is involved in cellular senescence in normal cells, especially epidermal keratinocytes.

Head and neck SCCs including oral cancer, that is derived from epidermal keratinocyte, is the sixth most common cancer globally, but the prognosis of oral SCCs have not been improved for decades in contrast to other malignant tumors, leaving behind the patients with impaired functions of swallowing, speech, and mastication. To overcome the difficulty of oral SCCs, it is critical to understand the mechanism of cellular senescence, as tumor suppression, of keratinocyte, being compared with SCCs.

The aim of the present study was to clarify whether cellular senescence contributes to the protection of carcinogenesis in NHEKs. We have investigated the effects of H_2O_2 on the expression of cellular senescence markers and tumor suppressor molecules involved in CDK inhibitors in NHEKs, compared to those in SCCs.

2. Materials and methods

2.1. Cell culture

NHEKs were purchased from Lonza Co., Ltd. (cat No. 00192627; Tokyo, Japan) and cultured in KGM2 medium (Lonza Co., Ltd.). The experiment were only used the 2–5 passage cells, which keep the characteristics of normal human keratinocytes, but not fibroblastic and/or mesenchymal cells. SCCs cells were maintained on culture dishes, in D-MEM with 10% fetal bovine serum. The cells were incubated in culture medium with or without H_2O_2 (800 μM) for 0–72 h. The cells were cultured at 37 °C in a water-jacketed 5% CO_2 incubator. In some experiments, cells were incubated in culture medium supplemented with 5-AzazC (10 μM) or menadione (10 μM).

2.2. Assay of SA-β-Gal activity

β-Gal Activity at ph 6.0 was detected with SA-β-Gal staining kit (Cell Signaling Technology, Inc.). The cells were washed with PBS and fixed in each dishes. The dished were added the β-Gal staining solution and incubate at 37 °C overnight in a dry incubator (EYELA low temperature incubator, LTI1000ED). The β-Gal positive cells stained blue color were counted under a microscope (200× total magnifications, Carl Zeiss, Jena, Germany).

2.3. RNA isolation and reverse-transcription polymerase chain reaction (PCR)

Total RNA was extracted from cells using the TRLzol reagent. First-strand cDNA was synthesized from 3 μg of total RNA using SuperScript II reverse transcriptase, according to the manufacturer's instructions (Invitrogen Carlsbad, CA, USA). To detect mRNA expression, we selected specific primer based on the nucleotide sequence of cDNA. The cDNA was amplified by PCR under the following condition: 30 s denaturation at 94 °C, 30 s annealing at 52–55 °C, and 1 min extension at 72 °C, for 28–35 cycles. PCR products were subjected to electrophoresis on 2% agarose gels and visualized after staining with ethidium bromide. The signal intensity of each PCR product was quantitatively measured with Scion image software (version 4.02, NIH, Bethesda, MD) and normalized to that GAPDH mRNA.

2.4. Western blot analysis

Cells were lysed in TNT buffer (Roche, Basel, Switzerland). Protein content was measured with a protein assay kit (Pierce, Hercules, CA, USA). Twenty micrograms of each protein was subjected to 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and separated proteins were then electrophoretically transferred to PVDF membrane at 75 V for 1.5 h at 4 °C. The membrane was incubated with the antibodies against Dnmt1, $p16^{INK4a}$, β-actin (Sigma Chem., St. Louis, MO, USA) p-Rb (Ser807/811), p53, $p21^{cip1}$ (Cell Signaling Technology, Inc.), in 5% skimmed milk solution plus 0.01% azide overnight at 4 °C. Blots were washed in TBST(10 mM tris–HCl, 50 mM, NaCl 0.25% Tween-20), then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or mouse IgG secondary antibodies and developed using an enhanced chemiluminescence system (GE Healthcare, Tokyo, Japan).

2.5. Methylation specific PCR assay

The NucleoSpin® Tissue (TAKARA Bio Inc., Tokyo, Japan) was used for DNA extraction. DNA was modified by the bisulfite reaction using MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit (TAKARA). After completion of the reaction, all unmethylated cytosines are determinate and converted to uracil, while methylated cytosines remain unchanged. To perform quantitative, the designed primers common nucleotide sequence in the DNA is unmethylated and methylated DNA. Bisulfite modified DNA was amplified with p16 gene-specific primers: 10 s denaturation at 98 °C, 30 s annealing at 54 °C, and 30 s extension at 72 °C, for 40 cycle. Subsequently, products of the PCR were amplified with p16 gene specific primers: 10 s denaturation at 98 °C, 30 s annealing at 54 °C, and 30 s extension at 72 °C, for 32–38 cycles. To detect ration of methylation in promoter sequence, we selected specific primer based on the nucleotide sequence of p16 promoter region in cDNA.

2.6. FACS analysis

NHEKs (1.0×10^6 cells/ml) and SCCs (1.0×10^6 cells/ml) were cultured on 10 cm dishes with or without H_2O_2 for 72 h. At the end of each treatment time, cells were collected. Finally the cells were resuspended at 0.1% Triton in PBS, propidium iodide (100 μg/ml) solution and 4 μl of RNase (10 mg/ml). Cell cycle distribution was analyzed by flow cytometry using BD FACS Calibur (BD Bioscience; San Diego, CA, USA).

2.7. Statistical analysis

Data were expressed as mean ± standard error. Differences were analyzed with one-way analysis of variance and Scheffe's multiple comparison tests. *P*-values of <0.05 were considered to be significant.

3. Results

3.1. ROS increased the expression of CDK inhibitors and the number of positive cells in SA-β-Gal activity in NHEKs, but not SCCs

Recently, we have reported that the treatment with H_2O_2 (800 μM) exerted the oxidation to DNA forming 8-oxo-7, 8-dihydroguanine, and induced EMT via TGF-β autocrine secretion in NHEKs [7,10]. However, little is known about the effect of ROS on cellular senescence of NHEKs in terms of the protection of tumor progression in NHEKs. We firstly performed a genome-wide screening of tumor suppressor genes following treatment with H_2O_2 using Gene Chip Human Genome U133 plus 2.0 arrays. H_2O_2-induced ROS stimulation for 72 h altered the mRNA expression of many cell cycle markers in NHEKs and SCCs (Supplemental Table 1). Cellular growth arrest of senescent cells is known to be mediated mainly by two CDK inhibitor pathways, $p21^{cip1}$/p53/ARF pathway and $p16^{INK4a}$/Rb/E2F pathway, indicating that both pathways are also referred as tumor suppressor [4]. ROS dramatically up-regulated the expression of CDK inhibitors such as $p21^{cip1}$ and $p16^{INK4a}$, but not p53 in NHEKs, which are associated with the cellular senescence markers. Furthermore, the expression of CDKs in cell cycling, especially CDK4 and CDK6 decreased in NHEKs. In contrast, ROS had little effects on these CDK inhibitors in SCCs. We subsequently focused on p53, $p21^{cip1}$, and $p16^{INK4a}$, which have been shown to be associated with cellular senescence. To clarify whether ROS induced cellular senescence with CDK inhibitors in NHEKs we examined the effect of H_2O_2 on the expression of a well-known senescence associated biomarker (SA-β-Gal) activity. The treatment with H_2O_2 significantly increased the number of NHEKs positive in SA-β-Gal activity in a time-dependent manner, indicating ROS induced cellular senescence in NHEKs (Fig. 1A). In contrast, H_2O_2 had little effect on the SA-β-Gal activity in SCCs.

H_2O_2 significantly increased the expression of $p21^{cip1}$ and $p16^{INK4a}$ in NHEKs in a time-dependent manner (Fig. 1B). In contrast, H_2O_2 had no effects on their expressions in SCCs. The upregulation of the CDK inhibitors was observed 3 h after the treatment with a concentration of H_2O_2 > 300 μM (data not shown). Similar to the PCR results, the increased expressions of their proteins were observed 6 h after treatment of H_2O_2 in NHEKs, whereas H_2O_2 had no effect on the expression of $p16^{INK4a}$ and reduced the $p21^{cip1}$ expression in SCCs.

Furthermore, the other ROS inducer menadione (10 μM) also increased the expression of mRNA and protein of $p16^{INK4a}$ in a time-dependent manner in NHEKs (Fig. 2A). The H_2O_2-induced upregulation of $p16^{INK4a}$ proteins was suppressed by the pretreatment with antioxidant drug N-acetylcysteine (NAC;10 μM) in NHEKs.

3.2. ROS suppressed the methylation of p16 promoter region in NHEKs, but not in SCCs

Interestingly, we found that H_2O_2 simultaneously decreased the expression of (cytosine-5)-DNA methyltransferase (DNMT) 1 in NHEKs, but not in SCCs (Fig. 1B). DNMT families are known to catalyze DNA methylation in mammalian cells. DNMT 1 is thought to play a major role in inducing and maintaining DNA

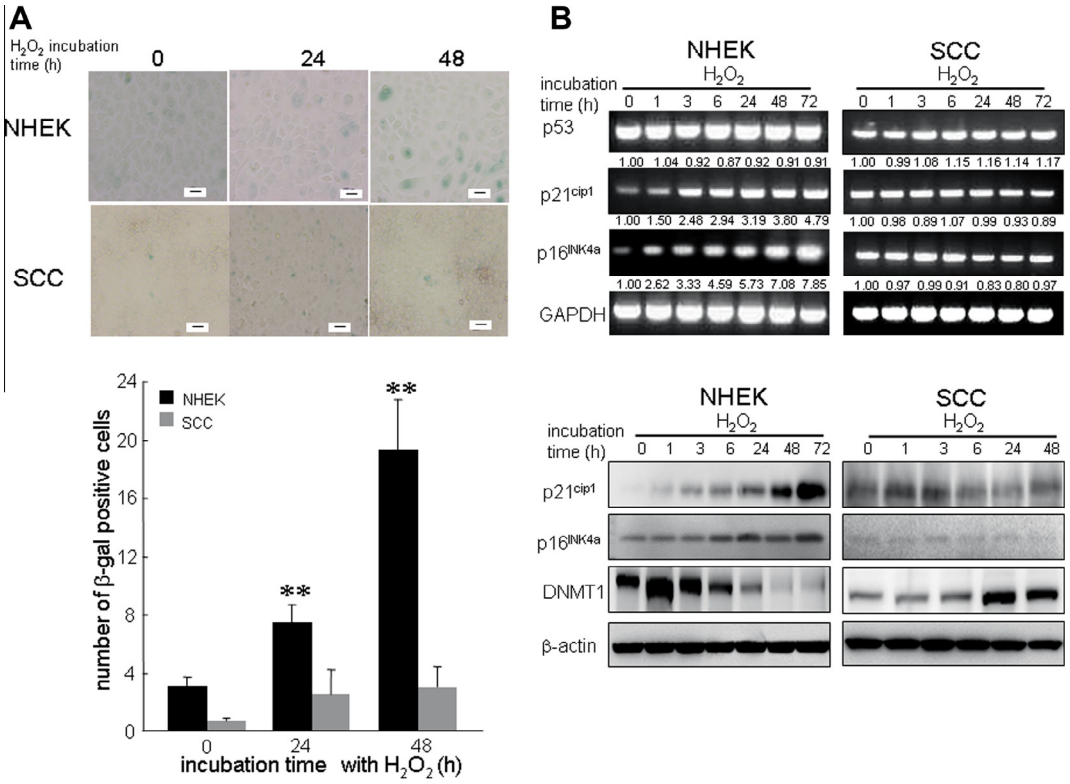

Fig. 1. ROS increased the number of positive cells in SA-β-Gal activity and the expression of CDK inhibitors in NHEKs. (A) The cells were incubated with H_2O_2 (800 μM) for 48 h and stained with SA-β-Gal staining for the indicated times. The panels indicate SA-β-gal staining images for the indicated times after H_2O_2 treatment, respectively. Bars indicate 100 μm. The SA-β-Gal-positive cells were counted on six wells. Data shown are the mean from six culture wells (mean ± SEM). $^{**}P < 0.01$, NHEKs vs SCCs group. (B) Expression of CDK inhibitors were analyzed semi-quantitatively RT-PCR and Western blot in NHEKs and SCCs. Numbers below the gels represent the intensity of each targeted mRNA relative to GAPDH mRNA. Western blotting carried out using targeted and β-actin antibodies in NHEKs and SCCs. Similar results were obtained in three independent experiments.

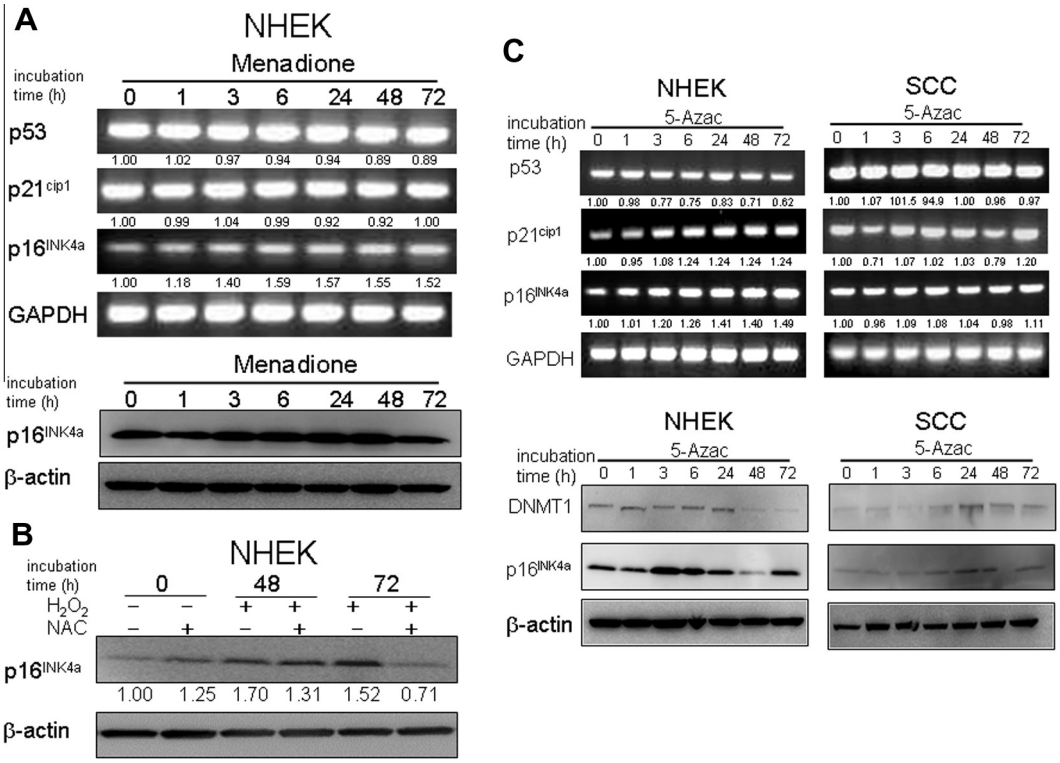

Fig. 2. ROS and methylation inhibitor induced the expression of p16 in NHEKs. (A) After treatment of with menadione (10 μM) the expression of CDK inhibitors was analyzed semi-quantitatively by RT-PCR and Western blot. Numbers below the gels represent the intensity of each targeted mRNA relative to GAPDH mRNA. (B) NHEKs were incubated H_2O_2 before and 72 h after treatment with N-acetylcysteine (NAC; 10 μM). (C) After treatment with a methylation inhibitor 5-Azac (10 μM) the expressions of CDK inhibitors and DNMT1 were analyzed semi-quantitatively RT-PCR and Western blot. Numbers below the gels represent the intensity of each targeted mRNA relative to GAPDH mRNA. Similar results were obtained in three independent experiments.

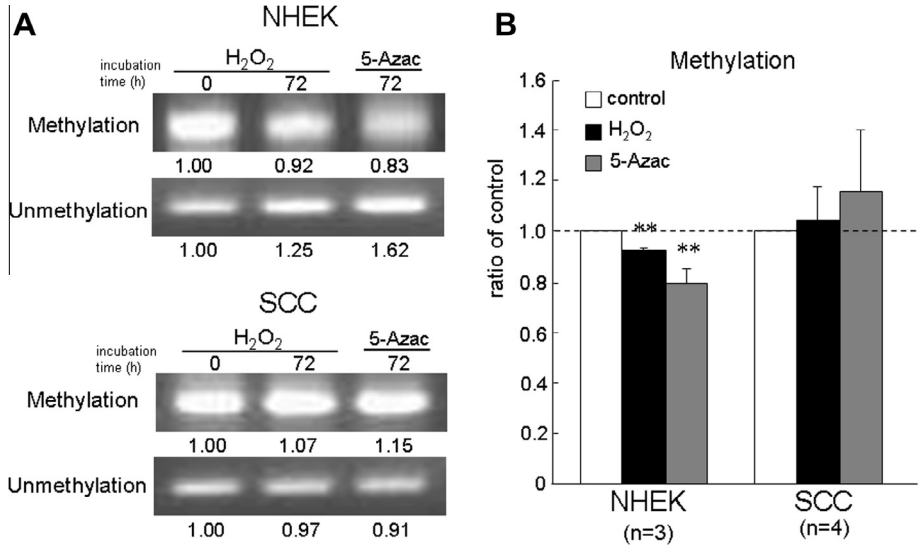

Fig. 3. ROS suppressed the methylation of p16 promoter region in NHEKs. (A) Bisulfite-treated DNA was used for PCR amplification using primers sets designed for methylated and unmethylated in p16 promoter. Numbers below the gels represent the intensity of each targeted mRNA relative to 0 h (control). (B) Methylation levels of p16 promoter region after H_2O_2 or 5-Azac (10 μM) treatment were analyzed semi-quantitatively by methylation specific PCR assay. Data shown are the mean from six culture wells (mean ± SEM). ** indicates $P < 0.01$ vs control.

methylation which is often observed in human cancer cells [11]. Therefore, we examined the effect of methylation inhibitor 5-Azac on the expression of CDK inhibitors in NHEKs. Similar to H_2O_2 treatment, 5-Azac upregulated the expressions of CDK inhibitors, especially $p16^{INK4a}$ in NHEKs in a time-dependent manner (Fig. 2B). In contrast, H_2O_2 had little effect on the expression of

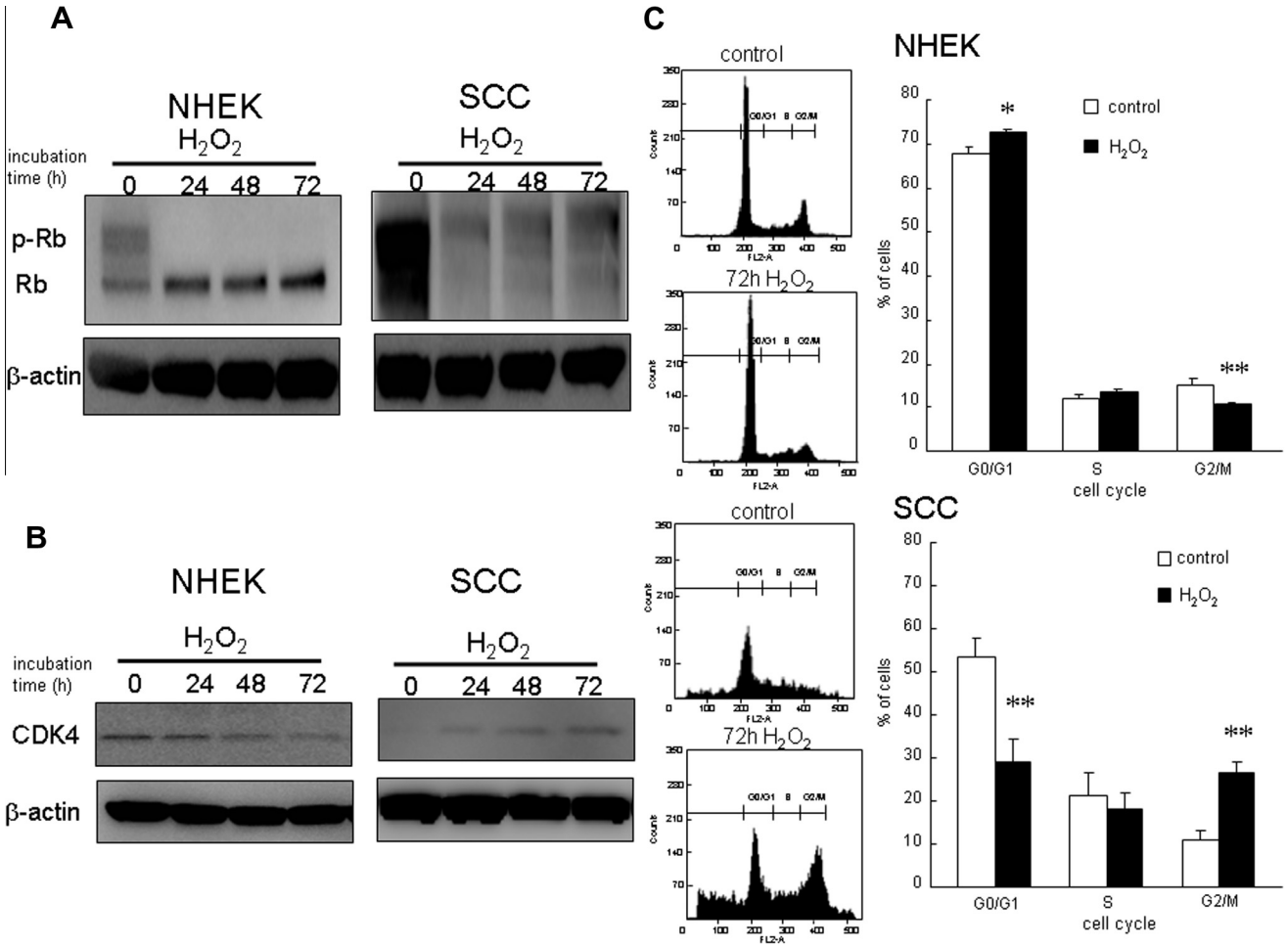

Fig. 4. ROS arrested in G0/G1 phase of cell cycle in NHEKs. (A) After the treatment with H_2O_2 (800 μM) in the cells expression of Rb proteins were analyzed by Western blot analysis. Similar results were obtained in three independent experiments. (B) After the treatment with H_2O_2 the expression of CDK4 was analyzed by Western blot analysis. Similar results were obtained in three independent experiments. (C) Distribution of cell cycle before (control) and 72 h after H_2O_2 treatment in the cells. Accumulated percentages of cells in each phase treated with H_2O_2. Data shown are the mean from six culture wells (mean ± SEM). ** indicates $P < 0.01$ vs control.

both CDK inhibitors in SCCs. The methylation of the promoter lesion in $p16^{INK4a}$ gene was reported to suppress the expression of p16 in human cells [12]. The results suggest that H_2O_2 inhibit the methylation of $p16^{INK4a}$ promoter, resulting in the upregulation of $p16^{INK4a}$ in NHEKs.

Then, we examined whether H_2O_2 affected the DNA methylation level of CpG in $p16^{INK4a}$ promoter regions of NHEKs. When MSP for amplification of methylated region on promoter regions of $p16^{INK4a}$ was performed, the frequency of 5-methylcytosine residues were significantly lowered in NHEKs 72 h after H_2O_2 treatment, while H_2O_2 also increased the frequencies of unmethylation of $p16^{INK4a}$ promoter regions in comparison with untreated NHEKs (Fig. 3). Similar to H_2O_2 treatment in NHEKs, 5-Azac inhibited the methylation of $p16^{INK4a}$ promoter regions in NHEKs. In contrast, neither H_2O_2 nor 5-Azac had little effects on the level of methylation in $p16^{INK4a}$ promoter regions in SCCs.

3.3. ROS suppressed arrested in G0/G1 phase of cell cycle in NHEKs

Given the prominent upstream role of $p16^{INK4a}$ in the negative regulation of Rb pathway [13], we examined the phosphorylation of Rb (p-Rb) and its requirement for $p16^{INK4a}$-mediated arrest of cell cycles in H_2O_2-treated NHEKs. Following the exposure to H_2O_2, there was a loss of Rb phosphorylation that is coincident with the upregulation of $p16^{INK4a}$ at 24 h after the treatment. The decreased the expression of p-Rb and as well as the conservation of total Rb proteins continued until 72 h (Fig. 4A). Furthermore, H_2O_2 decreased the expression of CDK4 in NHEKs, but not in SCCs (Fig. 4B), and the results coincident with the DNA microarray data (Supplemental Table 1), Cell cycle analysis revealed a slight, but significant increase in G0/G1 fraction and decrease in the G2/M fraction in the H_2O_2-treated NHEKs at 48 h (Fig. 4C). In contrast, H_2O_2 had no effects on the phosphorylation of Rb in SCCs, and cell cycle analysis demonstrated a decrease in G0/G1 fraction and an increase in G2/M in the H_2O_2-treatment SCCs at 48 h, indicating a continuous cell cycling.

4. Discussion

Cellular senescence is now considered to be an important tumor-suppression process, preventing DNA-damaged cells from undergoing aberrant proliferation [14,15]. Evading cellular senescence thus seems to be a fundamental task that all cancer cells, including squamous carcinoma cells, should resolve early on. Although the involvement of ROS in the initiation of cellular senescence has been reported in various physiological conditions and pathological processes [16], how ROS induce cellular senescence in normal keratinocytes remains unclear.

We stimulated ROS NHEKs as well as SCCs treated with H_2O_2, and examined the expressions of cellular senescence markers. In comparison between normal cells and cancer cells, the notable differences of their expressions in response to H_2O_2 are observed as follows: (a) SA-β-Gal-positive cells increased in NHEKs, but not SCCs. (b) The expression of $p16^{INK4a}$ and $p21^{cip1}$ increased in NHEKs, but not SCCs. (c) The expression of DNA methyltransferase (DNMT) was suppressed in NHEKs, but not SCCs. (d) The methylation inhibitor 5-Azac enhanced the expressions of $p16^{INK4a}$ and $p21^{cip1}$ in NHEKs, but not SCCs. (e) Methylation level in $p16^{INK4a}$ promoter was reduced with H_2O_2 in NHEKs, but not SCCs. (f) Rb was dephosphorylated with H_2O_2 in NHEKs, but not SCCs. (g) G1/G0 arrest was observed in NHEKs, but not SCCs. These results demonstrate that ROS induce cellular senescence in normal keratinocytes, which is mediated via the upregulation of $p16^{INK4a}$, and that the loss of cellular senescence observed in SCCs seems to be caused by the persistent methylation of $p16^{INK4a}$ promoter.

Numerous studies have elucidated the molecular mechanisms that direct cellular senescence [17,18]. Both $p16^{INK4a}$ and $p21^{cip1}$, whose expressions are invariably elevated in senescent cells, act as two critical pathways to senescence. It is widely accepted that $p16^{INK4a}$ is a tumor suppressor by inhibiting Rb phosphorylation and CDK, inducing cell cycle arrest in the G1/Go phase in response to potentially epigenotoxic or genotoxic stimuli [19]. Our results suggest that $p16^{INK4a}$, rather than $p21^{cip1}$, is a critical tumor suppressor to keratinocyte. To prevent malignant keratinocytes, SCCs, from proliferating and progressing, therefore, it is promising to induce cellular senescence in SCCs. DNA methylation status of $p16^{INK4a}$ promoter may be an effective target to upregulate the $p16^{INK4a}$ expression and induce cellular senescence in SCCs.

Gene expression is known to be modified with epigenetic regulation, the methylation and unmethylation of DNA bases. The levels of cytosine methylation of the gene promoter region are a critical determinant of its target-gene expression, and high levels of methylation are generally associated with repression of gene. Thus, DNA methylation provides an alternative mechanism for inactivation of tumor-suppressor genes during tumorigenesis [20,21]. DNA methylation in mammalian cells is catalyzed by members of cytosine (C)-DNA methyltransferase (DNMT) family. DNMT 1 is through to play a major role in the DNA methylation observed in human cancer cells. The methylation of 60–80% of cytosine residing in the dinucleotide sequence CpG is catalyzed by DNMTs [11,22]. Aberrant methylation of CpG islands is the main mechanism for $p16^{INK4a}$ inactivation in various human cancers [23]. Methylation of $p16^{INK4a}$ gene is an early event in carcinogenesis and has been shown to significantly increase the risk of malignant transformation of epithelial dysplasia in the stomach, oral cavity, and other organs in follow up cohort studies [24]. In fact, the extent of p16 methylation is reported as a prognosis predictor for precancerous lesions [25,26].

In the present study, ROS reduced the level of DNMT1 in NHEKs, resulting in the suppression of methylation of $p16^{INK4a}$ promoter and the subsequent increase in $p16^{INK4a}$ expression. The level of $p16^{INK4a}$ in the NHEKs also increased by treatment with a DNMT inhibitor. Therefore, epigenetic regulation of $p16^{INK4a}$ expression seems to be an essential mechanism of ROS to induce cellular senescence in normal keratinocytes, but not cancer cells SCCs.

It also plays an important role in tumor progression. Senescent cell-producing cytokines can stimulate premalignant epithelial cells to invade a basement membrane. Co-injection of senescent cells with cancer cells enhanced tumor formation in mice [27,28]. Hence, senescent cells are likely to promote malignant progression of precancerous cells and invasion of cancer cells. That may be why the aged population is susceptible to malignant tumorigenesis.

In conclusion, the present data imply that the protection in the ROS-induced carcinogenesis of NHEKs is caused by cellular senescence through the epigenetic regulation of $p16^{INK4a}$.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.123.

References

[1] L. Hayflick, The limited in vitro lifetime of human diploid cell strains, Exp. Cell Res. 37 (1965) 614–636.
[2] D.J. Baker, T. Wijshake, T. Tchkonia, N.K. LeBrasseur, B.G. Childs, B. van de Sluis, J.L. Kirkland, J.M. van Deursen, Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders, Nature 479 (2011) 232–236.
[3] F. Rodier, J. Campisi, Four faces of cellular senescence, J. Cell Biol. 192 (2011) 547–556.
[4] J. Campisi, Cellular senescence as a tumor-suppressor mechanism, Trends Cell Biol. 11S (2001) 27–31.
[5] T. Finkel, N.J. Holbrook, Oxidants, oxidative stress and the biology of ageing, Nature 408 (2000) 239–247.
[6] D.C. Radisky, D.D. Levy, L.E. Littlepage, H. Liu, C.M. Nelson, J.E. Fata, D. Leake, E.L. Godden, D.G. Albertson, M.A. Nieto, Z. Werb, M.J. Bissell, Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability, Nature 436 (2005) 123–127.
[7] T. Fukawa, H. Kajiya, S. Ozeki, T. Ikebe, K. Okabe, Reactive oxygen species stimulates epithelial mesenchymal transition in normal human epidermal keratinocytes via TGF-beta secretion, Exp. Cell Res. 318 (2012) 1926–1932.
[8] T. Lu, T. Finkel, Free radicals and senescence, Exp. Cell Res. 314 (2008) 1918–1922.
[9] Q. Chen, A. Fischer, J.D. Reagan, L.J. Yan, B.N. Ames, Oxidative DNA damage and senescence of human diploid fibroblast cells, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 4337–4341.
[10] S. Nakayama, H. Kajiya, K. Okabe, T. Ikebe, Effects of oxidative stress on the expression of 8-oxoguanine and its eliminating enzymes in human keratinocytes and squamous carcinoma cells, Oral Sci. Int. 8 (2011) 11–16.
[11] M.F. Robert, S. Morin, N. Beaulieu, F. Gauthier, I.C. Chute, A. Barsalou, A.R. MacLeod, DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells, Nat. Genet. 33 (2003) 61–65.
[12] N. Ohtani, K. Yamakoshi, A. Takahashi, E. Hara, Real-time in vivo imaging of p16gene expression: a new approach to study senescence stress signaling in living animals, Cell Div. 5 (2010) 1, http://dx.doi.org/10.1186/1747-1028-5-1.
[13] J. Lukas, D. Parry, L. Aagaard, D.J. Mann, J. Bartkova, M. Strauss, G. Peters, J. Bartek, Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16, Nature 375 (1995) 503–506.
[14] M. Ozturk, A. Arslan-Ergul, S. Bagislar, S. Senturk, H. Yuzugullu, Senescence and immortality in hepatocellular carcinoma, Cancer Lett. 286 (2009) 103–113.
[15] Q. Yan, N. Wajapeyee, Exploiting cellular senescence to treat cancer and circumvent drug resistance, Cancer Biol. Ther. 9 (2010) 166–175.
[16] T. Kuilman, C. Michaloglou, W.J. Mooi, D.S. Peeper, The essence of senescence, Genes Dev. 24 (2010) 2463–2479.
[17] N. Ohtani, Z. Zebedee, T.J. Huot, J.A. Stinson, M. Sugimoto, Y. Ohashi, A.D. Sharrocks, G. Peters, E. Hara, Opposing effects of Ets and Id proteins on $p16^{INK4a}$ expression during cellular senescence, Nature 409 (2001) 1067–1070.
[18] C.A. Schmitt, Cellular senescence and cancer treatment, Biochim. Biophys. Acta 1775 (2007) 5–20.
[19] D.A. Alcorta, Y. Xiong, D. Phelps, G. Hannon, D. Beach, J.C. Barrett, Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 13477–13742.
[20] T. Narimatsu, A. Tamori, N. Koh, S. Kubo, K. Hirohashi, Y. Yano, T. Arakawa, S. Otani, S. Nishiguchi, P16 promoter hypermethylation in human hepatocellular carcinoma with or without hepatitis virus infection, Intervirology 47 (2004) 26–31.
[21] R. Zhu, B.Z. Li, H. Li, Y.Q. Ling, X.Q. Hu, W.R. Zhai, H.G. Zhu, Association of p16INK4A hypermethylation with hepatitis B virus X protein expression in the early stage of HBV-associated hepatocarcinogenesis, Pathol. Int. 57 (2007) 328–336.

[22] M. Szyf, The role of DNA hypermethylation and demethylation in cancer and cancer therapy, Curr. Oncol. 15 (2008) 72–75.
[23] A. Merlo, J.G. Herman, L. Mao, D.J. Lee, E. Gabrielson, P.C. Burger, S.B. Baylin, D. Sidransky, 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers, Nat. Med. 1 (1995) 686–692.
[24] J. Cao, J. Zhou, Y. Gao, L. Gu, H. Meng, H. Liu, D. Deng, Methylation of p16 CpG island associated with malignant progression of oral epithelial dysplasia: a prospective cohort study, Clin. Cancer Res. 15 (2009) 5178–5183.
[25] D. Deng, Z. Liu, Y. Du, Epigenetic alterations as cancer diagnostic, prognostic, and predictive biomarkers, Adv. Genet. 71 (2010) 125–176.
[26] J. Zhou, J. Cao, Z. Lu, H. Liu, D. Deng, A 115-bp MethyLight assay for detection of p16 (CDKN2A) methylation as a diagnostic biomarker in human tissues, BMC Med. Genet. 12 (2011) 67, http://dx.doi.org/10.1186/1471-2350-12-67.
[27] B. Bhatia, A.S. Multani, L. Patrawala, X. Chen, T. Calhoun-Davis, J. Zhou, L. Schroeder, R. Schneider-Broussard, J. Shen, S. Pathak, S. Chang, D.G. Tang, Evidence that senescent human prostate epithelial cells enhance tumorigenicity: cell fusion as a potential mechanism and inhibition by $p16^{INK4a}$ and hTERT, Int. J. Cancer 122 (2008) 1483–1495.
[28] A. Krtolica, S. Parrinello, S. Lockett, P.Y. Desprez, J. Campisi, Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 12072–12077.