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# Nek6 overexpression antagonizes p53-induced senescence in human cancer cells

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**Key words:** Nek6, cellular senescence, p53, cell cycle, tumorigenesis

Nek6 is an NIMA-related kinase that plays a critical role in mitotic cell cycle progression. Recent studies have shown that Nek6 is upregulated in various human cancers, but the function of Nek6 in tumorigenesis is largely unknown. Here, we examined the role of Nek6 in cellular senescence. Our data revealed that Nek6 expression is decreased both in the replicative senescence of human normal fibroblasts and premature senescence induced by p53 expression in EJ human bladder cancer cells and H1299 human lung cancer cells. Interestingly, the enforced expression of Nek6 in EJ and H1299 cells completely suppresses p53-induced senescence, whereas the expression of kinase-dead Nek6 did not affect p53-induced senescence. Mechanistic studies revealed that cell cycle arrest in the $G_1$ and $G_2$/M phases, as well as the reduction of cyclin B and cdc2 protein level upon p53 expression were significantly reduced by Nek6 overexpression. In addition, p53-induced increases in intracellular levels of ROS were also inhibited in cells overexpressing Nek6. These results suggest that the downregulation of Nek6 expression is required for the onset of p53-induced cellular senescence and imply a possible role of Nek6 in tumorigenesis.

## Introduction



Cellular senescence is an irreversible growth arrest that occurs when a cell has exhausted its capacity to divide.[1] Senescent cells loss their proliferation potential and display characteristic phenotypes, such as enlarged and flattened morphology and the senescence-associated (SA) β-galactosidase activity at pH 6.0.[2] Normal mammalian cells enter the senescence stage called "replicative senescence" after a limited number of cell divisions. A number of recent studies demonstrated that various stresses such as oncogene activation, oxidative stress, DNA damage, and restoration of tumor suppressor function also induce cellular senescence.[3] This phenomenon, called "premature senescence", is thought to play an important role in tumor suppression and is considered to be a potential mechanism for controlling human cancers.[1,3] Consistent with this notion, two groups recently showed that restoration of p53 function in tumors lacking functional p53 induces tumor regression by premature senescence.[4,5] Therefore, identification of the regulatory proteins required for premature senescence of cancer cells is critical to understanding mechanisms of cellular senescence and developing anti-cancer treatments.

Nek6 is a serine/threonine kinase that belongs to the Neks (NIMA-related kinases) family, which has been implicated in mitosis control.[6] Previous studies suggest that Nek6 function is closely related to mitotic cell cycle progression. The protein level and the activity of Nek6 are increased in mitosis.[7,8] Interfering with Nek6 function by either overexpression of a dominant negative form of Nek6 or depletion of endogenous Nek6 using RNAi causes mitotic arrest, abnormal chromosome segregation and apoptosis.[6-8] In addition to its essential role in cell cycle control, we have previously shown that Nek6 is a novel target for the DNA damage checkpoint and the suppression of Nek6 function is essential for cell cycle arrest upon DNA damage.[9]

Interestingly, several recent studies suggest that Nek6 function is related to tumorigenesis. The Nek6 gene is localized on chromosome 9q33-34, a locus associated with several human cancers, such as bladder cancer, neuroblastoma and renal cell carcinoma.[10,11] Chen et al. showed that Nek6 expression was significantly upregulated in 70% of hepatic cell carcinomas.[12] It was recently shown that the protein level of Nek6 is increased in advanced-stage gastric cancers compared to early-stage samples.[13] More importantly, Nassirpour et al. recently showed that protein level and kinase activity of Nek6 are highly elevated in a variety of malignant human cancers, and a knockdown of Nek6 results in reduction of tumors in the nude mouse xenograft model.[14] These studies suggest that Nek6 function is important not only for normal cell cycle progression but also for cell transformation and tumor progression.

In this study, we examined the role of Nek6 in cellular senescence. Our data revealed that Nek6 expression is downregulated in both replicative and premature senescence of human cancer cells. In addition, ectopic overexpression of Nek6 completely abolished the p53-induced premature senescence of human

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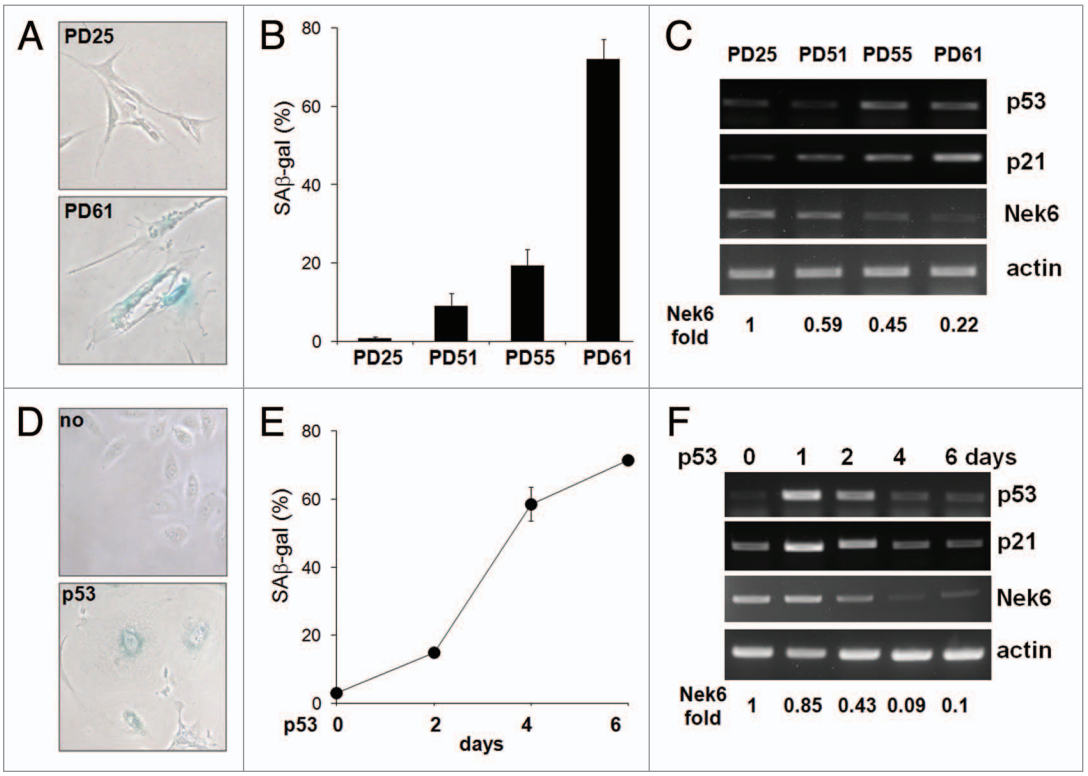


**Figure 1.** Nek6 expression in the replicative senescence of normal human fibroblasts and p53-induced senescence of human EJ bladder cancer cells. (A–C) IMR90 cells at indicated passages were stained with freshly prepared SA β-gal staining solution, cell images were captured using a microscope (A) and SA β-gal positive cells were counted and plotted (B). (C) Total RNA of IMR90 cells was isolated and semi-quantitative RT-PCR was performed using the indicated primers as described in Materials and Methods. The data shown are representative of three independent experiments. (D–F) EJ cells were infected with or without p53 adenovirus. Cell images were captured 6 days later under a microscope after SA β-gal staining (D). SA β-gal staining was performed at the indicated time points and the results are presented as mean values with standard deviations (E). (F) Semi-quantitative RT-PCR analysis was performed at the same time points as (E) using the indicated primers. The data shown are representative of three independent experiments.

cancer cells, suggesting that the inhibition of Nek6 activity is important for the onset of cellular senescence.

## Results

**Nek6 expression is downregulated during cellular senescence.** To investigate the role of Nek6 in senescence, we first examined whether the expression level of Nek6 is changed during replicative senescence. To examine Nek6 expression level in senescent cells, normal human lung fibroblasts IMR90 were serially cultured until they reached the senescence stage. Senescence-associated β-galactosidase (SA β-gal) activity, a specific marker of senescent cells,[2] started to increase after 51 population doublings (PD). A Flattened and enlarged morphology, and increase of SA β-gal activity confirmed that most of the IMR90 cells entered replicative senescence at PD61 (**Fig. 1A and B**). Interestingly, we found that the mRNA level of Nek6 in senescent IMR90 cells (PD61) was considerably lower than in early passage IMR90 cells (PD25), whereas p53 and p21 levels were increased in senescent IMR90 cells (**Fig. 1C**).

In addition to replicative senescence, various genotoxic drugs or the restoration of inactivated tumor suppressor function induces premature senescence of cancer cells.[3] To investigate the role of Nek6 in premature senescence, we examined Nek6 expression in p53-induced cancer cell senescence. Consistent with previous reports,[15-17] the adenovirus-mediated overexpression of p53 in p53-negative EJ human bladder carcinoma cells, in which p53 is nonfunctional, induced the rapid onset of premature senescence. In addition to morphological changes, SA β-gal positive cells increased in number beginning 4 days after p53 expression indicating that EJ cells entered the premature senescence state at this time (**Fig. 1D and E**). Semi-quantitative RT-PCR analysis results showed that mRNA levels of Nek6 decreased during senescence (**Fig. 1F**). In addition to the results in EJ cells, it has been previously reported that p53 expression induces premature senescence in H1299 human lung cancer cells.[18] We observed that mRNA levels of Nek6 also decreased in H1299 cells during p53-induce senescence (**Fig. S1**). These results suggest that the expression of Nek6 is downregulated during premature senescence as well as during replicative senescence.

**Overexpression of Nek6 suppresses p53-induced cancer cell senescence.** The reduction of Nek6 expression in both replicative and premature senescence raised the possibility that the downregulation of Nek6 expression may play a role in senescence. To examine whether Nek6 downregulation is required for the onset of senescence, we established EJ cell lines ectopically expressing Flag-Nek6 (EJ-Nek6) and confirmed the expression of Flag-Nek6 by western blotting using anti-Flag antibodies and semi-quantitative reverse transcriptase (RT)-PCR analyses (**Fig. 2A**). The RT-PCR results indicated that Nek6 mRNA levels increased about two-fold in EJ-Nek6 cells compared to EJ-control cells (Vector). Consistent with a previous report,[8] the ectopic expression of Nek6 did not significantly affect cell growth or cell cycle

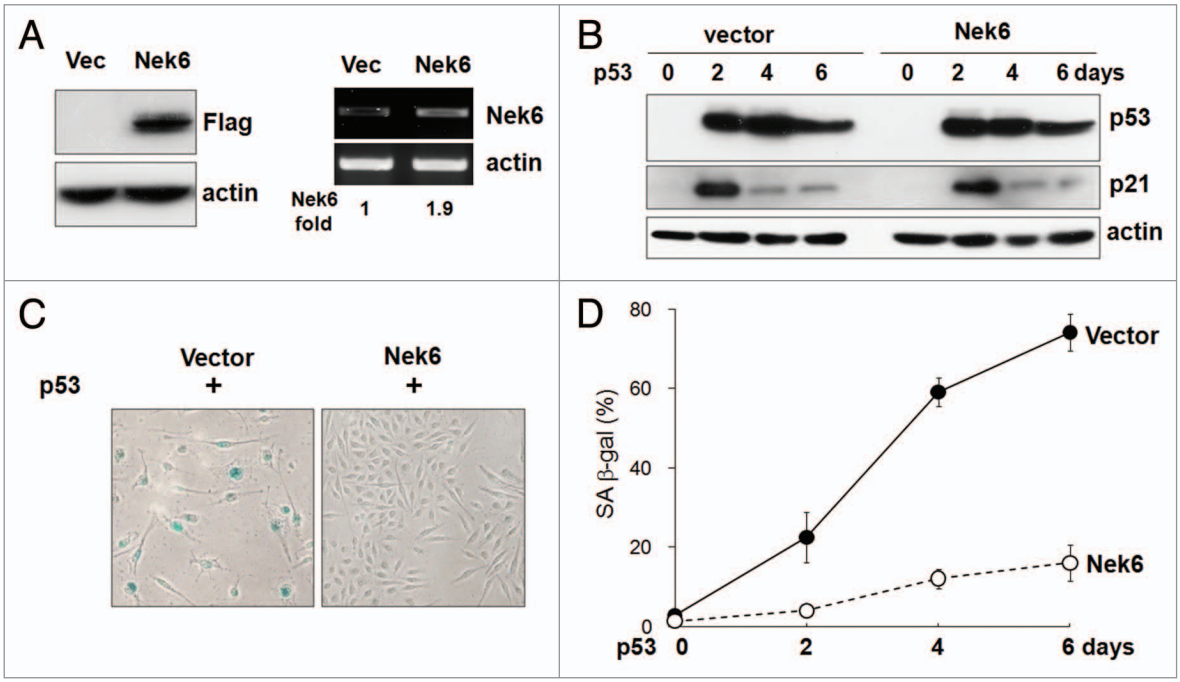


**Figure 2.** The overexpression of Nek6 inhibits p53-induced senescence of EJ cells. (A) Cell lysates from EJ-Nek6 and EJ-vector (Vec) cells were subjected to western blotting using anti-Flag and anti-actin antibodies. (B) Cell lysates were prepared at the indicated time points after p53 adenovirus infection and subjected to western blotting using the indicated antibodies. (C) EJ-vector and EJ-Nek6 cells were infected with p53 adenovirus and stained with freshly prepared SA β-gal staining solution 6 days after infection. (D) EJ-vector and EJ-Nek6 cells were infected with p53 adenovirus and analyzed for SA β-gal staining at the indicated time points. Experiments were repeated three times and the results were presented as mean values with standard deviations.



distribution (data not shown). Upon p53 adenovirus infection, p53 and p21 protein levels were increased in EJ-Nek6 cells to levels similar to those in the EJ control cells, which are transfected with the pFlag-vector, indicating that p53 function is not inhibited by Nek6 overexpression (**Fig. 2B**). However, while EJ-vector control cells became flattened and enlarged after p53 expression, EJ-Nek6 cells maintained normal morphology and grew continuously (**Fig. 2C**). In addition, whereas SA β-gal activity was significantly increased in EJ control cells starting 4 days after p53 expression, and a majority of EJ control cells showed SA β-gal activity at 6 day later, SA β-gal activity was significantly reduced in EJ-Nek6 cells (**Fig. 2D**) indicating that the onset of p53-induced senescence was suppressed by Nek6 overexpression.

**Nek6 kinase activity is required to inhibit p53-induced senescence.** Previous studies showed that the kinase activity of Nek6 is critical in executing its physiological role.[8,14] To further confirm the effect of Nek6 overexpression on p53-induced senescence, and to examine whether Nek6 kinase activity is required to inhibit p53-induced senescence, we established Nek6 wild type and kinase dead mutant expressing cell lines in EJ as well as H1299 human lung cancer cells using a retrovirus-mediated gene transfer as described in Materials and Methods. In order to avoid a possible artifact by using individual clone, we pooled resistant cells after antibiotics selection and Nek6 expression was confirmed by western blotting and RT-PCR analysis (**Fig. 3A**).

Consistent with previous data, SA β-gal activity upon p53 expression was completely inhibited in EJ cells infected with a retrovirus encoding wild type Nek6 (EJ-pBabe-Nek6), whereas EJ cells expressing kinase dead Nek6 (EJ-pBabe-Nek6 KK/MM) showed similar increases in SA β-gal activity and morphology changes as seen in EJ-vector control cells (EJ-pBabe) (**Fig. 3B and C**). Moreover, the inhibition of p53-induced senescence by wild type Nek6, but not by kinase dead Nek6, was also observed in H1299 cells (**Fig. 3B and C**). These data suggest that Nek6 overexpression can suppress p53-induced senescence in various cancer cells, and that kinase activity is essential to inhibit p53-induced senescence.

**The overexpression of Nek6 overrides p53-induced cell cycle arrest.** To investigate the underlying mechanism by which Nek6 overexpression inhibits p53-induced senescence, we next compared changes in cell cycle distribution after p53 expression in both EJ-Nek6 and EJ-vector control cells. Consistent with a previous report,[17] EJ-vector control cells were arrested in both $G_1$ (45% at 0 day, 72% at 6 days) and $G_2$/M (16% at 0 day, 25% at 6 days) after p53 expression, and the percentage of S phase cells was dramatically decreased starting 2 days after p53 expression (45% at 0 day, 16% at 2 days) (**Fig. 4A and B**). However, EJ-Nek6 cells showed much less $G_1$ arrest (40% at 0 day, 59% at 6 days), and the decrease in S phase cells after p53 expression was significantly less pronounced (49% at 0 day, 37% at 2 days) (**Fig. 4A and B**). Moreover, while the BrdU incorporation index, an indicator of cell proliferation potential, was also profoundly reduced in EJ-vector control cells beginning 2 days after p53 expression, EJ-Nek6 cells showed significant resistance to a decrease in BrdU incorporation (**Fig. 4C and D**). These results suggest that

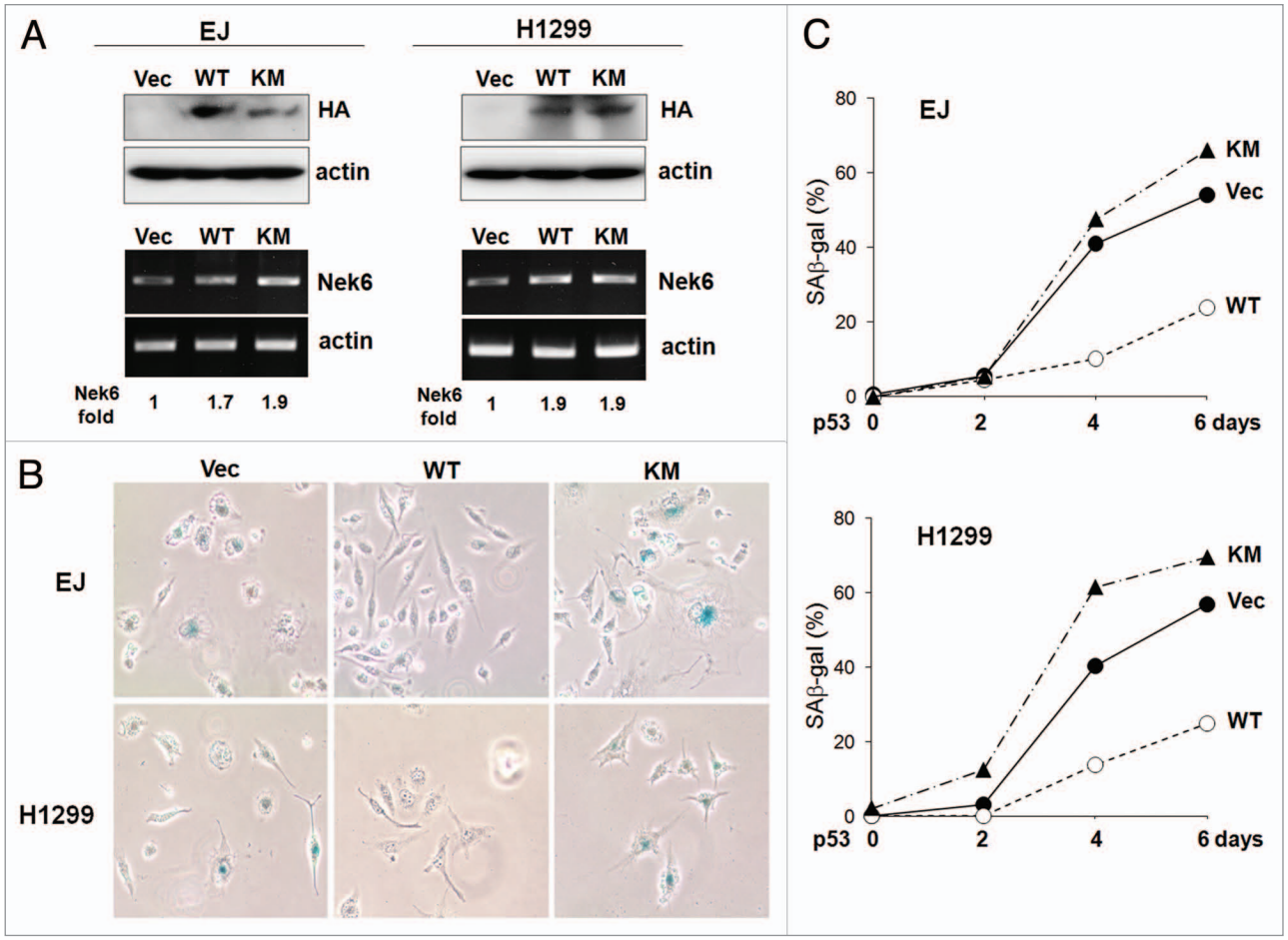


**Figure 3.** The overexpression of wild type Nek6, but not kinase-dead Nek6, inhibits p53-induced senescence of H1299 and EJ cells. (A) EJ and H1299 cells stably expressing the pBabe control (Vec), pBabe-HA-Nek6 wild type (WT) and pBabe-HA-Nek6 KK/MM (KM) were generated as described in Materials and Methods. Nek6 overexpression was confirmed by western blotting using anti-HA antibodies (upper part) and RT-PCR (lower part). (B and C) EJ and H1299 stable cells were infected with or without p53 adenovirus. Cell images were captured 6 days later under a microscope after SA β-gal staining (B). SA β-gal staining results performed at the indicated time points were presented as mean values with standard deviations (C).

Nek6 overexpression in EJ cells overrides p53-induced cell cycle arrest and maintains proliferative potential even after p53 expression.

**Nek6 inhibits both the p53-induced decrease of cyclin B and cdc2 levels and increase of ROS.** To investigate the mechanism by which Nek6 overexpression affects p53-induced cell cycle arrest, we examined the expression of cell cycle regulators. As shown in **Figure 5A**, the protein levels of mitotic cyclins (cyclin A and B) and cdc2 were decreased dramatically concomitant with the increase of p53 and p21 in EJ-vector cells. The decrease of protein levels was evident by 2 days after p53 expression, and cdc2 and the mitotic cyclins (cyclin A and B) were barely detectable 4 and 6 days later, respectively. However, EJ-Nek6 showed pronounced resistance to decreases in those cell cycle regulators after p53 expression. Specifically, although cyclin A showed a decrease similar to that seen in the EJ-vector cells, cylin B and cdc2 were clearly resistant to p53-induced decrease. These results suggest that Nek6 overexpression inhibits p53-induced senescence by suppressing the decrease of the $G_2$/M cell cycle regulators, cyclin B and cdc2.

Previously, it was shown that intracellular reactive oxygen species (ROS) are increased upon p53 expression and that this increase is essential for the onset of senescence in EJ cells.[15] To examine the effect of Nek6 overexpression on the p53-induced increase in intracellular ROS levels, we measured intracellular levels of ROS in both EJ-vector and EJ-Nek6 cells after p53 expression. As shown **Figure 5B**, the levels of ROS increased after p53-expression and peaked 4 days later in EJ-vector cells. However, ROS levels in EJ-Nek6 cells were only moderately increased 2 days after p53 expression and returned to basal levels 4 days later, suggesting that the p53-induced increase of intracellular ROS in EJ cells was suppressed by Nek6 overexpression.

## Discussion

In this study, we found that Nek6 is involved in the induction of cellular senescence. Although previous studies suggested that Nek6 is implicated in tumorigenesis, the role of Nek6 in senescence has never been examined. We found that Nek6 expression was reduced in senescent normal human fibroblast

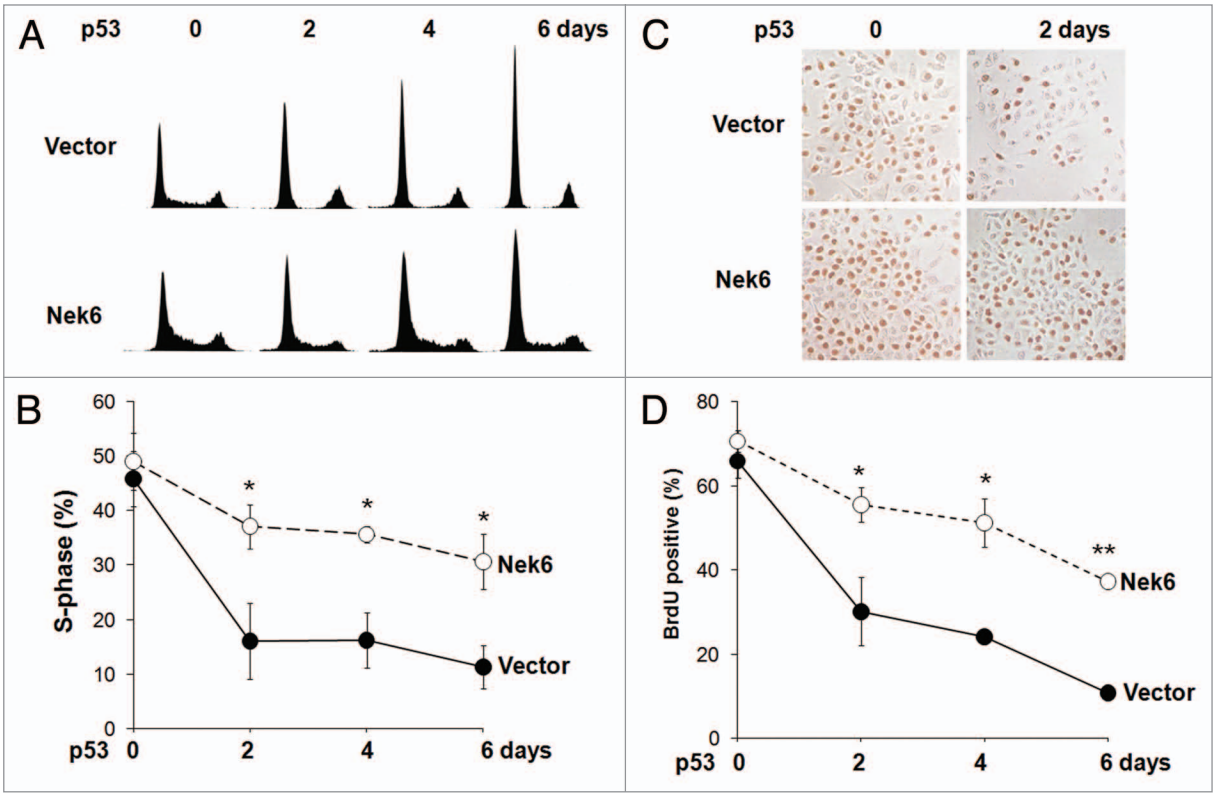


**Figure 4.** Nek6 inhibits p53-induce cell cycle arrest and loss of proliferation potential. (A) EJ-vector (Vector) and EJ-Nek6 (Nek6) cells were infected with p53 adenovirus and harvested at the indicated time points. Cells were fixed, stained with PI and then analyzed by flow cytometry as described in Materials and Methods. The data shown represent three independent experiments. (B) The percentage of cells in the S phase of sample (A) was determined with WINCYCLE software and plotted. Error bars indicate standard deviation. *p < 0.05 by Student's t-test. (C and D) BrdU incorporation of EJ-Nek6 (Nek6) and EJ-vector (Vector) cells was examined at 0, 2, 4 and 6 days after infection with p53 adenovirus as described in Materials and Methods. (A) BrdU incorporation assay images captured of the 2 day time point. (B) BrdU incorporation results of three independent experiments are presented as mean values with standard deviations. *p < 0.05; **p < 0.01 by Student's t-test.

IMR90 cells (**Fig. 1C**). The expression level of Nek6 was also significantly decreased during p53-induced senescence of EJ human bladder carcinoma cells (**Fig. 1F**). These results suggest that Nek6 expression is downregulated during both replicative and premature senescence. Moreover, complete inhibition of p53-induced senescence of EJ cells by Nek6 overexpression (**Fig. 2**) indicates that the reduction of Nek6 level is not a consequence of, but a causal event, in the onset of cellular senescence. The inhibition of p53-induced senescence by Nek6 overexpression was also observed in H1299 human lung carcinoma cells (**Fig. 3**), suggesting that Nek6 overexpression is able to antagonize p53-induced senescence in various human cancer cells. Whereas the overexpression of wild type Nek6 in EJ and H1299 cells effectively suppressed p53-induced senescence, the kinase-dead mutant of Nek6 failed to inhibit senescence upon p53 expression (**Fig. 3**), indicating that its kinase activity plays an essential role in Nek6-mediated senescence inhibition.

We noted that, although the increase of mRNA level was mild (about 2 fold), the ectopic expression of Nek6 in EJ and H1299 results the significant suppression of p53-induced senescence (**Figs. 2 and 3**). These results suggest that increased Nek6 expression may confer resistance to cells against cellular senescence, a major tumor suppression mechanism. Interestingly, recent studies indicate that Nek6 overexpression is related to human cancers. The enhanced expression of Nek6 has been demonstrated in cancer cell lines, tissue samples from human cancer patients, and in vivo animal models.[10,12-14,19] Specifically, Nassirpour, et al. and Jeon et al. recently demonstrated that Nek6 overexpression stimulates cell transformation and tumor growth in a mouse model.[14,19] These results indicate that Nek6 overexpression plays an important role in tumorigenesis. However, the detailed mechanism by which the overexpression of Nek6 contributes to tumorigenesis is not fully understood. In the present study, we showed that mild Nek6 overexpression can efficiently inhibit premature senescence induced by restoring p53 tumor suppressor function. The data presented here suggest that the increase of Nek6 expression in human cancer cells could promote cell transformation and tumorigenesis by inhibiting the onset of cellular senescence. Consistent with this notion, EJ cells overexpressing Nek6 maintain proliferative potential and grow continuously even in the presence of tumor suppressor p53 expression (**Fig. 4**).

Because Nek6 overexpression reduced the decrease of S phase and BrdU positive cells (**Fig. 4**), it is likely that Nek6 inhibits the onset of senescence by preventing p53-induced cell cycle arrest. Nek6 affect cell cycle control primarily by regulating mitotic cell cycle progression.[6] Consistent with this, Nek6 overexpression

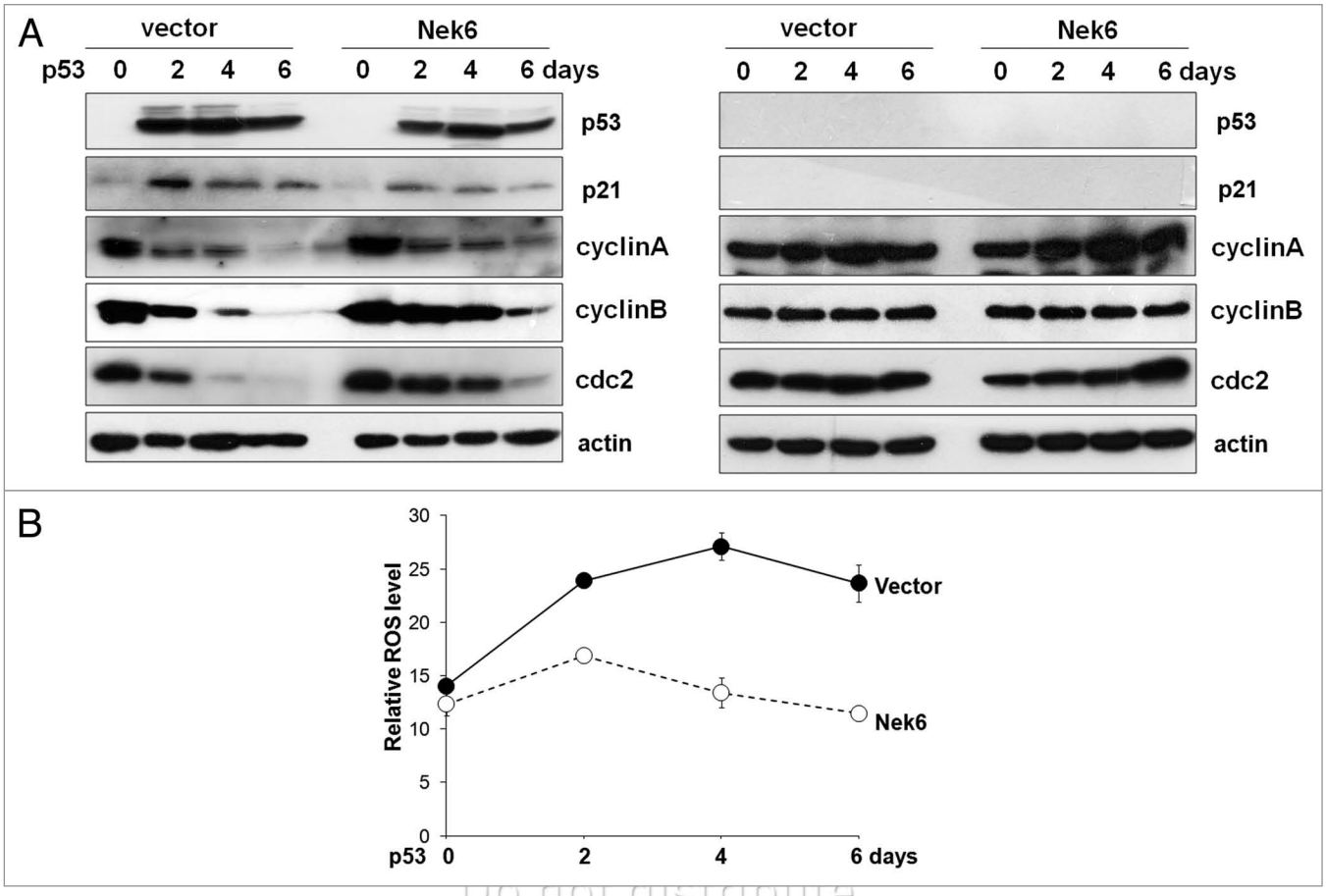




**Figure 5.** Effect of Nek6 overexpression on cell cycle regulatory proteins and intracellular levels of ROS upon p53 expression. (A) Cell lysates isolated from EJ-vector (Vector) and EJ-Nek6 (Nek6) cells after p53 adenovirus infection (left part) or without p53 adenovirus infection (right part) at the indicated time points were subjected to western blotting using the indicated antibodies. The data shown are representative of three independent experiments. (B) EJ-vector (Vector) and EJ-Nek6 (Nek6) cells were infected with or without p53 adenovirus and intracellular levels of ROS were determined as described in Materials and Methods. Experiments were repeated three times and the results were presented as mean values with standard deviations.

inhibited the p53-induced decrease of cyclin B and cdc2, which are critical $G_2$/M regulators, whereas the decrease of cyclin A upon p53 expression was not affected (**Fig. 5A**). It has been previously shown that p53 inhibits the $G_2$/M transition by decreasing levels of cyclin B and cdc2.[20,21] More importantly, Park et al. showed that the overexpression of cyclin B and cdc2 overrides p53-mediated $G_2$/M arrest.[21] Thus, it is possible to speculate that Nek6 overexpression could inhibit p53-induced cell cycle arrest by preventing a decrease in cyclin B and cdc2 protein levels. However, this does not completely rule out the possibility that the maintenance of cyclin B and cdc2 protein levels is the consequence of senescence suppression. Whether cyclin B and cdc2 are involved in Nek6-mediated suppression of senescence and how Nek6 regulates cyclin B and cdc2 levels remain to be defined in a future study.

Interestingly, we observed that the increase of intracellular ROS levels upon p53 expression was also inhibited by Nek6 overexpression (**Fig. 5B**). Accumulating evidence suggests that ROS play an important role in the induction of premature senescence.[3] Moreover, Jung, et al. showed that ROS induction is critical for the onset of p53-induced senescence in EJ cells.[15] It is possible that Nek6 overexpression may affect both p53-induced cell cycle arrest and ROS generation through independent mechanisms. Currently, it is not known if Nek6 can directly suppress intracellular ROS generation or if the inhibition of ROS induction is an indirect effect of antagonizing p53-induced cell cycle arrest. In addition to senescence-inducing activity, it has been recently reported that p53 is also able to suppress cellular senescence in some situations.[22,23] These studies suggest that interplay among the p53, ROS and mTOR signaling pathways is important to determine the onset of senescence in human cancer cells.[24,25] The identification of the physiological targets for Nek6 kinase would be helpful in understanding the molecular mechanism by which Nek6 suppresses the onset of p53-induced senescence and in understanding the complex network of the p53 signaling network.

In conclusion, this study revealed that the decrease of Nek6 expression is important for the onset of cellular senescence. Previously, we showed that Nek6 is a direct target of the DNA damage checkpoint, and the inhibition of Nek6 activity is required for proper cell cycle arrest upon genotoxic stress.[9] In conjunction with our previous study, these current results suggest

that Nek6 acts as an important target for tumor suppressor pathways during tumorigenesis. The overexpression of Nek6 could promote tumorigenesis, at least in part, by inhibiting cellular senescence and Nek6 could thus be a potential target for cancer treatment. Although the exact mechanism by which Nek6 inhibits cellular senescence remains to be elucidated, further studies will help to understand the role of Nek6 in tumorigenesis and provide efficient strategies for cancer treatment targeting Nek6.

## Materials and Methods

**Cell culture, plasmids and establishment of Nek6 stable cell lines.** IMR90 normal human lung fibroblasts were obtained from Coriell Cell Repositories (Camden, NJ, USA) and cultured in Eagle's minimum essential medium supplemented with 10% FBS. EJ human bladder carcinoma cells and H1299 human lung carcinoma cells were maintained with DMEM containing 10% FBS. To induce premature senescence, EJ cells were infected with a recombinant adenovirus encoding wild type p53 at 50–100 m.o.i for 3 h as described previously in reference 21.

To generate the Flag-Nek6 expression plasmid containing the neomycin selection marker, the Nek6 coding sequence from pCMV5-Flag-Nek6,[26] was subcloned into the EcoRI site of the pCMV-Tag2A vector (Stratagene, La Jolla, CA). The Flag-Nek6 plasmid or Flag-vector was transfected into EJ cells using Lipofectin reagent (Invitrogen, Carlsbad, CA) and cells were selected with 500 μg/ml of neomycin for 2 weeks.



Retroviral expression constructs for Nek6 wild type and K74M/K75M kinase dead mutant (pBabe-Nek6 and pBabe-Nek6 KK/MM)[8] were kindly provided by Dr. Min-Jean Yin (Pfizer Inc., San Diego, CA). Retroviral Nek6 constructs were transfected into ecotropic Bosc cells with pantropic retroviral packaging constructs and cell-free viral supernatants were used to infect EJ and H1299 cells. After puromycin selection, resistant cells were pooled and used for all following experiments.

**Senescence associated (SA)-β-galactosidase staining.** Cells were fixed with 0.25% glutaraldehyde and SA β-galactosidase staining was performed at pH 6.0 as described previously.[2] After staining, at least 300 cells in several fields were examined and SA β-gal positive cells were counted. These experiments were repeated three times and the results were presented as mean values with standard deviations.

**Semi-quantitative RT-PCR analyses.** For semi-quantitative reverse transcriptase (RT)-PCR analyses, total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was obtained using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and oligo-dT primers (Promega, Madison, WI). PCR was carried out with AccuPower PCR premix (Bioneer Co., Taejon, Korea) using cDNA as template. PCR products were separated by electrophoresis in 2% agarose gels. PCR conditions were as follows: one cycle at 95°C for 5 min, followed by 22 cycles at 95°C for 45 sec, 55°C for 1 min and 72°C for 1 min. The primers employed in RT-PCR were as follows: p53 forward primer 5'-ATG GAG GAG CCG CAG TCA GAT-3', p53 reverse primer 5'-ACC TGG GTC TTC AGT GAA CCA TTG-3', p21 forward primer 5'-ACT GTG ATG CGC TAA TGG C-3', p21 reverse primer 5'-ATG GTC TTC CTC TGC TGT CC-3', Nek6 forward primer 5'-TAG CAT AAG CCT TGA CAG GTC AC-3', Nek6 reverse primer 5'-AGA AGA TCG AGC AGT GTG ACT AC-3', actin forward primer 5'-ATG GAT GAT GAT ATC GCC GCG-3', and actin reverse primer 5'-TCT CCA TGT CGT CCC AGT TG-3'. Experiments were repeated at least twice and relative band intensities were calculated after normalization to the actin signal.

**Western blot analysis and antibodies.** Cells were lysed in RIPA buffer and subjected to western blot analysis as described previously in reference 27. Antibodies for p53, cyclinA, cyclinB and cdc2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for p21 were purchased from Oncogene Science (Cambridge, MA). Levels of actin were monitored as an internal loading control using anti-actin (Sigma) antibodies.

**BrdU incorporation assay.** EJ-Nek6 and EJ-vector cells infected with p53 adenovirus were seeded on coverslips. After 0, 2, 4 and 6 days, cells on the coverslips were labeled with 10 μM of BrdU for 1 hr. The labeled cells were detected using a BrdU Staining Kit (Invitrogen) as described in the manufacturer's instructions. After staining, at least 300 cells in several fields were examined and the BrdU-positive cells were counted. These experiments were repeated three times and the results were presented as mean values with standard deviations.

**Cell cycle analysis.** To determine the cell cycle distribution, $1 \times 10^6$ cells were seeded into a 100 mm dish. After p53 adenovirus infection, cells were trypsinized at the indicated time points and fixed with 70% ethanol. Subsequently, cells were stained with propidium iodide (PI), and flow cytometric analysis was performed using an EPICS XL cytometer and WINCYCLE software (Beckman Coulter Inc.). A total of 10,000 events were analyzed for each sample, and the experiment was repeated at least three times.

**Measurement of intracellular ROS level.** To measure intracellular levels of ROS, cells were stained with 50 μM of dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma-Aldrich Co., Ltd.,) for 30 min and then harvested. The fluorescent intensities were quantified using an EPICS XL cytometer (Beckman Coulter Inc.). Experiments were performed in triplicate, and the results were presented as mean values with standard deviations.

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### Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/JeeCC9-23-sup.pdf

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