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Knockdown of the Dickkopf 3 gene induces apoptosis in a lung adenocarcinoma

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Abstract. The expression of the Wnt-antagonist Dickkopf gene (*DKK*) is downregulated in several types of tumors as a consequence of epigenetic DNA modification; four *DKK* members, *DKK1*, *DKK2*, *DKK3*, and *DKK4*, have been identified. In this study, we investigated another function of *DKK3* in non-small cell lung cancer H460 cells, in which *DKK3* was hypermethylated (44%) but still expressed, by interfering with *DKK3* expression using *DKK3*-silencing RNA (SiRNA). We found that knockdown of *DKK3* expression by *DKK3* SiRNA transfection led to the detachment of H460 cells from the bottom of the culture plate and caused apoptosis. The expression of cyclin-dependent kinases D1 and E were increased by *DKK3* knockdown, indicating that cells with blocked *DKK3* expression entered the apoptotic pathway. We also found that the intracellular level of reactive oxygen species was higher in cells with blocked *DKK3* expression than in normal H460 cells, and levels of p53, p21, and Bax were also increased by the gene knockdown. These results indicate that *DKK3* acts as an antiapoptotic molecule by decreasing the intracellular level of reactive oxygen species.

Introduction

The mammalian Dickkopf gene (*DKK*) encodes a class of extracellular signaling molecules that control the fate of a cell during embryonic development and regulate tissue homeostasis (1,2). Four *DKK* members have been identified, *DKK1*, *DKK2*, *DKK3*, and *DKK4*, whose functions are known to antagonize canonical Wnt/ß-catenin signaling (2-6).

Of the four members, *DKK3*, a putative Wnt antagonist, is generally downregulated in human cancers, such as lung cancer (7-9), renal clear cell carcinoma (10), pancreatic cancer (11), leukemia (12), prostate cancer (13,14), bladder cancer (15), melanoma (16), and gastrointestinal tumors (17), and reportedly functions as a putative tumor-suppressive molecule (18-20). In many of these cancers, the failure of its normal expression is closely associated to CpG island methylation on the *DKK3* promoter; thus, many studies of *DKK3* have focused on the methylation-related field (7,8,10,12,14,15,17,21).

Exposure to cellular stress, such as ultraviolet light irradiation or reactive oxygen species (ROS), can trigger the p53 tumor suppressor to induce two different cellular responses: cell growth arrest and apoptosis. The choice between these cellular responses is dependent on the type of cell and stress as well as on the action of p53 coactivators. The prevention or progression of cancer is known to be greatly dependent on the p53 tumor suppressor protein. The ability of p53 to eliminate excess, damaged, or infected cells by apoptosis is vital for proper regulation of cell proliferation (22,23). The protein (p53) senses internal stress signals that promote its nuclear accumulation in an active form and in turn determines whether cells die or exhibit growth arrest. The growth-inhibitory activities of p53 prevent the proliferation of cells with damaged DNA or with the potential for neoplastic transformation. In addition, p53 contributes to cellular processes such as differentiation, DNA repair, and angiogenesis, which also appear to be vital for tumor suppression (22-26).

In this study, we demonstrated that *DKK3* knockdown prevents normal cell growth of non-small cell lung cancer (NSCLC) adenocarcinoma cell line H460 and induces apoptosis. We also found that the increased cytotoxicity following *DKK3* knockdown may be a result of the high intracellular level of ROS beyond the cell's capacity to combat oxidative stress.

Materials and methods

Cells and their cultivation. The NSCLC H460 cell line used in the study was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Hyclone Laboratories, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin.

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Cells were incubated at 37˚C in a humidified atmosphere with 95% air and 5% CO_2. They were inoculated at an initial density of $1x10^5$ cells in a 6-well culture plate and incubated for further study.

Silencing-RNA targeting of DKK3. Cells incubated for 1 day were transfected with Stealth RNA targeting *DKK3* (*DKK3* silencing RNA (SiRNA), 5'-AGCUGCUGCUAAAGCAUC AUCAGAA) purchased from Invitrogen (Carlsbad, CA, USA) or with scrambled SiRNA as a negative control (NC; Bioneer Co., Daejeon, Korea) at a concentration of 100 nM by using the Lipofectamine RNAi MAX reagent (Invitrogen). Cells were incubated for another 1-4 days after transfection.

Sodium bisulfite modification. Bisulfite-modified genomic DNA (gDNA) was prepared by using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. For the bisulfite reaction, 400 ng gDNA and 130 μl CT conversion reagent were added. Sample tubes were placed in a thermal cycler (MJ Research, Waltham, MA, USA) and the following steps were performed, 10 min at 98˚C and 2 h 30 min at 64˚C, and then stored at 4˚C. The resultant DNA was purified by using the reagent contained in the EZ DNA Methylation-Gold kit. Converted samples were added to a Zymo-Spin IC column containing 600 μl of M-binding buffer and mixed by inverting the column several times. The column was centrifuged at full speed for 30 sec and the flow-through was discarded. The column was washed by adding 200 μl M-wash buffer and centrifuged at full speed. Then, 200 μl M-desulphonation buffer was added to the column and incubated at room temperature (20-30˚C) for 15-20 min. After incubation, the column was centrifuged at full speed for 30 sec. Finally, the column was washed by adding 200 μl M-wash buffer and centrifuged at full speed (this step was repeated once more). The converted gDNA was eluted by adding 20 μl M-elution buffer into the column. DNA samples were finally stored at -20˚C for further study.

Pyrosequencing analysis. Polymerase chain reaction (PCR) was carried out in a volume of 50 μl with ≤20 ng converted gDNA, 5 μl 10x Taq buffer, 5 U Hot/Start Taq polymerase (Enzynomics, Daejeon, Korea), 4 μl of each 2.5 mM dNTP mixture, 2 μl of 10 pmol/μl Primer-S, and 2 μl of 10 pmol/μl biotinylated-Primer-As. The amplification was carried out according to the general guidelines suggested for pyro-sequencing, denaturing at 95˚C for 15 min, 45 cycles at 95˚C for 40 sec, 55˚C for 40 sec, 72˚C for 40 sec, and final extension at 72˚C for 10 min. The PCR product (5 μl) was confirmed by electrophoresis on 3% agarose gel and visualized by ethidium bromide staining. A single-strand DNA (ssDNA) template was prepared from 20-25 μl of the biotinylated PCR product by using streptavidin Sepharose HP beads (Amersham Biosciences, Uppsala, Sweden) following the PSQ 96 sample preparation guide and using multichannel pipettes. Then, 15 pmol of the respective sequencing primer set was added for analysis. Sequencing was performed on a PyroMark ID system with the Pyro Gold reagents kit (Biotage, Charlottesville, VA, USA) according to the manufacturer's instructions without further optimization. The methylation percentage was calculated by the average of the degree of methylation at 5 or 6 CpG sites formulated by pyrosequencing.

Complementary DNA synthesis and PCR amplification. Total RNA was isolated from H460 cells by using an RNA extraction kit (Qiagen, Valencia, CA, USA). Complementary DNAs (cDNAs) were prepared by using the PCR premix kit purchased from Intron Biotechnology (Daejeon, Korea), and the resultant cDNAs were used as templates for PCR amplification with forward and reverse primers as follows, *DKK3*-F, 5'-GTTGA GGAACTGATGGAGGACA; *DKK3*-R, 5'-TTGCACACAT ACACCAGGCTGT; ß-actin-F, 5'-ATGTGCAAGGCCCGC TTCG; ß-actin-R, 5'-TTAATGTCACGCACGATTTCC. For amplifying the GC-rich *DKK3* template, a specific buffer, i-GC capture solution (Intron Biotechnology), was added to the PCR mixture. PCR conditions for *DKK3* were as follows, denaturing at 94˚C for 30 sec, 40 cycles at 94˚C for 30 sec, 61˚C for 30 sec, 72˚C for 1 min; and final extension at 72˚C for 5 min. The amplified PCR products were analyzed by agarose gel (1%) electrophoresis and photographed.

Microscopy, analysis of cell survival fraction, and MTT cell proliferation assay. Total cells, floating cells in the medium supernatant, and attached cells at the bottom of the culture plate were photographed by light microscopy (Leica Microsystems, Westchester, IL, USA). Images were captured with a Cannon Power Shot S45 digital camera system. For analysis of the cell survival fraction, trypsinized cells were harvested and the cell number was directly counted. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay, cells (1,000-3,000 cells) were seeded intp 96-well plates in triplicate. After 24, 48, and 72 h, 100 μl of MTT was added to each well and plates were incubated for 4 h at 37˚C. The medium was removed, and MTT crystals were solubilized in dimethyl sulfoxide, after which the spectrophotometric absorbance of each sample was measured at 570 nm by using the Promega Glomax 96 microplate luminometer and analyzed using Microplate manager (Promega, Madison, WI, USA).

Colony forming assay. After transfecting cells for 1 day, trypsinized cells were collected and plated on new 35-mm culture dishes at a density of $5x10^3$ cells/plate. After 7 days, cells were stained with 0.5% crystal violet and photographed.

Flow cytometric detection. After cells were fixed with 70% ethanol at 4˚C for 30 min in the dark and washed with phosphate-buffered saline (PBS), they were stained with propidium iodide (PI, 50 μg/ml). DNA content was measured with FACScan (Epics XL; Beckman Coulter Counter, Fullerton, CA, USA). A minimum of 10,000 cells was counted per sample. The percentage of cells in each cell phase was determined by using the Phoenix Multicycler software (Phoenix Flow System, San Diego, CA, USA).

Western blot analysis. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except for cyclin E, phospho-Akt (pAkt), and ß-actin, which were purchased from Cell Signaling (Beverly, MA, USA), and for $p21^{WAF1/Cip1}$ (p21), purchased from Abcam (Cambridge, MA, USA). The protein concentration was determined by using a Lowry kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated on a 10-12% sodium

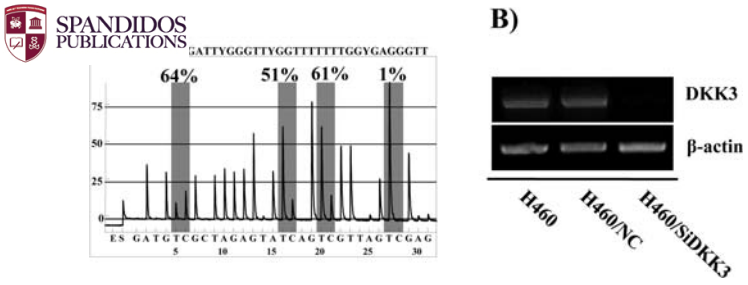

Figure 1. Induction of G2/M arrest in H460 cells by irradiation. (A) FAScan analysis of H460 and A549 cells. (B) RT-PCR analysis of *DKK3* after transfecting *DKK3* SiRNA or negative scrambled SiRNA. ß-Actin was used as an internal control.

dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia, Piscataway, NJ, USA). Blots were blocked for 2 h at room temperature with blocking buffer (10% non-fat milk in PBS buffer containing 0.1% Tween-20), and the membrane was incubated for 1 h with the specific antibodies. After washing with the blocking buffer, the membrane was incubated with a horseradish peroxidase-labeled secondary antibody and visualized using a Westzol enhanced chemiluminescence detection kit (Intron Biotechnology).

Measurement of ROS. Carboxydichlorofluorescein diacetate (DCFH-DA) is a nonpolar compound that converts to a membrane-impermeable nonfluorescent polar derivative (DCFH) by cellular esterase after incorporation into cells. The trapped DCFH was rapidly oxidized to fluorescent 2',7'-dicloro-fluorescein (DCF) by intracellular hydrogen peroxide. Trypsinized cells (~1x10^5 cells) were washed, resuspended with PBS, and treated with DCFH-DA at a final concentration of 10 μM. Cells were incubated for 30 min in the dark at 37˚C and the ROS level was directly measured by using flow cytometry.

Results

Methylated CpG islands on the DKK3 promoter. In many cancer cells, the failure of *DKK3* expression is closely associated to methylation on the *DKK3* promoter (7,8,10,12,14,15,17). Thus, we determined the extent of *DKK3* methylation in a NSCLC H460 cell line. In the bisulfite pyrosequencing method for methylation analysis of *DKK3*, we selected four CpG sites, as shown in Fig. 1A. After performing PCR amplification, the methylation percentages were calculated by averaging the degree of methylation at the four CpG sites. The average of the extent of methylation was ~44%, and *DKK3* was still expressed (Fig. 1B). We also found that the introduction of *DKK3* SiRNA into H460 cells caused complete repression of *DKK3* expression (Fig. 1B).

Cytotoxic effect of DKK3 SiRNA on cell growth. To investigate whether *DKK3* is involved in the signaling pathway for cell growth and apoptosis in NSCLC cell line H460, *DKK3* was suppressed by SiRNA in H460 lung adenocarcinoma cells. As shown in Fig. 2A, cells treated with *DKK3* SiRNA failed to attach onto the culture plate and then floated. Cells treated with *DKK3* SiRNA showed significantly inhibited growth (by 70%;

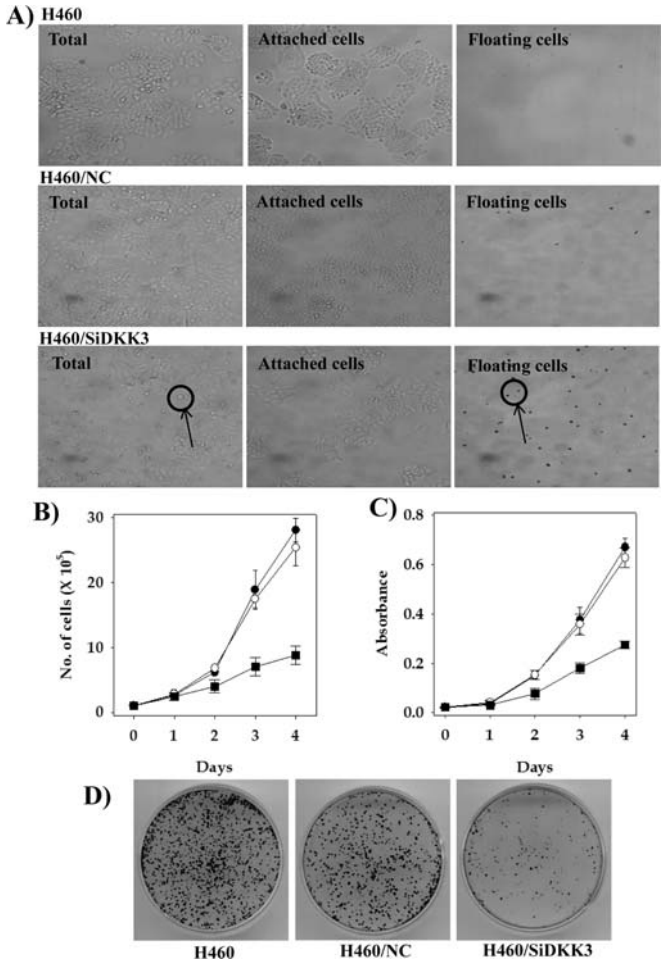

Figure. 2. Growth impairment on transfection of *DKK3* SiRNA. (A) Microscopic views. Total cells (total), cells in medium supernatant (floating cells), and those attached onto culture plate (attached cells) were photographed. Detached (floating) cells seen in the sections of total and floating cells are indicated by arrows. (B) Comparison of the surviving fractions with and without *DKK3* SiRNA transfection. Closed circles, normal H460; open circles, H460/NC; closed squares, H460/*DKK3* SiRNA. (C and D) MTT cell proliferation assay. Symbols are as the same as depicted in B.

Fig. 2B) compared with the parental H460 cells in which *DKK3* was still expressed. This lower cell number seen in the H460/*DKK3* SiRNA cells may have resulted from cell detachment, because numerous floating cells in the supernatant were observed. This abnormal growth pattern of was also confirmed by MTT cell proliferation and colony forming assays (Fig. 2C and D).

Induction of apoptosis by DKK3 downregulation. To evaluate the cytotoxic effect induced by *DKK3* downregulation in H460 cells, cells were analyzed by flow cytometry after PI staining. The *DKK3*-negative cells (H460/*DKK3* SiRNA) showed a similar pattern to the *DKK3*-positive cells (parental H460) on the first day but entered the apoptotic phase after 2 days.

As shown in Fig. 3, a significant difference was observed in the level of apoptosis. Whereas the parental H460 and H460/NC SiRNA cells showed only <3% apoptosis, the apoptosis increased progressively through day 3 in the case of the H460/*DKK3* SiRNA cells (reaching up to 24%). However, no great differences were present in the other cell cycle phases.

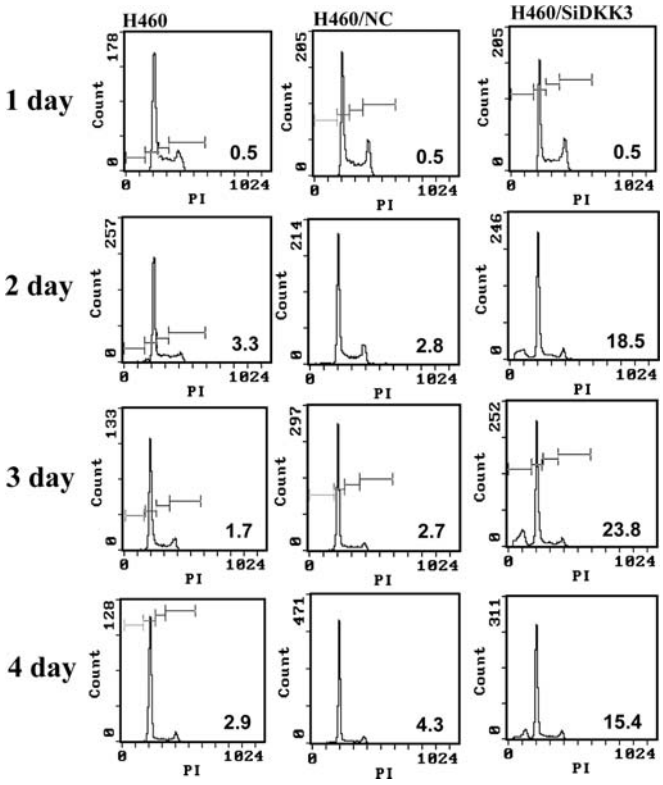

Figure. 3. Flow cytometric analysis. Cells were transfected with or without *DKK3* SiRNA, incubated for 1-4 days, and then PI-stained after harvesting. Apoptotic cell fractions are indicated in each diagram.

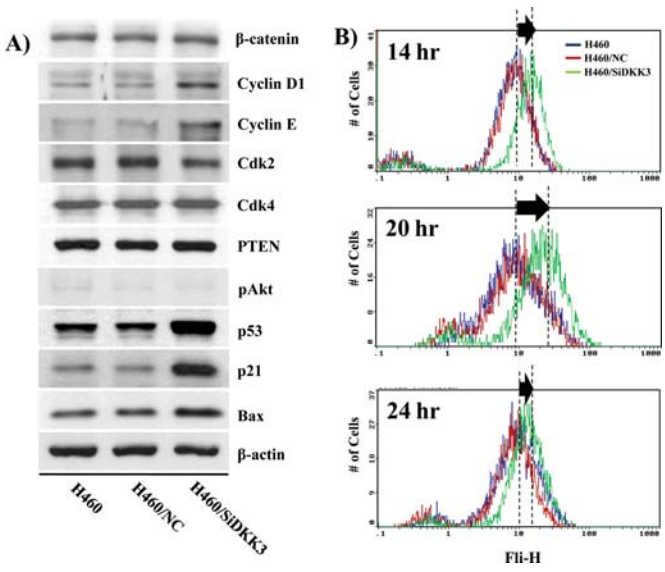

Figure. 4. Western blot analysis and measurement of the intracellular ROS level. (A) Cells were grown for 24 h after transfection and the intracellular expression patterns of proteins were compared. (B) ROS determination by DCFH-DA treatment.

These data indicate that the cell detachment and abnormal growth pattern seen in H460/*DKK3* SiRNA cells were a result of the apoptosis induced by downregulation of *DKK3*.

Signaling pathway involved in apoptosis and determination of the ROS level. We investigated the apoptosis-related protein expression in parental H460, H460/NC SiRNA, or H460/*DKK3* SiRNA cells (Fig. 4A). Blockage of *DKK3* resulted in an increase in the levels of cyclin D1 and E but not the levels of Cdk2 and Cdk4. Furthermore, no differences in the phosphatase and tensin homolog (PTEN) and pAkt levels were seen between the parental H460 and the H460/*DKK3* SiRNA cells, indicating that the apoptotis seen in the H460/*DKK3* SiRNA cells was independent of the PTEN/pAkt signaling pathway. Interestingly, the levels of p53 and p21 were greatly elevated in the H460/*DKK3* SiRNA cells, suggesting that they suffered from oxidative stress. We also monitored the expression level of Bax, which was translocated to the nucleus after the induction of apoptosis, by Western blotting. As expected, the Bax level was higher in the H460/*DKK3* SiRNA cells than in the parental H460 cells.

A signaling pathway for p53 induction by increased ROS levels has been established. Thus, we investigated whether *DKK3* modulates endogenous ROS levels in H460 cells. ROS measurements in the H460 cells with or without *DKK3* SiRNA were performed by the uptake of reduced fluorescent indicator DCFH-DA, which is known to be oxidized by hydrogen peroxide (27). In this study, oxidized DCF was determined by flow cytometry. The level of ROS was significantly elevated by the blockage of *DKK3*, and the greatest difference in the ROS level was seen at 20 h after transfecting *DKK3* SiRNA (Fig. 4B). These results suggest a function of *DKK3* in maintaining the intracellular ROS level at an appropriate level.

Discussion

DKK3 expression is downregulated in several tumors as a consequence of epigenetic DNA modification (1,5,8,10,12, 14,15,17). The loss of *DKK3* expression was first observed in numerous immortalized tumor-derived cell lines (24). Immortalization, or escape from cellular senescence, is an early event in malignant transformation (25). *DKK3* could act as a tumor suppressor gene by mediating the effects of senescence stimuli. In accordance with this hypothesis, *DKK3* expression was found to be elevated in organs with predominantly growth-arrested postmitotic cells, such as the heart and brain (26), and also in senescent prostate epithelial cells (28). More recently, a breast cancer xenotransplantation model demonstrated that a single adenoviral-mediated intratumoral injection of a *DKK3* expression vector efficiently discontinued tumor growth, with the induction of apoptosis in these cells (23). These results suggest that *DKK3* has an important tumor-suppressive function that either prevents tumor initiation or attenuates cancer progression. In this study, however, we also suggest that, *DKK3* has a tumor-suppressive function not only when it is present in the normal state but also when its expression is completely blocked. In fact, lung adenocarcinorma H460 cells showed an apoptotic response when *DKK3* SiRNA was transfected, and thus, endogenous *DKK3* expression was blocked. As shown in Fig. 1, *DKK3* was methylated to the 44% level but still expressed. On transfecting *DKK3* SiRNA, *DKK3* expression was not detectable. In this case, H460/*DKK3* SiRNA cells showed a significant growth impairment such as characteristic cell detachment from the culture plate and decreased cell number as well as apoptosis.

Expression of p53 is rapidly upregulated after ionizing radiation- or ROS-induced DNA damage, followed by

ional activation of several downstream genes such yclin G, and GADD45 whose products function as regulators of diverse aspects of cell growth. Indeed, the p53 protein is a critical regulator of cellular growth and controls both proliferation and apoptotic response of cells (29-31).

ROS are produced as a normal by-product of cellular metabolism and function as signaling molecules that are involved in numerous signaling pathways regulating cell proliferation, senescence, apoptosis, necrosis, and autophagy (32,33). Stress, such as exposure to ROS and chemotoxic substrates, usually activates the intrinsic apoptotic pathway. This pathway is controlled by the activation of the ATM/ATR set of kinases that sense single- and double-strand damage, followed by the phosphorylation of p53, and in turn activated p53 participates in the DNA repair process (34-36). As shown in Fig. 4B, the intracellular ROS level was higher in the H460/*DKK3* SiRNA cells than in the parental H460 or H460/NC SiRNA cells, indicating that the blockage of *DKK3* leads to an increase in the cellular ROS level, and thus, the cell may suffer from high oxidative stress. Therefore, to endure oxidative stress, H460 cells with no detectable *DKK3* expression operate a specific repair mechanism related to p53 and p21 through their activation.

The p53-dependent G1- and G2-arrest response may allow cells to repair DNA damage induced by ROS, ionizing radiation, and other genotoxic agents prior to transit through the cell cycle to initiate DNA synthesis, replication, and mitosis. When the ROS stress exceeds a cell's capacity to endure DNA damage-induced stress, the cell undergoes an apoptotic response. As shown in Fig 4A, a significant increase in the expression of p53 and p21 was found in the H460/*DKK3* SiRNA cells, without detectable *DKK3*. Interestingly, cyclin D1 and E were also induced, indicating that the elimination of endogenous *DKK3* by transfecting *DKK3* SiRNA triggers cells to proceed through the G1 and G2 cell cycles even when experiencing high oxidative stress, and not attenuate the cell cycle to repair the damage. Bax was the first member of the group induced by p53 (37). Under various oxidative stresses, Bax forms a homodimer and releases cytochrome c from the mitochondria (38), resulting in caspase-9 activation (39). The requirement of Bax in p53-mediated apoptosis appears to be cell-type dependent (37-39). We confirmed that Bax was also induced by transfecting *DKK3* SiRNA, with the result that the cells operated the apoptotic cycle via p53 activated by the increased ROS level.

In conclusion, this study is the first to present another function of *DKK3* in maintaining the intracellular ROS level at an appropriate level and thus enabling cells to grow normally.

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