Biochemical and Biophysical Research Communications 443 (2014) 49–55

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Disturbance of DKK1 level is partly involved in survival of lung cancer cells via regulation of ROMO1 and γ-radiation sensitivity

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ARTICLE INFO

Article history:
Received 6 November 2013
Available online 20 November 2013

Keywords:
Cell survival
DKK1
ROMO1
NSCLC
Radiation resistance
ROS

ABSTRACT

Dickkopf1 (DKK1), a secreted protein involved in embryonic development, is a potent inhibitor of the Wnt signaling pathway and has been postulated to be a tumor suppressor or tumor promoter depending on the tumor type. In this study, we showed that *DKK1* was expressed differently among non-small-cell lung cancer cell lines. The *DKK1* expression level was much higher in A549 cells than in H460 cells. We revealed that blockage of *DKK1* expression by silencing RNA in A549 cells caused up-regulation of intracellular reactive oxygen species (ROS) modulator (ROMO1) protein, followed by partial cell death, cell growth inhibition, and loss of epithelial–mesenchymal transition property caused by ROS, and it also increased γ-radiation sensitivity. *DKK1* overexpression in H460 significantly inhibited cell survival with the decrease of ROMO1 level, which induced the decrease of cellular ROS. Thereafter, exogenous *N*-acetylcysteine, an antioxidant, or hydrogen peroxide, a pro-oxidant, partially rescued cells from death and growth inhibition. In each cell line, both overexpression and blockage of *DKK1* not only elevated p-RB activation, which led to cell growth arrest, but also inactivated AKT/NF-kB, which increased radiation sensitivity and inhibited cell growth. This study is the first to demonstrate that strict modulation of *DKK1* expression in different cell types partially maintains cell survival via tight regulation of the ROS-producing *ROMO1* and radiation resistance.

1. Introduction

The Wnt/β-catenin signal is a critical and strictly regulated pathway of physiological processes underlying normal embryonic development and tissue homeostasis. This pathway is also involved in an intricate network of signaling that serves as a negative regulator of osteogenesis in adult animals [1,2]. Deregulated activation of the Wnt/β-catenin signal is an early event in many human cancer types, and it is thought to be associated with an aggressive phenotype of several cancers [3]. Dickkopf1 (DKK1) is the predominant secretory antagonist of the Wnt/β-catenin signal, suggesting that if DKK1 were not precisely regulated, it could result in tumor formation and progression. In fact, several clinical studies demonstrated that DKK1 was down-regulated in breast, melanoma, and colon cancer [4,5]. Other study showed that oncogenic Wnt/β-catenin signaling pathway is down-regulated by DKK1 [6]. Qiao et al. also showed that conditioned media from human mesenchymal stem cells, with high levels of DKK1, inhibited growth of human MCF-7 breast cancer cells *in vitro* and *in vivo* by inhibiting or down-regulating the Wnt/β-catenin signaling pathway [7]. In turn, removing DKK1 from the medium by adding a neutralizing antibody nullified these inhibitory effects. This result strongly suggests that DKK1 can function as a suppressor of tumor growth in a paracrine fashion. However, DKK1 expression levels are elevated in a wide variety of cancers. Several reports also suggest that DKK1 overexpression is associated with cancer progression and poor prognosis; for example, there have been several reports of DKK1 up-regulation in malignant cancers including multiple myeloma, hepatoblastoma, and Wilms' tumor [8,9]. DKK1 expression in myeloma cells plays a major role in osteoblastic differentiation in patients with multiple myeloma [9].

An enhanced level of reactive oxygen species (ROS) from the mitochondria has been investigated in many cancer cells [10,11]. Previous studies showed that a strictly regulated cellular level of ROS is essential for the proliferation of tumor cell growth, and *ROMO1* expression triggered ROS production in the mitochondria and thus cell growth [12,13]. Therefore, cell growth inhibition

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http://dx.doi.org/10.1016/j.bbrc.2013.11.038

by *ROMO1* suppression with silenced RNA (siRNA) can be rescued by exogenous oxidant such as hydrogen peroxide (H_2O_2). In addition, ROS production caused by *ROMO1* overexpression is associated with the invasiveness of hepatic tumor cells [14]. Together, these findings suggest that *ROMO1* expression-induced ROS production plays a critical role in redox signaling and thus cell proliferation in cancer cells.

In this study, we revealed that DKK1 negatively regulates the expression of *ROMO1* and thus modulates cellular ROS in non-small-cell lung cancer (NSCLC) cell lines. Disturbance of cellular DKK1 levels is partially involved in cell death, cell growth inhibition, and loss of the epithelial–mesenchymal transition (EMT) property via ROMO1-induced imbalance of intracellular ROS and resistance to γ-radiation.

2. Materials and methods

2.1. Cell culture and irradiation

All NSCLC cell lines used in the study were obtained from Korea Cell Line Bank (Seoul, Korea) and grown in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 95% air/5% CO_2. Cells were inoculated at a density of 1×10^5 cells in a T-25 flask, incubated for 1 day, and then irradiated with a dose of 2 Gy from a ^{60}Co γ-ray at a dose rate of 0.2 Gy/min.

2.2. Construction of DKK1 and ROMO1 expression vector and PCR amplification

An 801-bp insert of human *DKK1* and 240-bp insert of human *ROMO1* were amplified from human lung cancer cell poly(A) mRNA by RT-PCR to construct the *DKK1* and *ROMO1* gene expression vector. Total RNA was isolated from H460 or A549 cells, with the use of TRIzol reagent (Invitrogen) RNA extraction. To generate first-strand complementary DNA (cDNA) from the total RNA (1 μg) using oligo-dT, we used a cDNA synthesis kit (Intron Biotechnology). Resultant cDNAs served as templates for PCR amplification with the following forward and reverse primers (DKK1, *Eco*RI [forward]: 5′-ATATGAATTCATGATGGCTCTGGGCG-3′; *Xho*I [reverse]: 5′-ATATCTCGAGTTAGTGTCTCTGACAAG-3′; ROMO1, *Eco*RI [forward]: 5′-ATATCTCGAGATGCCGGTGGCCGTG-3′; *Xho*I [reverse]: 5′-ATATCTCGAGTTAGCATCGGATGCC-3′). The DKK1 and ROMO1 cDNA inserts were cloned into the mammalian expression vector pcDNA3.1 (Invitrogen). The DKK1 and ROMO1 gene expression vector was transfected into the cells using Lipofectamine 2000 (Invitrogen), followed by selection with 400 μg/ml G418 (Calbiochem). For PCR analysis of other gene expression, we analyzed *β-actin* (forward: 5′-CATCCTCACCCTGAAGTACCC-3′; reverse: 5′-AGCCTGGATAGCAACGTACATG-3′) and *GAPDH* (forward: 5′-ATGGGGAAGGTGAAGG-3′; reverse: 5′-TTACTCCTTGGAGGCC-3′). The PCR conditions for *DKK1*, *ROMO1*, *β-actin*, and *GAPDH* were as follows: denaturating at 94 °C for 5 min; followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; and final extension at 72 °C for 10 min. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis and then photographed under ultraviolet light.

2.3. Western blot analysis

Western blot analysis was performed with primary antibodies specific for human DKK1, p53, ROMO1, cyclin D1, NF-kB, and retinoblastoma protein (Santa Cruz Biotechnology), p-AKT, AKT, E-cadherin, $p21^{Waf1/cip1}$, phosphorylated retinoblastoma protein (p-RB: serine 608, 780, and 807/811), β-actin (Cell Signaling Technology), Vimentin (Thermo Fisher Scientific), N-cadherin and anti-Snail (Abcam). Protein concentration was determined with a protein assay (Bio-Rad). Equal amounts of protein were separated on a 10–15% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia). The blots were blocked for 1 h at room temperature with blocking buffer (10% nonfat milk in PBS containing 0.1% Tween 20; TBS). The membrane was incubated overnight in a cold chamber with specific antibodies. After being washed with TBS, the membrane was incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase linked secondary antibody (Cell Signaling Technology) and visualized with the Westzol enhanced chemiluminescence detection kit (Intron Biotechnology).

2.4. Silencing RNA targeting of DKK1

Cells were transfected with three different Stealth RNA targeting *DKK1* genes (Invitrogen; primer sequences: 5′-CACUAAACCAGCUAUCCAA-3′/5′-UUGGAUAGCUGGUUUAGUG-3′) or with Stealth RNAi Negative Control Medium GC at a concentration of 80 nM of Lipofectamine RNAi MAX reagent (Invitrogen). The cells were incubated for 72 h after transfection, and then *DKK1* expression was determined by reverse transcription RT-PCR.

2.5. Flow cytometry analysis

Cells (1×10^6 cells in 200 μl PBS) were collected, washed with PBS, and fixed with 70% ethanol at 4 °C for 2 h in the dark. Fixed cells were washed with PBS and stained with propidium iodide (50 μg/ml). The DNA content was measured with a FACScan (EPICS XL; Beckman Coulter Counter). A minimum of 10,000 cells was counted for each sample. The percentage of cells in each phase was determined by Phoenix Multicycler Software (Phoenix Flow System).

2.6. Colony-forming assay

For the colony-forming assay, transfected suppression and overexpression cells were plated in 35-mm culture dishes at 1×10^3 cells per plate and allowed to attach overnight. Cells were left untreated or exposed to a 2 Gy dose of radiation and then incubated for 10–14 days post-irradiation and stained with 0.5% crystal violet. Colonies (i.e., groups $\geqslant$ 50 cells) were counted. Clonogenic survival was expressed as a percentage relative to the untreated controls.

2.7. Microscopic analysis

All attached cells at the bottom of the culture plate were photographed by light microscopy (Leica Microsystems). Images were captured with a Canon Power Shot S45 digital camera system.

2.8. Detection of intracellular ROS

Intracellular ROS was measured by using 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes), as described previously [15].

2.9. Senescence-associated β-galactosidase (SA-β-Gal) activity

Staining for SA-β-Gal activity was performed using bromo-4-chloro-3-indolyl-β-D-galactosidase according to the manufacturer's instructions (Intron Biotechnology). Quantification of SA-β-Gal-positive cell was obtained by counting five random fields per dish and calculating the percentage.

2.10. Invasion and migration assay

Cell invasion and migration were performed as described previously [16] using Matrigel-coated invasion/migration chambers (BD Biosciences) according to the manufacturer's instructions. The stained cells were counted under a light microscope in four randomly chosen fields, with results expressed as means of triplicates from a representative of two independent experiments.

3. Results

3.1. Different expression pattern of DKK1 in H460 and A549 cells

Within NSCLC lines, large cell carcinoma H1299 cells (p53 null mutant) and adenocarcinoma A549 cells (p53 wild type) have different invasive and resistance characteristics to γ-radiation as compared with large cell carcinoma H460 cells (p53 wild type) [17–19]. In a preliminary study, to identify new genes involved in ionizing radiation resistance we compared expression patterns of 30,000 human genes between H460 and A549, using a DNA chip (data not shown). *DKK1* showed very different expression patterns between H460, H1299, and A549 cells. We confirmed the expression level of *DKK1* in H460, H1299, and A549 cells by RT-PCR analysis and Western blot analysis. *DKK1* was expressed at high levels in A549 or H1299 cells but at low levels in H460 cells (Fig. 1A). To investigate the involvement of epigenetic modification on *DKK1* expression, we analyzed the methylation pattern of *DKK1* using the bisulfite pyrosequencing method in A549 and H460 cells, both of which are p53 wild types. In CpG sites at all positions (4 positions) tested in H460 and A549 cells, the average extent of the methylation was approximately 1–6%. Moreover, the degree of methylation was similar in A549 and H460 cells (supplementary data 1). Therefore, we concluded that different *DKK1* expression patterns among NSCLC lines is due to mechanisms other than DNA epigenetic modification, such as different micro RNA.

To investigate whether the appropriate *DKK1* expression level is involved in the signaling pathway for cell growth, cell death, and radiation resistance in NSCLC cells, *DKK1* expression was suppressed by siRNA in A549 and *DKK1* was overexpressed in H460 cells using pcDNA3.1 expressing vectors (Fig. 1B).

3.2. DKK1 expression is associated with cell death, cell growth, and radiation resistance in NSCLC cells

Apoptotic cell death was assayed by FACS analysis, and cell growth and radiation resistance were determined by colony-forming assay. Blockage of *DKK1* expression with siRNA significantly induced cellular ROS in *DKK1*-overexpressing A549 cells (Fig. 2A). As ROS increased due to *DKK1* suppression, apoptotic cell death and cell growth inhibition were significantly induced. We investigated whether this is partially caused by an increase in cellular ROS induced by DKK1 blockage. Treatment with *N*-acetylcysteine, an ROS-scavenging antioxidant, significantly rescued the apoptotic cell death and growth inhibition induced by DKK1 blockage (Fig. 2B and C). The colony-forming assay also showed that blockage of *DKK1* expression also significantly sensitized the cellular response to γ-radiation (Fig. 2D). Our findings clearly showed that DKK1 negatively regulates *ROMO1*, which has been show to regulate cell proliferation and serum-deprived apoptotic cell death [13,20]. Suppression of *DKK1* expression in A549 cells increased *ROMO1* expression and, in turn, increased cellular ROS (Fig. 2E).

In H460 large carcinoma cells with low *DKK1* expression, enforced *DKK1* overexpression also induced cell death with the decrease of intracellular ROS (Fig. 3A and B). However, exogenous pro-oxidant H_2O_2 significantly rescued apoptotic cell death caused by *DKK1* overexpression, ROMO1 suppression, and ROS decline. Cell growth was also greatly inhibited by increased cellular DKK1 in H460 cells, and cell growth inhibition was partially recovered by pro-oxidant H_2O_2 (Fig. 3C). DKK1 overexpression in H460 cells also increased the γ-radiation sensitivity (Fig. 3D). In contrast to DKK1 blockage, enforced DKK1 overexpression decreased *ROMO1* expression and cellular protein levels, which decreased intracellular ROS (Fig. 3E). We also showed that the *ROMO1* expression level in H460 and A549 cells was similar, meaning proper ROMO1-induced ROS is essential for cell survival (Fig. 3F). These results indicate that disturbance of cellular level of DKK1 (both overexpression and knockdown) is partially associated with cell death and cell growth arrest via *ROMO1* regulation. We also confirmed that both enforced *ROMO1* overexpression or knockdown significantly inhibited cell growth and induced cell death of H460 and A549 lung cancer cells (supplementary data 2). Together, these findings strongly suggest that in order to optimize cell growth and survival in each NSCLC line, its characteristic level of DKK1, which functions as a modulator of cellular ROS level via strict regulation of *ROMO1* expression, must be maintained.

3.3. Disturbance of DKK1 level inactivated p-RB and AKT/NF-kB and induced loss of EMT property

To identify downstream targets of DKK1 that regulate cell proliferation and cell resistance in A549 cells, we analyzed cellular levels of p53, $p21^{Waf1/cip1}$, and p-RB, which are known to be primarily responsible for cell growth arrest and thus cellular senescence initiation [21]. In DKK1-suppressed A549 cells, p53 and $p21^{Waf1/cip1}$, which be accumulated in cells and thus suppress tumors, were not induced. However, phosphorylation at serine 608, 780, and 807/811 residues of p-RB was significantly inhibited, which prevents the detachment of E2F from p-RB and thus

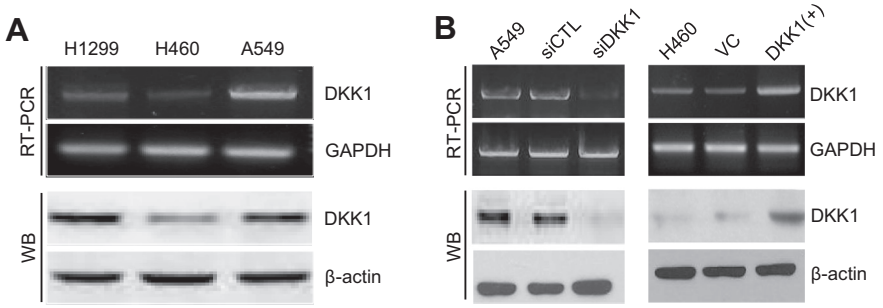

Fig. 1. RT-PCR and Western blot analysis of (A) DKK1 gene and protein in control non-small-cell lung cancer cell lines; (B) proteins in A549 cells that suppressed *DKK1* expression with siRNA or H460 cells that overexpressed *DKK1* with pcDNA3.1 gene expression vectors. Cells (1×10^5) were transfected with 10 nM siRNA or 2 μg pcDNA3.1/DKK1 gene expression vector and grown for 72 h; thereafter, cells were harvested for the analysis.

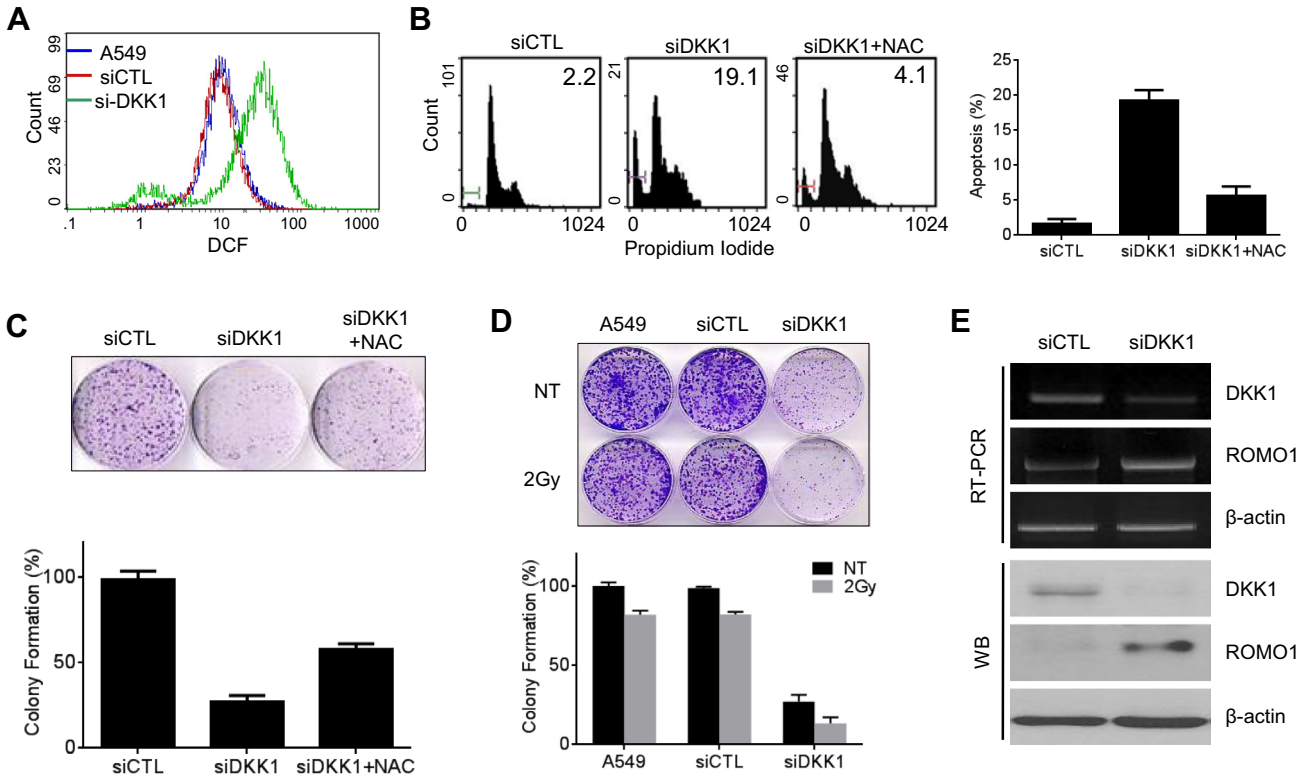

Fig. 2. Comparison of cell cytotoxicity using colony-forming assay and flow cytometry analysis in DKK1-suppressed A549 cells. (A) Measurement of intracellular ROS change in DKK1-blocked cells by DCFH-DA treatment using FACS. (B) Hypodiploid content in DKK1-suppressed A549 cells with siRNA. (C) Recovery effect of *N*-acetylcysteine (10 μM) treatment on DKK1-suppressed A549 cells. (D) Colony-forming assay of DKK1-suppressed A549 cells after γ-radiation (2 Gy). (E) RT-PCR and Western blot of ROMO1 in the DKK1-suppressed A549 cells. *N*-acetylcysteine was treated at 6 h after transfection.

suppresses cell proliferation (Fig. 4A). However, disturbance of the cellular DKK1 level by overexpression resulted in a significant elevation of p53 and $p21^{Waf1/cip1}$, according to Western blot. Consequently, enforced overexpression of *DKK1* in H460 cells also significantly inhibited the phosphorylation of serine 608, 780, and 807/811 residues in p-RB. Decrease of cyclin D1 and inactivation of p-ERK were also observed in both DKK1-suppressed and -overexpressed cells (Fig. 4A). These results strongly suggest that the inactivation of p-RB due to an imbalance of DKK1 level is partly involved in the initiation of cellular senescence by cell growth arrest (Fig. 4B).

We also used Western blot analysis to investigate activation (phosphorylation) levels of AKT and NF-kB, which can affect cell resistance and proliferation in cancer cell lines. When cellular DKK1 was overexpressed or suppressed, AKT and NF-kB inactivation (dephosphorylation) was significantly induced in A549 and in H460 cells, thus making cells resistant to γ-radiation. The EMT is an important initiation pathway in cancer metastasis. Therefore, we investigated whether characteristic maintenance of cellular DKK1 level in each cell line was essential for the EMT pathway. The cellular levels of E-cadherin, N-cadherin, Vimentin, and Snail, important EMT markers, were markedly changed by disturbance of *DKK1* expression. E-cadherin, which adheres contiguous epithelial cells, was significantly induced and its negative regulator Snail was markedly reduced (Fig. 4C). N-cadherin and Vimentin, which regulate mesenchymal cell migration, were highly reduced. Based on these changes in EMT markers, disturbance of *DKK1* expression in A549 and H460 cells also significantly decreased the migration/invasion capacity of cells (Fig. 4D and E) and altered cell morphology, from spindle- to cobblestone-shaped cells, which are typical characteristics of EMT alleviation (Fig. 4B). However, exogenous antioxidant (*N*-acetylcysteine) or pro-oxidant (H_2O_2) treatment significantly recovered the attenuated migration/invasiveness caused by disturbance of cellular DKK1 levels in each cell line. These results indicate that the imbalance of ROS level caused by *DKK1* suppression or overexpression is involved in the EMT of cancer cells.

4. Discussion

Depending on cell origin, great differences in gene expression patterns exist among NSCLC cell lines with regard to histology and responses to γ-radiation and anticancer drugs [17,18]. These gene expression patterns not only contribute to differences in tumorigenicity of lung cancer but also intrinsic resistance to chemotherapy and radiotherapy. In addition, specific genes have different functions according to the tumor cell types. We previously reported that EFEMP1 functions as a tumor suppressor in NSCLC, although it has been reported as a tumor promoter in glioma and pancreatic cancer [22–24]. Likewise, TSPYL5 is a tumor suppressor in gastric cancer cells but a tumor promoter in lung cancer cells [18,25].

In this study, we revealed the different expression patterns of DKK1, a Wnt antagonist, by comparing two NSCLC lines, H460 and A549, which both have functional wild-type p53, but very different γ-radiation sensitivity and metastasis. In spite of high level of DKK1, Wnt antagonist, A549 lung adenocarcinoma cells have high metastatic characteristics and poor prognosis. For this reason we considered that perhaps DKK1 have a function completely different from Wnt antagonist in NSCLC. The Wnt signaling pathway is essential for normal developmental and physiological processes,

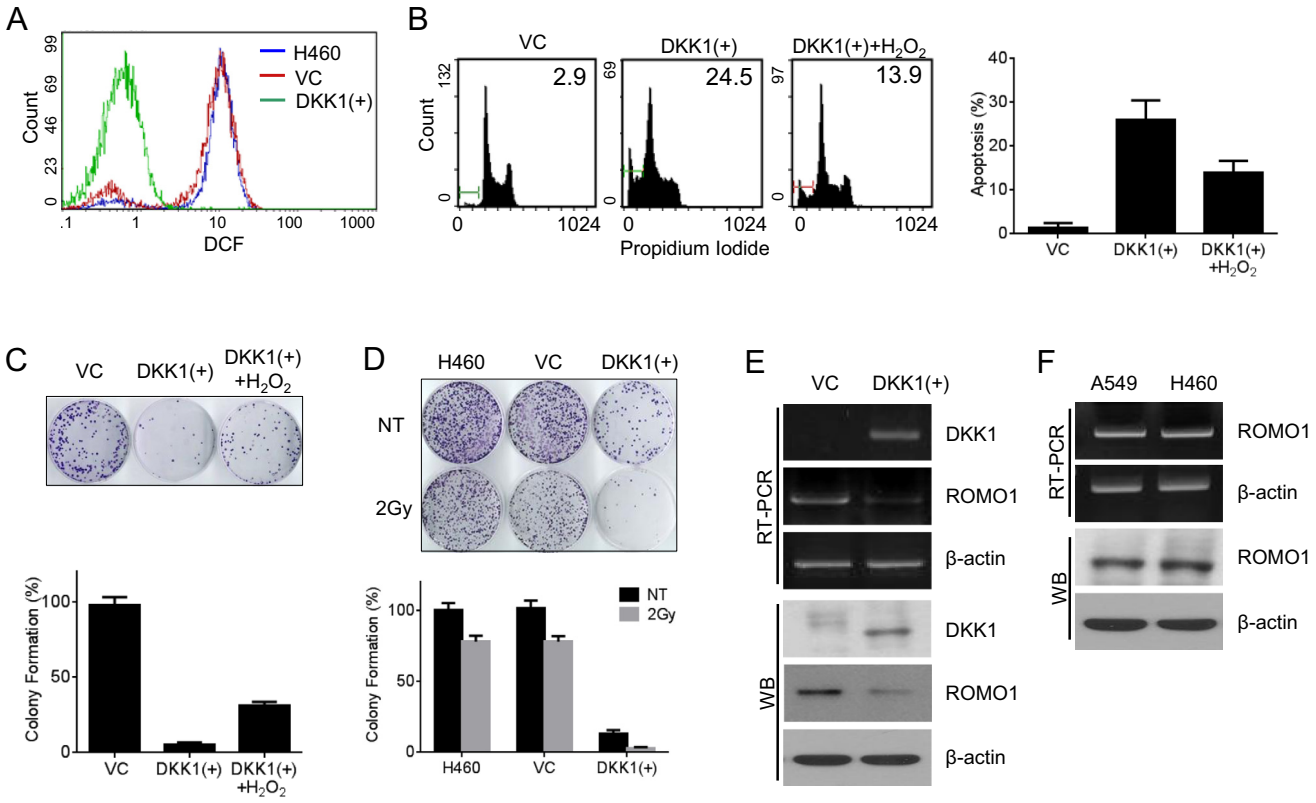

Fig. 3. Comparison of cell cytotoxicity using colony-forming assay and flow cytometry analysis in DKK1-overexpressing H460 cells. (A) Measurement of intracellular ROS change in DKK1-overexpressing H460 cells by DCFH-DA treatment using FACS. (B) Hypodiploid content in DKK1-overexpressing H460 cells with siRNA. (C) Recovery effect of H_2O_2 (10 μM) treatment on DKK1-suppressed H460 cells. (D) Colony-forming assay of DKK1-suppressed H460 cells after γ-radiation (2 Gy). (E) RT-PCR and Western blot of ROMO1 in the DKK1-overexpressing H460 cells. H_2O_2 was treated at 6 h after transfection. (F) RT-PCR and Western blot analysis of ROMO1 level in A549 and H460 cells.

such as tissue morphogenesis and angiogenesis [26]. Thus, the loss or gain of Wnt pathway components results in diseases, such as cancer or cardiovascular disorders. Due to the significance of Wnt signaling, Wnt antagonists have been considered as potential treatments for vascular disorders and cancer. Members of the DKK protein family (DKKs) are well-known Wnt antagonists. Four DKKs have been identified, DKK1, DKK2, DKK3, and DKK4, and their expression is down-regulated in several types of tumors as a consequence of epigenetic DNA modification [27,28]. We showed previously that in large cell carcinoma H460 cells, DKK3 suppression increased intracellular ROS, which induced apoptotic cell death and elevated p53 and $p21^{Waf1/cip1}$ levels, thus inhibiting cell proliferation [16]. However, the signaling pathway or modulator that regulates intracellular ROS level was not identified.

The main source of ROS production is the mitochondria. Endogenous ROS produced in the mitochondrial respiratory chain alters the intracellular redox potential, triggers DNA damage and genetic mutations, and contributes to tumor development and poor prognosis [29,30]. ROMO1, a novel protein localized in the mitochondria, was found to trigger ROS production at the cellular level [12]. Increased ROS has been observed in various cancer cell lines, suggesting that the increased *ROMO1* expression during cancer progression causes persistent oxidative stress to tumor cells and thus may increase their malignancy. The previous study also showed that ROS-producing ROMO1 was indispensable for the proliferation of cancer cells as well as normal cells [12]. Until now, however, the regulators or mechanisms related to *ROMO1* expression in cancer cells was poorly understood. Our findings indicate that *ROMO1* is expressed in both H460 and A549 cells, and DKK1 can negatively regulate *ROMO1* expression and thus strictly modulate the cellular level of ROS, which is indispensable for the proliferation of lung cancer cells. Therefore, ROS imbalance caused by the disturbance of *DKK1* expression in each cancer cell line plays an important role in cell death and cell growth inhibition. However, our results clearly showed that the level of *ROMO1* expression was similar in H460 and A549, whereas *DKK1* expression was higher in A549 than in H460 cells. The reason may be that gene expression patterns differ according to tumor cell type or cellular pathophysiological or environmental conditions. The cellular DKK1 levels required to modulate ROS production via *ROMO1* expression are quite different among cell lines. DKK1 is one of several factors maintaining the proper ROS level for cell growth and contributing to cell survival and the EMT property of tumor via strict regulation of intracellular ROS level.

Persistent accumulation of DNA damage caused by various stresses such as ROS imbalance appears to be a major contributor to cell growth arrest and thus cellular senescence. Cell growth arrest and senescence initiation is controlled by $p53/p21^{WAF1/Cip1}$ induction, the typical signal pathway, or by $p16^{INK4a}$/p-RB) activation. Because of $p16^{INK4a}$ knockdown in A459 and H460 cells, we investigated $p53/p21^{WAF1/Cip1}$ induction. In spite of cell growth inhibition caused by DKK1 down-regulation, elevated levels of the tumor suppressors p53 and $p21^{Waf1/cip1}$ were not observed. On the contrary, p-RB was significantly inactivated in response to blockage of *DKK1* expression, irrespective of $p16^{INK4a}$ deficiency. Moreover, the physiological imbalance of DKK1 level by overexpression also decreased the levels of p-RB in a $p16^{INK4a}$-independent manner. This means that another pathway for p-RB activation by strict DKK1 modulation might exist.

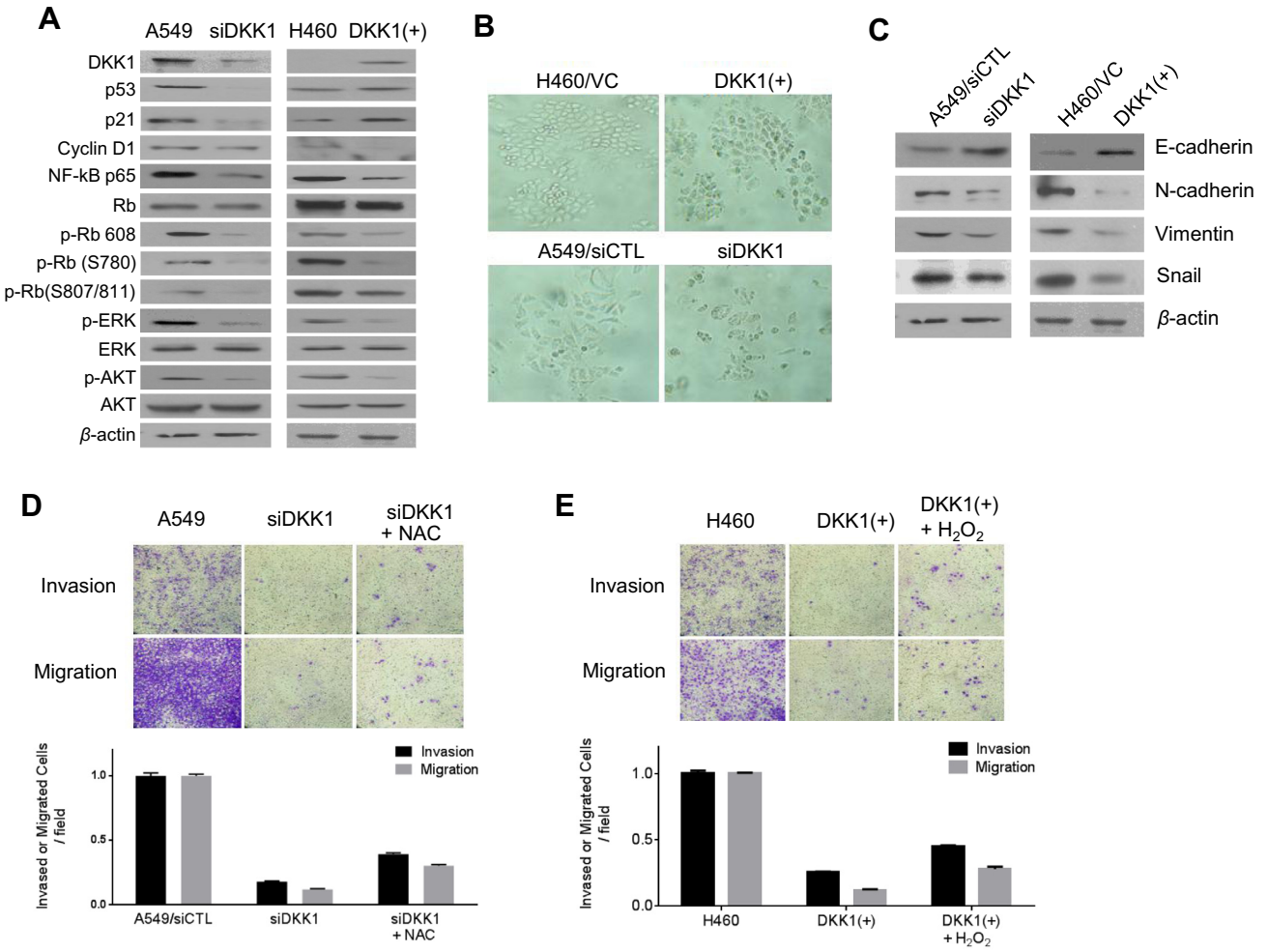

Fig. 4. Western blot analysis, β-Gal staining, and assay of EMT property change in DKK1-suppressed A549 and DKK1-overexpressing H460 cells. (A) Western blot analysis. (B) β-Gal staining in DKK1-suppressed A549 and -overexpressing H460 cells. (C) Western blot analysis of EMT markers in DKK1-suppressed A549 and -overexpressing H460 cells. (D) Invasion/migration assay in DKK1-suppressed A549 cell (E) and DKK1-overexpressing H460 cell. A549 cells (1×10^5) were transfected with 100 nM siRNA and H460 cells were transfected with 2 μg pcDNA3.1/DKK1 gene expression vector and grown for 72 h; thereafter, cells were harvested for the analysis. *N*-acetylcysteine and H_2O_2 were administered at 6 h after transfection.

In conclusion, this was the first study to demonstrate that *DKK1* plays a role in cell growth and resistance to cytotoxic agents, such as γ-radiation, via regulation of $p16^{INK4a}$-independent p-RB activation and AKT/NF-kB activation.

Acknowledgments

This research was supported by grants from the Ministry of Science, ICT, and Future Planning (Nuclear Research and Development Program) of the Republic of Korea and by a creative program of the Korea Atomic Energy Research Institute.

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.2013.11.038.

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