

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

Mina Sasaki^{a,b}, Hiroshi Kajiya^{a,*}, Satoru Ozeki^b, Koji Okabe^a, and Tetsuro Ikebe^b

^aDepartment of Physiological Science and Molecular Biology, Fukuoka Dental College, Fukuoka 8140193, Japan

^bDepartment of Oral and Maxillofacial Surgery, Fukuoka Dental College, Fukuoka 8140193, Japan

***Corresponding author:**

Hiroshi Kajiya, Ph.D., Department of Physiological Science and Molecular Biology, Fukuoka Dental College, Tamura 2-15-1, Sawara-ku, Fukuoka 8140193, Japan

Tel: +81-92-801-0411 ext. 660

Fax: +81-92-801-4909

E-mail: kajiya@college.fdcnet.ac.jp

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 NHEK, we examined the effect of hydrogen peroxide (H_2O_2) on the expression of cellular senescence-associated molecules in NHEK, 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 NHEK, but not SCCs. The expression of cyclin-dependent kinase (CDK) inhibitors, especially $p16^{INK4a}$ was upregulated in NHEK treated with H_2O_2 . Interestingly, H_2O_2 suppressed the methylation of $p16^{INK4a}$ promoter region in NHEK, 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 NHEK 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 NHEK.

Key words: reactive oxygen species; normal human epidermal keratinocyte; cyclin-dependent kinase inhibitors; cellar senescence

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].

ROS, such as superoxide anions, hydroxyl radicals, and H₂O₂ 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 NHEK through TGF- β 1 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 SCC has 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 SCC, it is critical to understand the mechanism of cellular senescence, as tumor suppression, of keratinocyte, being compared with SCC.

The aim of the present study was to clarify whether cellular senescence contributes to the protection of carcinogenesis in NHEK. We have investigated the effects of H₂O₂ on the expression of cellular senescence markers and tumor suppressor molecules involved in CDK inhibitors in NHEK, compared to those in SCCs.

2. Materials and methods

2.1. Cell Culture

NHEK were purchased from Lonza Co., Ltd. (cat no.00192627;Tokyo, Japan) and cultured in KGM2 medium (Lonza Co., Lt.). 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. SCC cells were maintained on culture dishes, in D-MEM with 10% fetal bovine serum. The cells were incubated in culture medium with or without H₂O₂ (800 μM) for 0-72h. The cells were cultured at 37 °C in a water-jacketed 5% CO₂ incubator. In some experiments, cells were incubated in culture medium supplemented with 5-AzazC (10 μM) or menadion (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 TRIZOL 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 sec denaturation at 94 °C, 30 sec annealing at 52-55 °C, and 1 min extension at 72 °C, for 28-35cycles. 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 (10mM Tris-HCl, 50mM, NaCl 0.25% Tween-20), then incubated for 1h 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 demethylated 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 sec denaturation at 98 °C, 30 sec annealing at 54 °C, and 30 sec extension at 72 °C, for 40 cycle. Subsequently, products of the PCR were amplified with p16 gene specific primers: 10 sec denaturation at 98 °C, 30 sec annealing at 54 °C, and 30 sec extension at 72 °C, for 32-38 cycles. To detect ratio of methylation in promoter sequence, we selected specific primer based on the nucleotide sequence of p16 promoter region in cDNA.

2.6. FACS analysis

NHEK (1.0x10⁶ cells/ml) and SCC (1.0x10⁶ cells/ml) were cultured on 10cm dishes with or without H₂O₂ 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 μ g/mL) solution and 4 μ l 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 H₂O₂ (800 μM) exerted the oxidation to DNA forming 8-oxo-7, 8-dihydroguanine, and induced EMT via TGF-β autocrine secretion in NHEK [7, 10]. However, little is known about the effect of ROS on cellular senescence of NHEK in terms of the protection of tumor progression in NHEK. We firstly performed a genome-wide screening of tumor suppressor genes following treatment with H₂O₂ using Gene Chip Human Genome U133 plus 2.0 arrays. H₂O₂-induced ROS stimulation for 72 h altered the mRNA expression of many cell cycle markers in NHEK 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 NHEK, which are associated with the cellular senescence markers. Furthermore, the expression of CDKs in cell cycling, especially CDK4 and CDK6 decreased in NHEK. 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 NHEK we examined the effect of H₂O₂ on the expression of a well-known senescence associated biomarker (SA-β-Gal) activity. The treatment with H₂O₂ significantly increased the number of NHEK positive in SA-β-Gal activity in a time-dependent manner, indicating ROS induced cellular senescence in

NHEK (Fig. 1A). In contrast, H₂O₂ had little effect on the SA-β-Gal activity in SCCs.

H₂O₂ significantly increased the expression of p21^{cip1} and p16^{INK4a} in NHEK in a time-dependent manner (Fig. 1B). In contrast, H₂O₂ 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 H₂O₂>300μM (data not shown). Similar to the PCR results, the increased expressions of their proteins were observed 6h after treatment of H₂O₂ in NHEK, whereas H₂O₂ had no effect on the expression of p16^{INK4a} and reduced the p21^{cip1} expression in SCCs.

Furthermore, the other ROS inducer menadion (10μM) also increased the expression of mRNA and protein of p16^{INK4a} in a time-dependent manner in NHEK (Fig. 2A). The H₂O₂-induced upregulation of p16^{INK4a} proteins was suppressed by the pretreatment with antioxidant drug N-acetylcystein (NAC;10μM) in NHEK.

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

Interestingly, we found that H₂O₂ simultaneously decreased the expression of (cytosine-5)-DNA methyltransferase (DNMT) 1 in NHEK, 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 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 NHEK. Similar to H₂O₂ treatment, 5-Azac upregulated the expressions of CDK inhibitors, especially p16^{INK4a} in NHEK in a time-dependent manner (Fig. 2B). In contrast, H₂O₂ had little effect on the expression of both CDK inhibitors in SCCs. The methylation of the promoter lesion in p16^{INK4a} gene was reported to be suppressed the expression of p16 in human cells [12]. The results suggest that H₂O₂ inhibit the methylation of p16^{INK4a} promoter, resulting in the upregulation of p16^{INK4a} in NHEK.

Then, we examined whether H₂O₂ affected the DNA methylation level of CpG in p16^{INK4a} promoter regions of NHEK. 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 NHEK 72h after

H₂O₂ treatment, while H₂O₂ also increased the frequencies of unmethylation of p16^{INK4a} promoter regions in comparison with untreated NHEK (Fig. 3). Similar to H₂O₂ treatment in NHEK, 5-Azac inhibited the methylation of p16^{INK4a} promoter regions in NHEK. In contrast, neither H₂O₂ nor 5-Azac had little effects on the level of methylation in p16^{INK4a} promoter regions in SCCs.

33. 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 H₂O₂-treated NHEK. Following the exposure to H₂O₂, there was a loss of Rb phosphorylation that is coincident with the upregulation of p16^{INK4a} at 24h 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, H₂O₂ decreased the expression of CDK4 in NHEK, but not in SCCs (Fig. 4B), and the results coincident with the DNA microarray data (Table 1), Cell cycle analysis revealed a slight, but significant increase in G0/G1 fraction and decrease in the G2/M fraction in the H₂O₂-treated NHEK at 48h (Fig.4C). In contrast, H₂O₂ 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 H₂O₂-treatment SCCs at 48h, 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 [18], how ROS induce cellular senescence in normal keratinocytes remains unclear.

We stimulated ROS NHEK as well as SCCs treated with H₂O₂, 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 H₂O₂ are observed as follows: (a) SA-β-Gal-positive cells increased in NHEK, but not SCCs. (b) The expression of p16^{INK4a} and p21^{cip1} increased in NHEK, but not SCCs. (c) The expression of DNA methyltransferase (DNMT) was suppressed in NHEK, but not SCCs. (d) The methylation inhibitor 5-Azac enhanced the expressions of p16^{INK4a} and p21^{cip1} in NHEK, but not SCCs. (e) Methylation level in p16^{INK4a} promoter was reduced with H₂O₂ in NHEK, but not SCCs. (f) Rb was dephosphorylated with H₂O₂ in NHEK, but not SCCs. (g) G1/G0 arrest was observed in NHEK, 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 thought 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 [22, 23]. Aberrant methylation of CpG islands is the main mechanism for p16^{INK4a} inactivation in various human cancers [24]. 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 [25]. In fact, the extent of p16 methylation is reported as a prognosis predictor for precancerous lesions [26, 27].

In the present study, ROS reduced the level of DNMT1 in NHEK, 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 NHEK 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 [28, 29]. 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 NHEK is caused by cellular senescence through the epigenetic regulation of p16^{INK4a}.

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Figure legends

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 H₂O₂ (800 μ M) for 48h and stained with SA- β -Gal staining for the indicated times. The panels indicate SA- β -gal staining images for the indicated times after H₂O₂ treatment, respectively. Bars indicate 100 μ m. The SA- β -Gal-positive cells were counted on six wells. Data shown are the mean from six culture wells (mean \pm SEM). ** p < 0.01, NHEK versus SCC 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 NHEK

(A) After treatment of with menadion (10 μ M) 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. NHEK were incubated H₂O₂ before and 72h after treatment with N-acetylcystein (NAC; 10 μ M). (B) After treatment with a methylation inhibitor 5-Azac (10 μ M) 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.

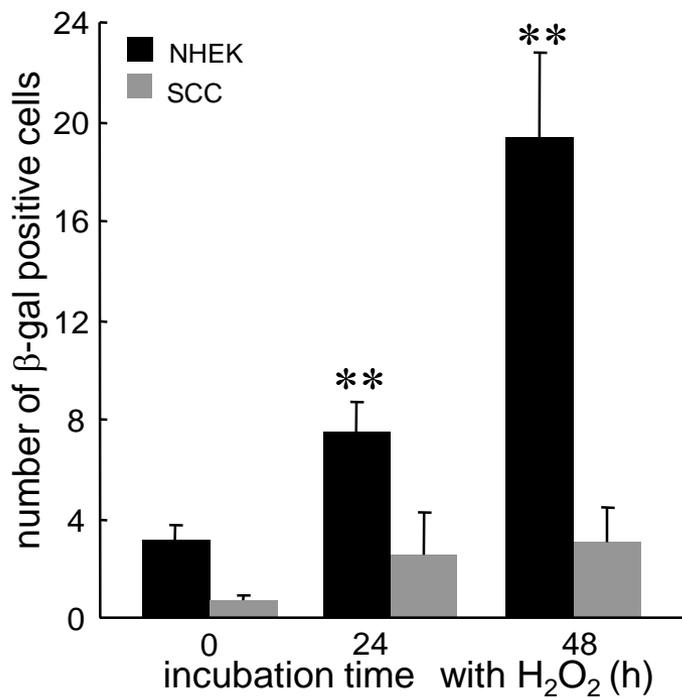
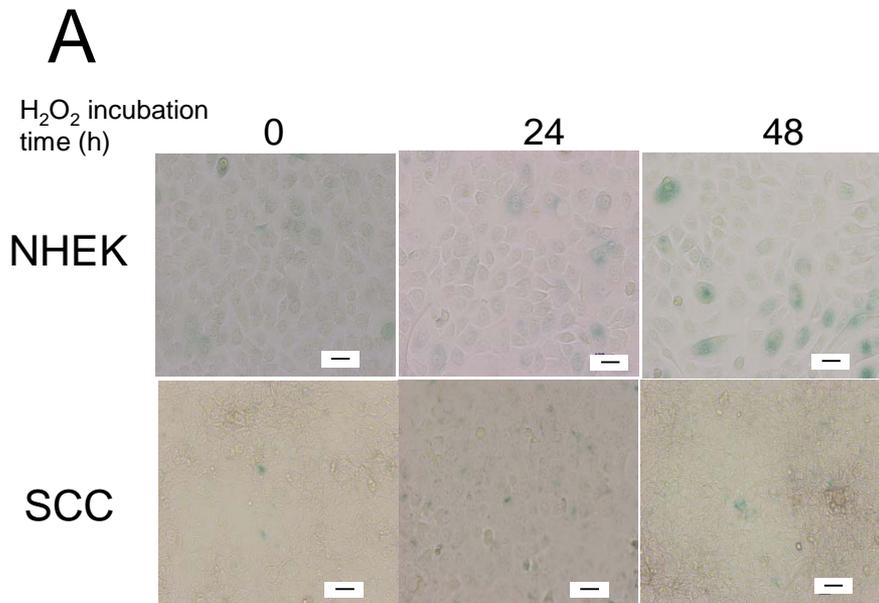
Fig. 3. ROS suppressed the methylation of p16 promoter region in NHEK.

(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 0h (control). (B) Methylation levels of p16 promoter region after H₂O₂ or 5-Azac (10 μ M) 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.

Fig. 4. ROS arrested in G0/G1 phase of cell cycle in NHEKs.

(A) After the treatment with H₂O₂ (800μM) 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 H₂O₂ 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 72h after H₂O₂ treatment in the cells. Accumulated percentages of cells in each phase treated with H₂O₂. Data shown are the mean from six culture wells (mean±SEM). ** indicates $P<0.01$ vs control.



B

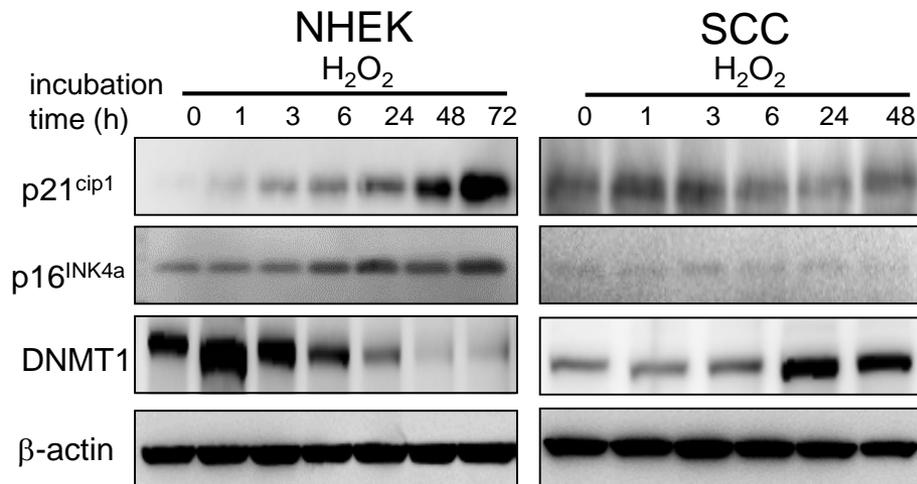
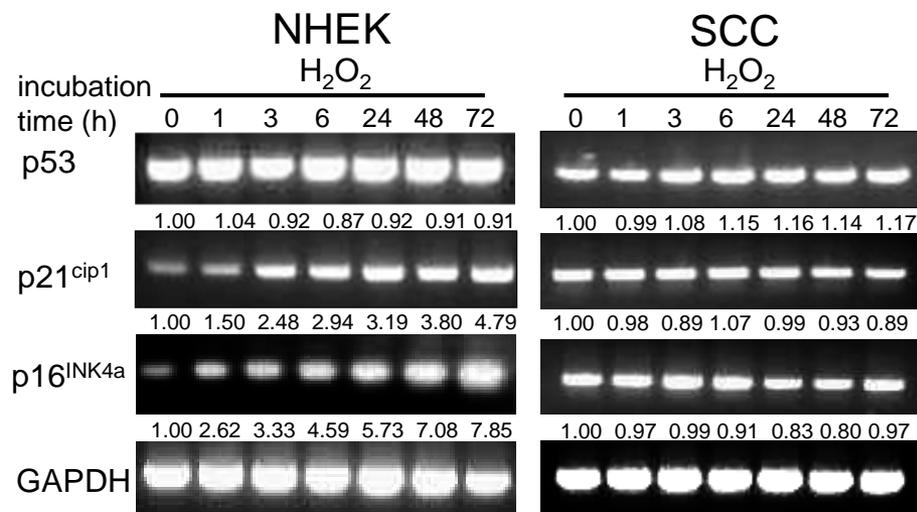
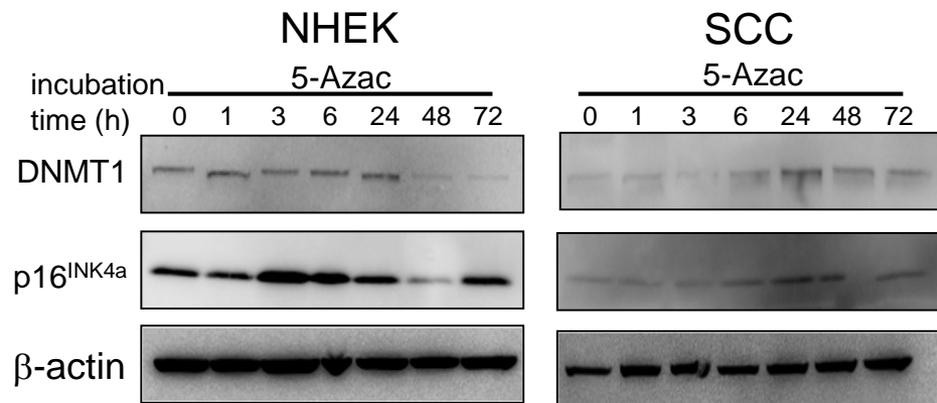
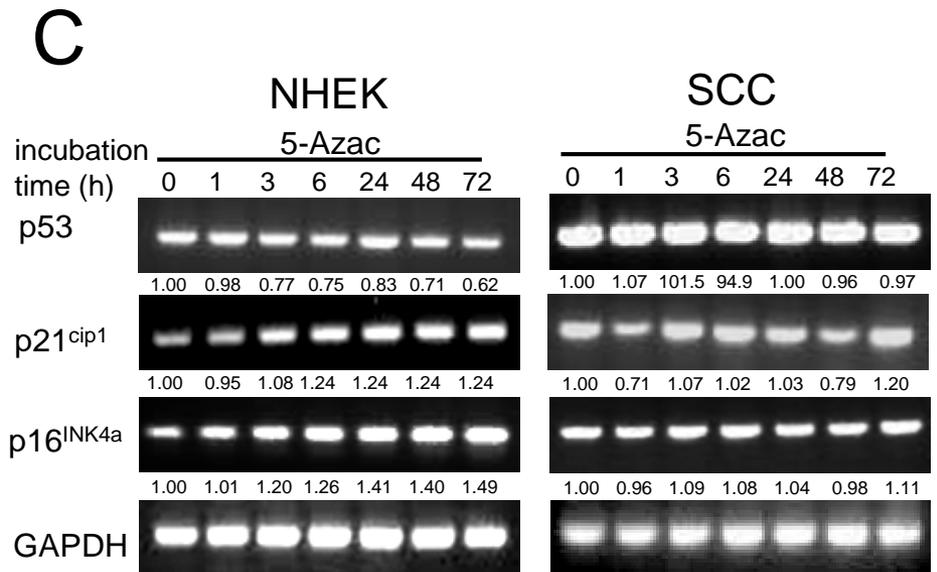
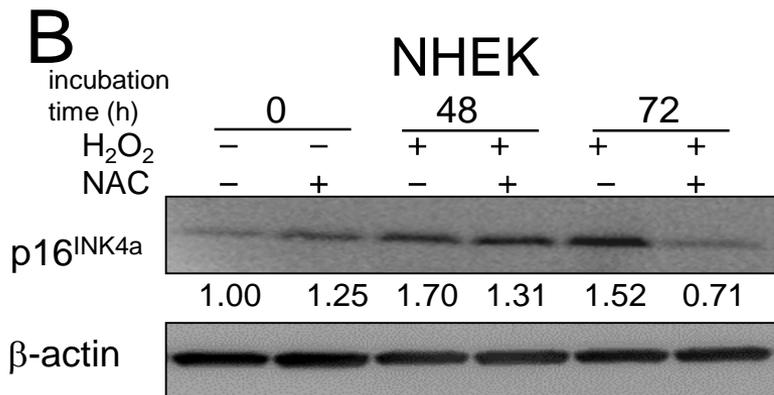
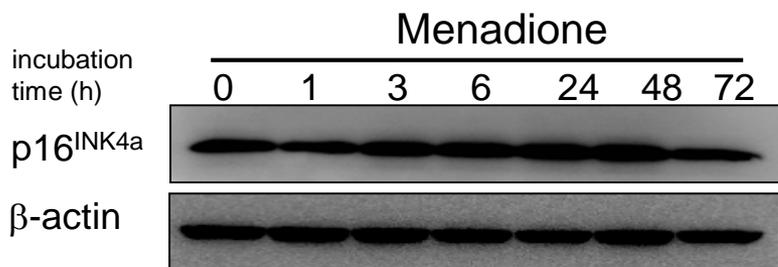
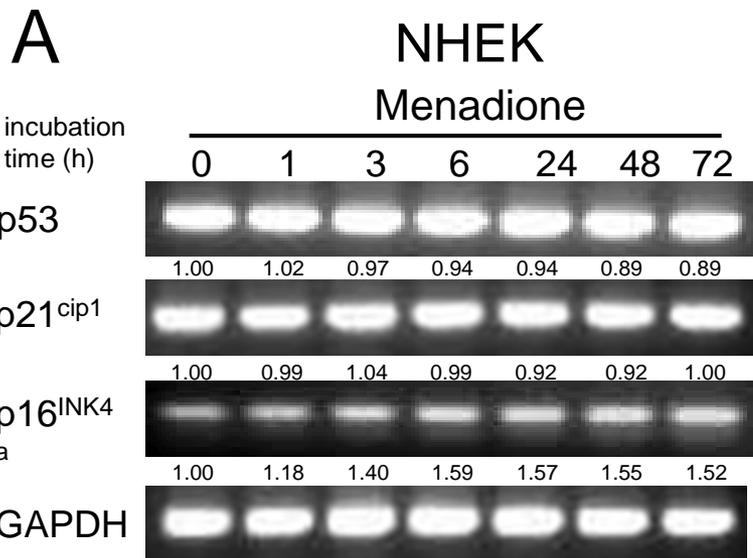


Fig. 1



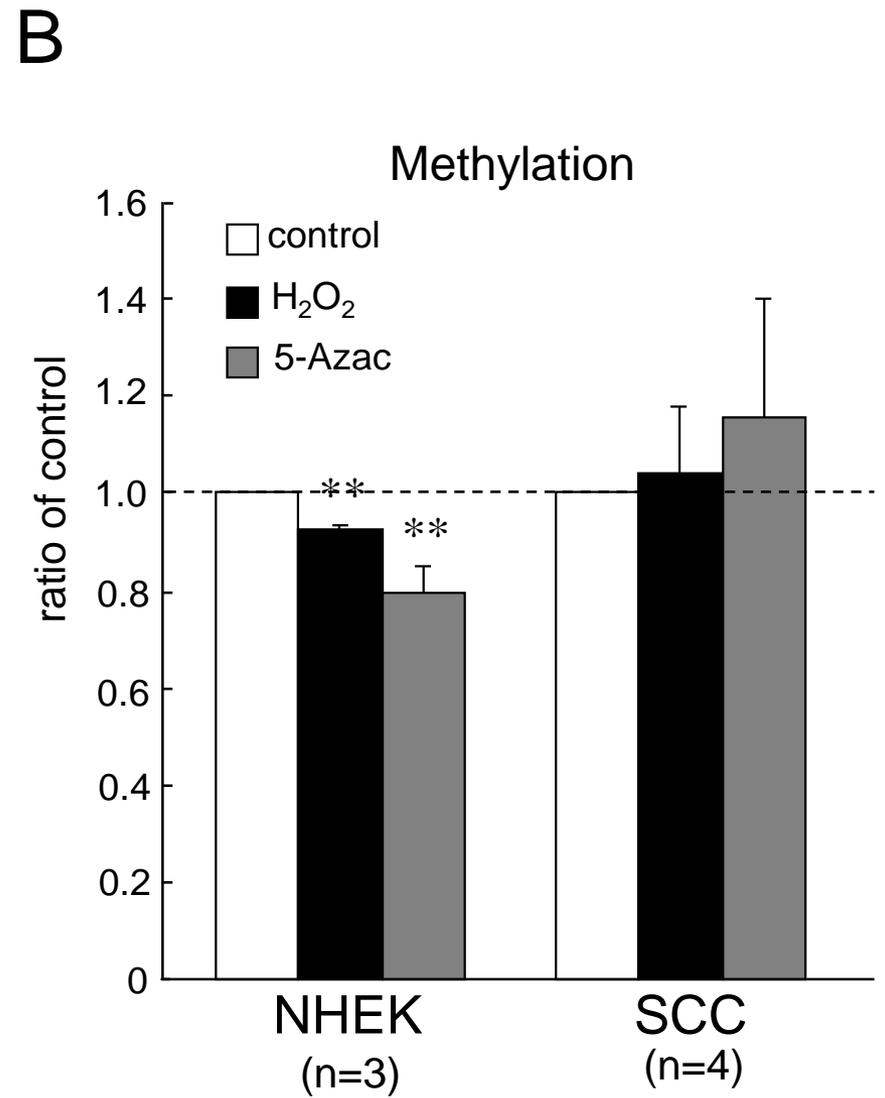
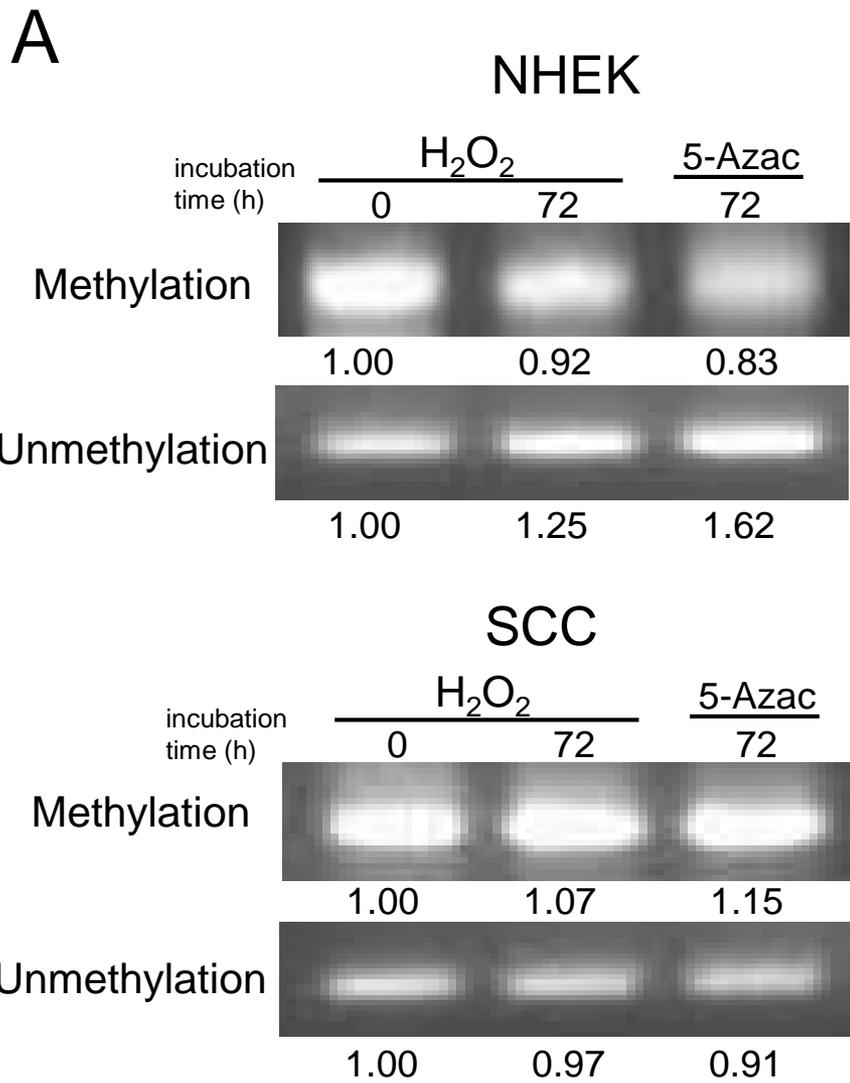
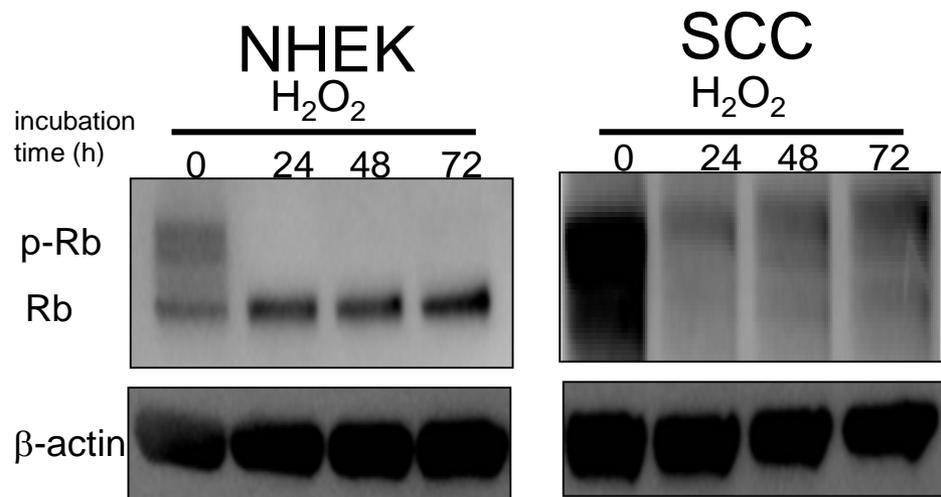
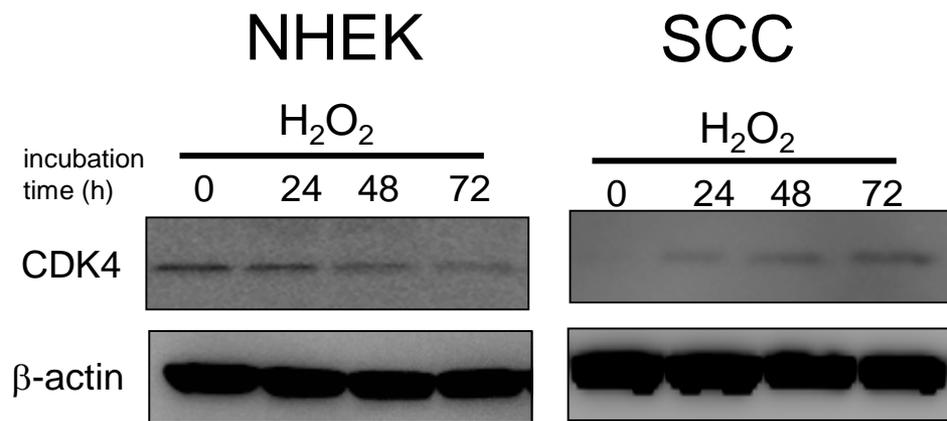
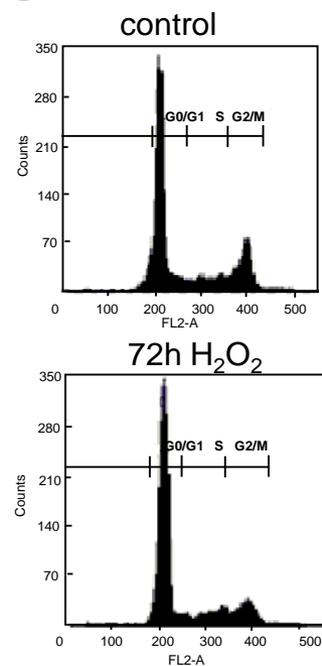
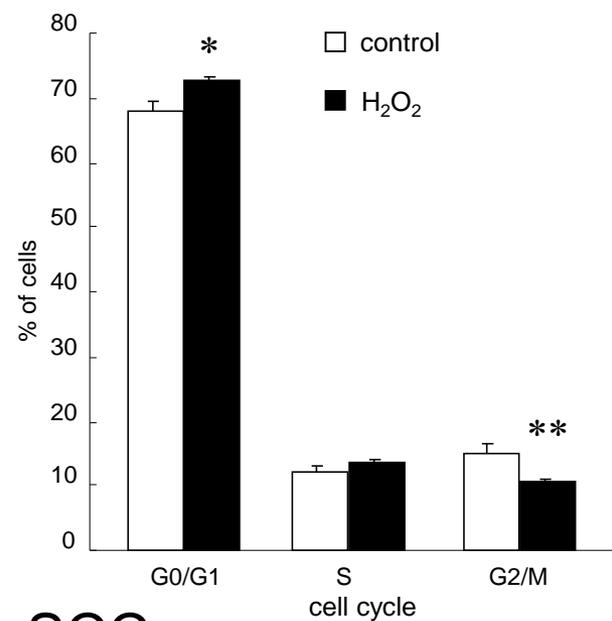
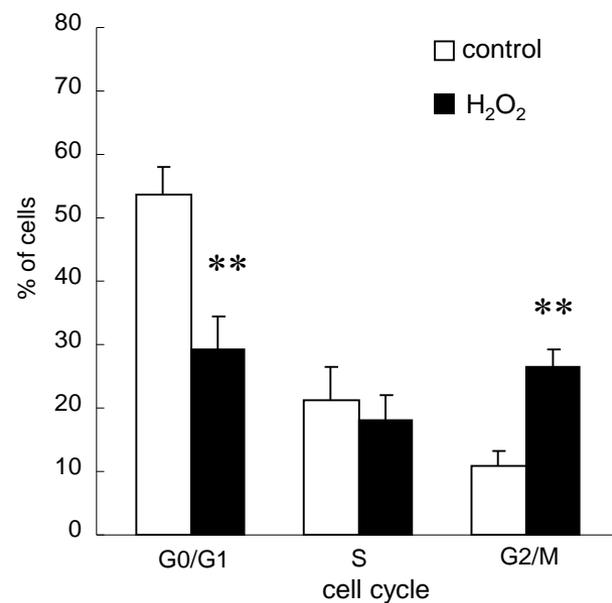
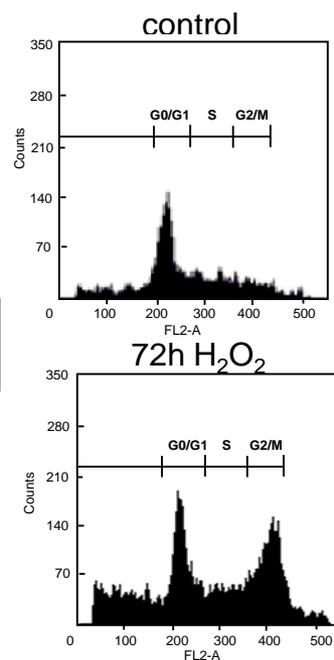


Fig. 3

A**B****C****NHEK****SCC**

Supplement table1. Expressions of cell senescence markers increased by reactive oxygen species

Entrez gene symbol	GeneBank accession No.	Fold change	
		NHEK	SCC
p53	NM_000546	0.6308215	1.2258246
p21^{cip1}	NM_078467	2.7392757	1.2457686
p16^{INK4}	NM_058197	1.7523187	0.940313
CDK6	NM_001259	0.4435822	1.2457686
CDK4	NM_000075	0.5053371	1.2258246
CDK2	NM_001798	0.9827361	0.9403130

After H₂O₂ treatment for 72 hours, the expressions of cellular senescence markers in NHEKs and SCCs were evaluated using DNA microarray analysis.