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The inhibition of Nek6 function sensitizes human cancer cells to premature senescence upon serum reduction or anticancer drug treatment

Hye Jin Jee [a,b], Hyun-Ju Kim [a,b], Ae Jeong Kim [a,b], Naree Song [a,b], Minjee Kim [a,b], Hye-Jeong Lee [a,c], Jeanho Yun [a,b,*]

[a] Department of Biochemistry, College of Medicine, Dong-A University, Busan 602-714, South Korea
[b] Mitochondria Hub Regulation Center, College of Medicine, Dong-A University, Busan 602-714, South Korea
[c] Department of Pharmacology, College of Medicine, Dong-A University, Busan 602-714, South Korea

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ABSTRACT

The induction of premature senescence in cancer cells was proposed as an effective cancer treatment strategy. In this paper, we show that the inhibition of Nek6 expression by Nek6 siRNA-mediated knockdown or the overexpression of a dominant negative form of Nek6 (Nek6KM) induced premature senescence as well as cell death under reduced serum conditions in multiple cancer cell lines, including both p53 wild-type and p53 mutant/null backgrounds. Moreover, cancer cells expressing Nek6KM exhibited significantly increased premature senescence upon treatment with the anticancer drugs doxorubicin (DOX) and camptothecin (CPT). Significantly, the overexpression of Nek6KM also inhibited tumor growth and promoted premature senescence *in vivo* in a xenograft mouse model. Taken together, our results further confirm that Nek6 plays an important role in the premature senescence of cancer cells, suggesting that Nek6 may be a potential therapeutic target for human cancers.

1. Introduction

Chemotherapy remains the primary treatment for most human cancers. These drugs reduce the proliferative potential of cancer cells through either apoptotic cell death or premature senescence [1]. Although apoptosis has been considered the main response to chemotherapeutic drugs, recent studies have indicated an important role for premature senescence in cancer treatment [2]. Neoadjuvant chemotherapy induces premature senescence in tumor tissues obtained from breast and lung cancer patients [3,4]. Importantly, Schmitt et al. demonstrated that anticancer drugs induce premature senescence *in vivo* and that tumor prognosis following anticancer chemotherapy correlates to the degree of premature senescence [5]. These results suggest that premature senescence is a critical determinant of clinical outcome in response to anticancer drug treatments.

Premature senescence is accompanied by various phenotypic changes that are similar to those of replicative senescence, including morphological changes, increased senescence-associated β-galactosidase (SA β-gal) activity, cell cycle arrest, and the induction of reactive oxygen species (ROS) [6,7]. While replicative senescence is induced by the exhaustion of proliferative potential, premature senescence can be induced by cellular stresses, such as DNA damage, oxidative stress, and oncogene activation [6,7]. Previous studies demonstrated that premature senescence acts as a barrier to tumorigenesis by eliminating damaged or oncogene-activated cells [8]. Therefore, the acquisition of resistance to premature senescence is an essential step in tumor development. The identification of central regulators of senescence pathways is a crucial step toward understanding how cancer cells escape premature senescence and developing efficient strategies for inducing senescence in cancer cells.

We previously identified Nek6, a mitotic kinase belonging to the Nek (NIMA-related kinase) family, as a novel regulator of the onset of premature senescence. Nek6 expression was decreased during replicative and p53-induced premature senescence [9]. Importantly, the overexpression of Nek6 suppressed p53-induced senescence. In a subsequent study, we showed that Nek6 also suppressed anticancer drug-induced premature senescence [10]. Interestingly, previous studies showed that Nek6 is upregulated in various human cancers [11–15]. Recently, it has been shown that Nek6 is able to stimulate tumorigenesis *in vitro* and *in vivo* [11,12]. In conjunction with recent reports, our results suggest that

* Corresponding author at: Department of Biochemistry, College of Medicine, Dong-A University, Busan 602-714, South Korea. Tel.: +82 51 240 2919; fax: +82 51 241 6940.
E-mail address: yunj@dau.ac.kr (J. Yun).

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Nek6 may promote tumorigenesis, at least in part, by suppressing the premature senescence of cancer cells. In this study, to further understand the role of Nek6 in premature senescence, we examined the effects of Nek6 inhibition on premature senescence in human cancer cells. We found that the inhibition of Nek6 function by either knockdown of Nek6 expression using siRNA or the overexpression of a kinase-dead mutant of Nek6 (Nek6KM) sensitized human cancer cells to serum deprivation or anticancer drug-induced premature senescence. Moreover, the overexpression of Nek6KM inhibited tumor growth in a mouse xenograft model and increased premature senescence in tumor tissues. These results further confirm that Nek6 confers resistance against premature senescence in human cancer cells and that Nek6 is a potential target for cancer treatment.

2. Materials and methods

2.1. Cell culture, treatments, and establishment of stable cell lines

A549, H596, H1299, and EJ cells were maintained in DMEM containing 10% FBS (Sigma, St. Louis, MO). The retroviral expression constructs for wild-type and K74M/K75M kinase-dead mutant Nek6 (pBabe-Nek6 and pBabe-Nek6 KK/MM) [16] were kindly provided by Dr. Min-Jean Yin (Pfizer Inc., San Diego, CA). The retroviral Nek6 constructs were transfected into Bosc23 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 subsequent experiments. Doxorubicin (DOX) and camptothecin (CPT) were purchased from Sigma. To induce premature senescence, cells were treated with 20 nM of DOX or CPT for 16 h and incubated in drug-free culture medium for the indicated times.

2.2. Nek6 RNA interference and RT-PCR

A pool of siRNAs specific for Nek6 (ON-TARGETplus SMARTpool Human Nek6, L-004166000005) and control siRNA (ON-TARGETplus Non-targeting siRNA, D-0018100105) were obtained from Dharmacon (Lafayette, CO). To knock down Nek6 expression, 4×10^5 cells were seeded into a 60 mm dish and transfected with siRNA using the DharmaFECT1 transfection reagent (Dharmacon) according to the manufacturer's instructions. For semi-quantitative reverse transcriptase (RT)-PCR analyses, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) 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, South Korea) using cDNA as a template. PCR products were separated by electrophoresis in 2% agarose gels. The PCR conditions were as follows: one cycle at 95 °C for 5 min and 22 cycles at 95 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min. The primers employed in RT-PCR were as follows: Nek6 forward primer 5′-TAGCATAAGCCTTGACAGGTCAC-3′, Nek6 reverse primer 5′-AGAAGATCGAGCAGTGTGACTAC-3′, actin forward primer 5′-ATGGATGATGATATCGCCGCG-3′, and actin reverse primer 5′-TCTCCATGTCGTCCCAGTTG-3′. The experiments were repeated at least twice, and relative band intensities were calculated after normalization to the actin signal.

2.3. 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 [17]. After staining, at least 300 cells in several visual fields were examined, and the number of SA β-gal-positive cells was determined. These experiments were repeated three times, and the results were presented as the mean values with standard deviations.

2.4. Annexin V cell death assay

Cells were stained using the Annexin V-FICT Apoptosis Detection Kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Stained cells were analyzed by flow cytometry.

2.5. Western blot analysis and antibodies

Cells were lysed in RIPA buffer and subjected to western blot analysis, as described previously [18]. Antibodies for Nek6 were purchased from Gene Tex Inc. (Irvine, CA). Antibodies for cyclin B and cdc2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for p21 were purchased from Oncogene Science (Cambridge, MA). Actin was used as an internal loading control using anti-actin (Sigma) antibodies.

2.6. Cell cycle analysis

To determine the cell cycle distribution, 1×10^6 cells were seeded into a 100 mm dish. The cells were treated with DOX; 4 days later, the cells were trypsinized and fixed with 70% ethanol. Subsequently, the cells were stained with propidium iodide (PI), and flow cytometry analysis was performed using an EPICS XL cytometer and WINCYCLE software (Beckman Coulter Inc., Brea, CA). A total of 10,000 events were analyzed for each sample, and the experiment was repeated at least three times.

2.7. Measurement of intracellular ROS level

To measure intracellular levels of ROS, cells were stained with 50 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma) for 30 min at the indicated time points after DOX treatment. Cells were trypsinized, and the fluorescent intensities were quantified using an EPICS XL cytometer (Beckman Coulter Inc.) Experiments were performed in triplicate, and the results are presented as the mean values with standard deviations.

2.8. In vivo mouse studies

Five- to six-week-old BALB/c NOD/SCID mice were purchased from the Animal Laboratory of the Korea Research Institute of Bioscience and Biotechnology (Taejeon, South Korea) and maintained under pathogen-free conditions. All procedures were performed according to a protocol (DIACUC-09-19) approved by the Dong-A Institutional Animal Care and Use Committee. Suspensions of 2×10^6 EJ cells stably expressing wild-type (Nek6WT) or KK/MM (Nek6KM) Nek6 in 100 μl of PBS buffer were injected subcutaneously into five mice per group. The tumor size was measured using electronic digital calipers every week. To measure the senescence-associated (SA) β-galactosidase activity in the tissue, tumors were surgically dissected at day 45. Tumor samples were freshly frozen in Tissue-Tek cryopreservation medium and sectioned using a LEICA CM3050 S cryostat (Leica Microsystems Inc., Buffalo, IL). SA β-galactosidase staining was performed using freshly prepared SA β-gal staining solution.

3. Results

3.1. The knockdown of endogenous Nek6 expression induces premature senescence under reduced serum conditions

To address whether the inhibition of Nek6 function could induces premature senescence in human cancer cells, we first examined the effect of the RNAi-mediated knockdown of Nek6 in cancer cells with both p53 wild-type (A549, H596) and p53 mutant/null backgrounds (H1299, EJ). Western blot and semi-quantitative RT-PCR analysis confirmed that the level of Nek6 protein and mRNA were markedly suppressed in all four cell lines after transfection with a pool of siRNA against Nek6 (siNek6). Meanwhile, the transfection of a negative control siRNA (NC) did not alter either Nek6 protein or mRNA levels (Fig. 1A and Supplementary Fig. 1), indicating that Nek6 expression is successfully and specifically suppressed by siRNA transfection. Nek6 knockdown did not induce premature senescence under ordinary culture conditions (DMEM containing 10% FBS) in any of the four cancer cell lines examined (data not shown). However, under reduced serum culture conditions (1% FBS), Nek6 knockdown causes decreased cancer cell proliferation after 6 days compared with control cultures; in addition, the cells are enlarged and exhibit a flattened morphology (Fig. 1B). Cells entering premature senescence exhibit distinct features, including reduced proliferation and dramatic changes to their morphology [7]. An annexin V/PI staining assay demonstrates a mild increase of cell death in Nek6 knockdown cancer cells under reduced serum conditions (Fig. 1C). However, senescence-associated β-galactosidase (SA β-gal) staining activity, which is a reliable marker for cellular senescence [17], was increased by approximately 2.5- to 17-fold under reduced serum conditions in all Nek6 knockdown cancer cells, including both wild-type p53 and p53 null backgrounds (Fig. 1C). In order to rule out a possible off-target effect of siRNA, we divided four individual siRNA oligonucleotides in the Nek6 siRNA pool into two sets and transfected into cancer cells. Nek6 western blot and SA β-gal staining analysis confirmed that

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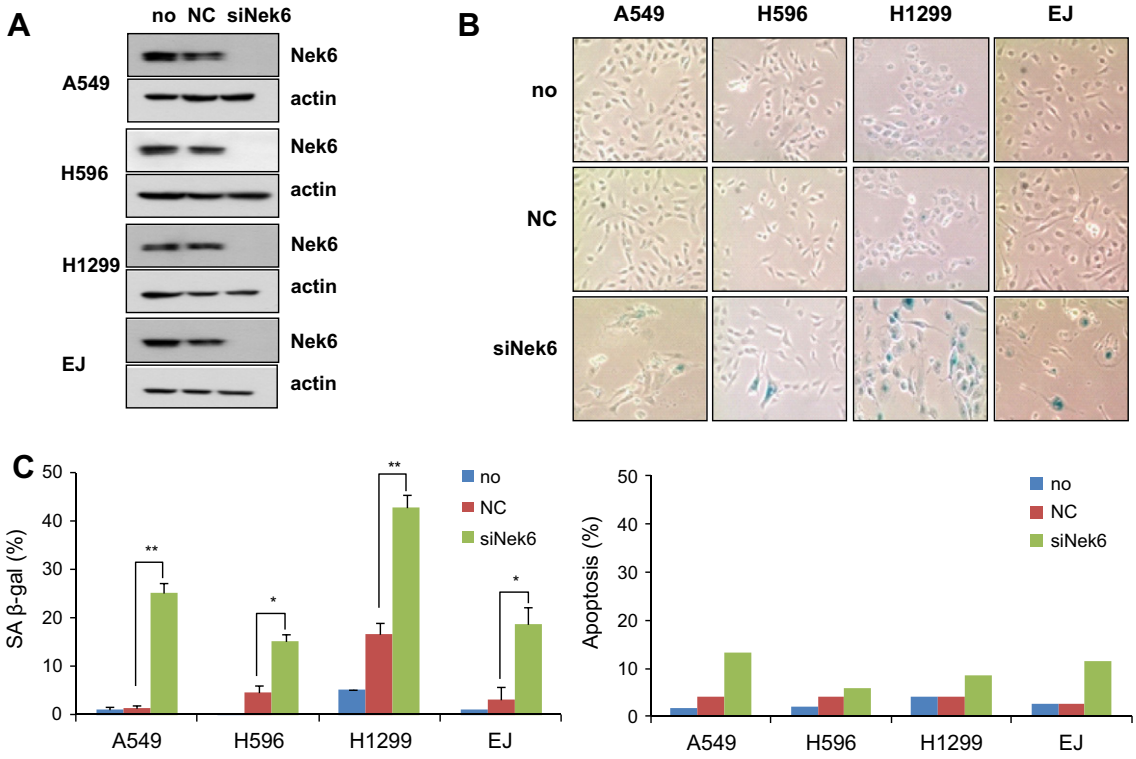

Fig. 1. The induction of premature senescence upon Nek6 knockdown in human cancer cells. (A–C) A549, H596, H1299, and EJ cells were transfected with either negative control siRNA (NC) or a siRNA pool targeting Nek6 (siNek6). (A) Total lysates were prepared 2 days after transfection, and western blot analysis was performed using anti-Nek6 and anti-actin antibodies. (B) After 6 days of culture in 1% FBS medium, cells were stained with freshly prepared SA β-gal staining solution. Cell images were captured under a microscope. The data shown are representative of three independent experiments (upper panel). (C) The percentages of SA β-gal-positive cells in three independent experiments are presented as the mean values with standard deviations (lower panel). $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t*-test. To analysis cell death, cells were stained using the Annexin V-FITC apoptosis kit (BD Biosciences, NJ) after 6 days of culture in 1% FBS medium. Stained cells were analyzed by flow cytometry.

both Nek6 siRNA sets induced Nek6 knockdown and the increase of SA β-gal staining activity (Supplementary Fig. 2). These results suggest that the knockdown of Nek6 expression sensitizes cancer cells to stress-induced premature senescence and cell death regardless of their p53 status.

3.2. The overexpression of a dominant negative form of Nek6 induces premature senescence under reduced serum conditions

To further confirm the above findings, we inhibited Nek6 function by overexpressing a kinase-dead Nek6 (Nek6KM), which acts as dominant negative mutant [16]. To this end, we established cell lines overexpressing wild-type (Nek6wt) or kinase-dead Nek6 (Nek6KM) using retrovirus-mediated gene transfer. To control for clonal variation, we pooled resistant cells after antibiotic selection. The overexpression of HA-tagged Nek6wt or Nek6KM was confirmed by western blot analysis using anti-Nek6 antibodies (Fig. 2A).

Consistent with the results of Nek6 knockdown experiments, Nek6KM expression did not induce premature senescence under ordinary culture conditions in the p53 mutants or wild-type cancer cells. However, all cancer cells overexpressing Nek6KM exhibited morphological changes and growth retardation, similar to Nek6 knockdown cells under reduced serum conditions (Fig. 2B). SA β-gal staining activity was also increased by approximately 2.4- to 9.4-fold under reduced serum conditions, while the overexpression of wild-type Nek6 did not have a similar effect (Fig. 2C). The annexin V/PI staining assay indicates that cell death is moderately increased in Nek6KM cells after culture in reduced serum conditions (Fig. 2C). These results further confirm that the inhibition of Nek6 function sensitizes human cancer cells to premature senescence as well as cell death under stress conditions in a p53-independent manner.

3.3. The inhibition of Nek6 function sensitizes human cancer cells to anticancer drug-induced premature senescence

The induction of premature senescence under reduced serum conditions after either Nek6 knockdown or the overexpression of a Nek6 dominant negative mutant (Nek6KM) in multiple cancer cells raised the possibility that the inhibition of Nek6 function sensitizes cancer cells to premature senescence. To further test this theory, we investigated whether the inhibition of Nek6 function sensitizes cancer cells to anticancer drug-induced premature senescence.

To induce anticancer drug-induced premature senescence, we treated Nek6wt and Nek6KM cells with sublethal doses of doxorubicin (DOX) or camptothecin (CPT). Premature senescence was assessed by SA β-gal staining 6 days after DOX or CPT treatment. As shown in Fig. 3A, Nek6KM cells exhibited approximately 1.7- to 2.7-fold greater premature senescence than vector-transfected cells upon DOX treatment, while Nek6wt expression suppressed DOX-induced premature senescence, as we had shown previously [10]. Moreover, cancer cells overexpressing Nek6KM also showed significantly higher premature senescence after CPT treatment (Fig. 3B). The annexin V/PI staining assay indicated that cell death was moderately increased in response to DOX treatment in Nek6KM cells (Fig. 3C). These results suggest that the inhibition of Nek6 function through Nek6KM expression sensitizes cancer cells to anticancer drug-induced premature senescence.

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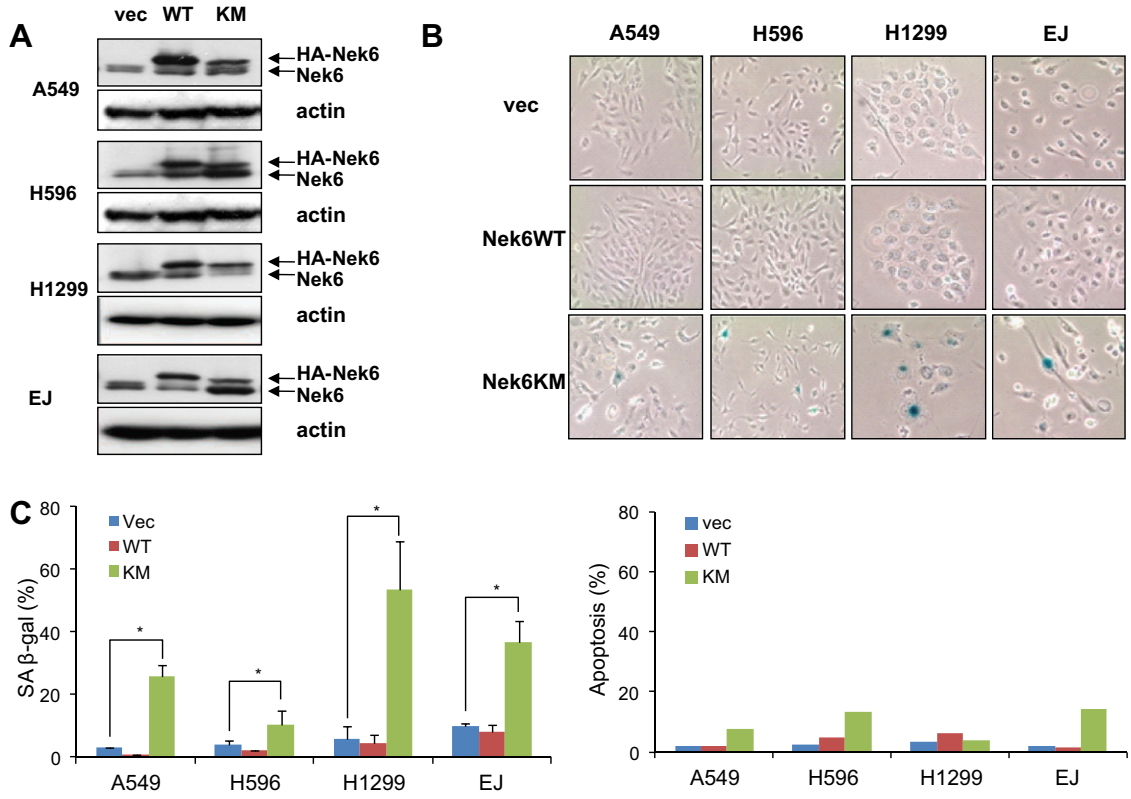

Fig. 2. The induction of premature senescence upon Nek6KM overexpression in human cancer cells. (A) A549, H596, H1299, and EJ cells stably expressing empty pBabe (Vec), pBabe-HA-Nek6 wild type (Nek6WT), and pBabe-HA-Nek6 KK/MM (Nek6KM) [9] were generated. Cell lysates were subjected to western blotting using anti-Nek6 and anti-actin antibodies. (B) Cells were cultured in 1% FBS medium for 6 days and stained with freshly prepared SA β-gal staining solution. The data shown are representative of three independent experiments (upper panel). (C) The percentages of SA β-gal-positive cells in three independent experiments are presented as the mean values with standard deviations (lower panel). $^{*}P < 0.05$ by Student's *t*-test. Cell death was measured using the Annexin V-FITC apoptosis kit (BD Biosciences, NJ) according to the manufacturer's protocol.

3.4. Cancer cells overexpressing Nek6KM are more susceptible to G2/M cell cycle arrest after DOX treatment

Cell cycle arrest is one of the typical steps in the onset of premature senescence in response to various genotoxic stresses [8]. Nek6 plays an essential role in mitotic cell cycle progression [16,19–21]. To investigate how the inhibition of Nek6 function sensitizes cancer cells to premature senescence, we first analyzed cell cycle arrest after DOX treatment. Cell cycle analysis revealed that cancer cells expressing Nek6KM exhibited much greater G2/M cell cycle arrest compared to vector control cells (Fig. 4A). Quantitative analysis showed that G2/M arrest in Nek6KM cells in response to DOX treatment was approximately 3-fold higher than in vector control cells (Fig. 4B). These results suggest that the inhibition of Nek6 function sensitizes cancer cells to DOX-induced cell cycle arrest.

Cyclin B and cdc2/cdk1, which are essential components for G2/M cell cycle phase progression, have been shown to be key regulators of premature senescence after genotoxic treatment [1]. To investigate the mechanism by which the inhibition of Nek6 function affects DOX-induced cell cycle arrest, we examined cyclin B and cdc2 protein level using western blotting analysis after DOX treatment. Interestingly, the protein levels of cyclin B and cdc2 were decreased more rapidly in response to DOX treatment in Nek6KM cells than in vector control cells (Fig. 5). In vector control cells, cyclin B and cdc2 levels were usually decreased by 4 days after DOX treatment, but Nek6KM cells showed clear decreases in cyclin B and cdc2 levels at 2 days after DOX treatment. In contrast, semi-quantitative RT-PCR analysis revealed that the mRNA levels of cyclin B and cdc2 decreased upon DOX treatment in both Nek6KM and vector control cells in a similar manner (Supplementary Fig. 3). These results suggest that the inhibition of Nek6 function increases G2/M cell cycle arrest by stimulating a decrease in cyclin B and cdc2 protein levels through post-transcriptional regulation.

3.5. Cancer cells overexpressing Nek6KM are more susceptible to increased intracellular levels of ROS after DOX treatment

Previous studies showed that the induction of intracellular ROS levels plays an important role in the onset of premature senescence [6]. To examine whether the overexpression of Nek6KM influences ROS levels after DOX treatment, we measured intracellular ROS levels in both vector control and Nek6KM cell lines after DOX treatment. As shown in Fig. 6, the increase in ROS levels in response to DOX treatment was much more evident in Nek6KM cells than in vector control cells. Quantitative analysis revealed that the ROS levels of Nek6KM cells were approximately 1.5- to 1.9-fold higher than those of vector control cells at 4 days after DOX treatment. These results suggest that the inhibition of Nek6 function stimulates an increase in ROS and cell cycle arrest after DOX treatment.

3.6. The inhibition of Nek6 function increases premature senescence in vivo

To further confirm that the inhibition of Nek6 function sensitizes cancer cells to premature senescence under physiological conditions, we extended our studies to tumor xenograft mouse models. Because we observed that the overexpression of the Nek6 dominant negative mutant Nek6KM sensitized cancer cells

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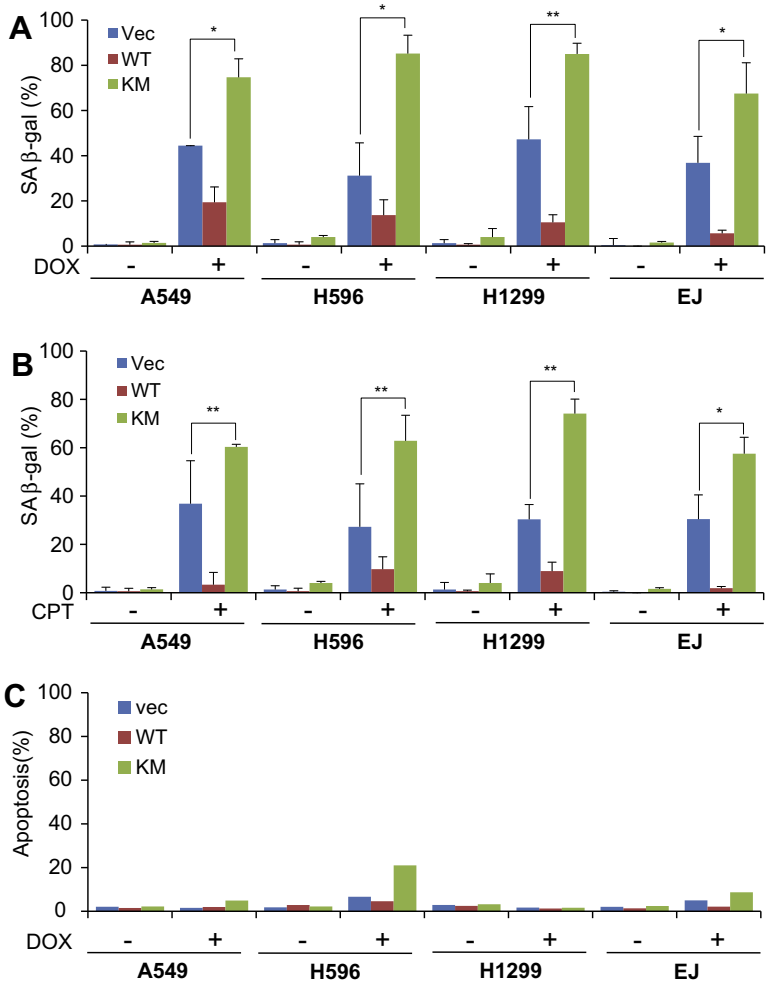

Fig. 3. Increased anticancer drug-induced premature senescence in Nek6KM cell lines. A549, H596, H1299, and EJ cells stably expressing empty pBabe (Vec), wild-type Nek6 (WT), or Nek6 KK/MM (KM) were treated with 20 nM of doxorubicin (DOX) (A) or 20 nM of camptothecin (CPT) (B) for 16 h. After 6 days of culture in drug-free medium, cells were stained with freshly prepared SA β-gal staining solution. The percentages of SA β-gal-positive cells in three independent experiments are presented as the mean values with standard deviations (lower panel). $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t*-test. (C) Cell death was measured 6 days after DOX treatment using the Annexin V-FITC apoptosis kit (BD Biosciences, NJ) according to the manufacturer's protocol.

to premature senescence, we expected that cancer cells expressing Nek6KM might undergo premature senescence under the nutrient starvation conditions present in a solid tumor.

To examine whether the inhibition of Nek6 function also sensitizes cancer cells to premature senescence *in vivo*, NOD/SCID mice were subcutaneously injected with EJ cells expressing either wild-type Nek6 or Nek6KM. EJ-Nek6KM cells showed impaired tumor formation compared with EJ cells expressing wild-type Nek6 (Fig. 7A). Interestingly, the number of SA β-gal-stained cells was markedly increased in tumor tissues derived from EJ-Nek6KM cells, while SA β-gal-positive cells were rarely found in tumor tissues derived from EJ-Nek6wt cells (Fig. 7B). These results further support the theory that the inhibition of Nek6 function sensitizes human cancer cells to premature senescence *in vivo*.

4. Discussions

We have previously demonstrated that Nek6 is able to inhibit the p53- and anticancer drug-induced premature senescence of cancer cells [9,10]. Therefore, we investigated the effect of the inhibition of Nek6 function on premature senescence in cancer cells. We found that the knockdown of Nek6 expression using siRNA against Nek6 induced premature senescence as well as under reduced serum conditions (1% FBS) (Fig. 1 and Supplementary Fig. 1). Because we previously demonstrated that Nek6 kinase activity is essential for Nek6-mediated senescence inhibition [9], it is expected that the inhibition of Nek6 kinase activity would also induce premature senescence in cancer cells. Indeed, the overexpression of a Nek6 dominant negative mutant (Nek6KM) also induced premature senescence under reduced serum conditions (Fig. 2). In addition, the overexpression of Nek6KM significantly sensitized cancer cells to doxorubicin (DOX)- and camptothecin (CPT)-induced premature senescence (Fig. 3). Nek6 RNAi or overexpression of the kinase dead Nek6 mutant induced cell death in several types of cancer cells [11,12,16,22]. Consistent with these reports, we also observed that both the knockdown of Nek6 expression and the overexpression of Nek6KM induced cell death under reduced serum conditions and upon DOX treatment. However, the level of cell death was lower than the level of SA β-gal staining observed in most of the cancer cells (Figs. 1–3, and Supplementary Fig. 2). Thus, these results suggest that the inhibition of Nek6 function stimulates premature senescence as well as cell death, at least under the experimental conditions described in the present study.

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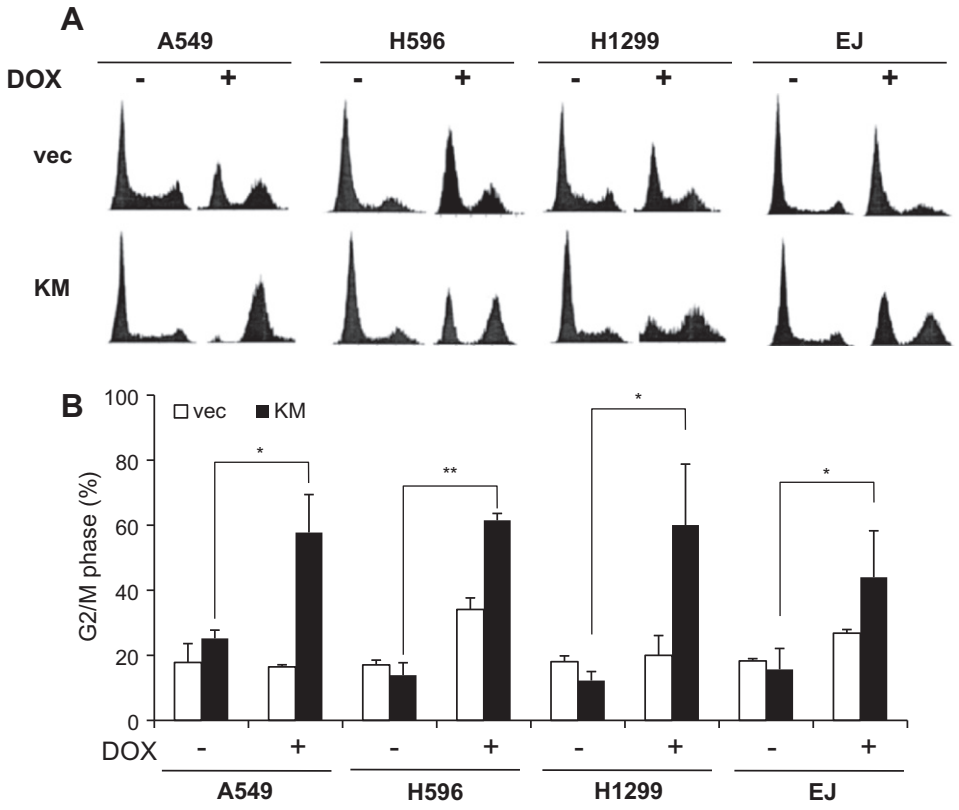

Fig. 4. Increased G2/M cell cycle arrest after DOX treatment in Nek6KM cell lines. A549, H596, H1299, and EJ cells stably expressing empty pBabe (Vec) or Nek6 KK/MM (KM) were treated with or without 20 nM DOX and harvested 4 days later. Cells were fixed, stained with PI, and analyzed using flow cytometry. The data shown are representative of three independent experiments. The percentage of cells in G2/M phase in three independent experiments is presented as the mean values with standard deviations. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t*-test.

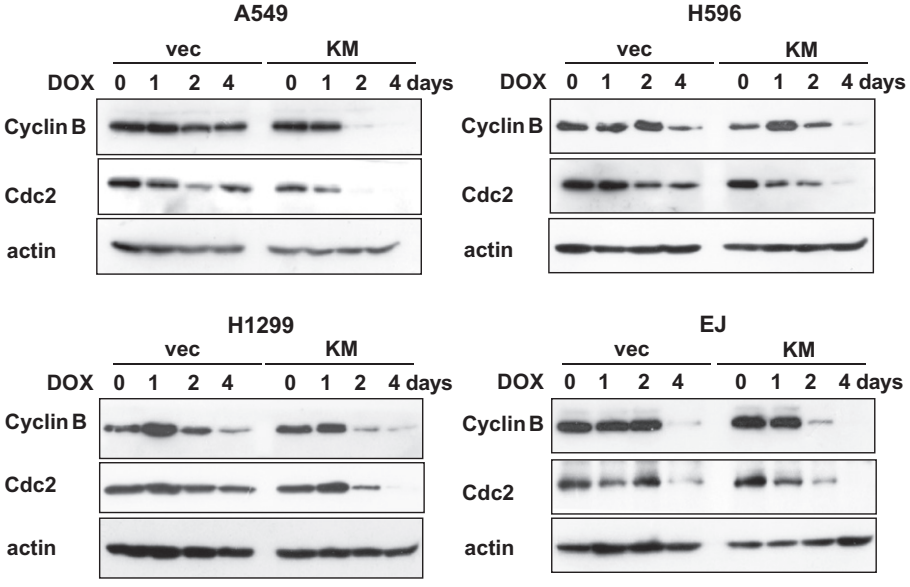

Fig. 5. The effect of Nek6KM overexpression on cell cycle regulatory protein expression upon DOX treatment. A549, H596, H1299, and EJ cells stably expressing empty pBabe (Vec) or Nek6 KK/MM (KM) were treated with or without 20 nM DOX and harvested at the indicated timepoints. Cell lysates were subjected to western blotting using the indicated antibodies. The data shown are representative of three independent experiments.

Recent studies reported that Nek6 levels are increased in various human cancers [11–15]. Moreover, it has been shown that the overexpression of Nek6 is able to promote tumorigenesis, while the inhibition of Nek6 function by siRNA in HeLa cells inhibits its tumor growth in a xenograft mouse model [11,12]. These studies indicated that Nek6 could stimulate tumorigenesis, but the

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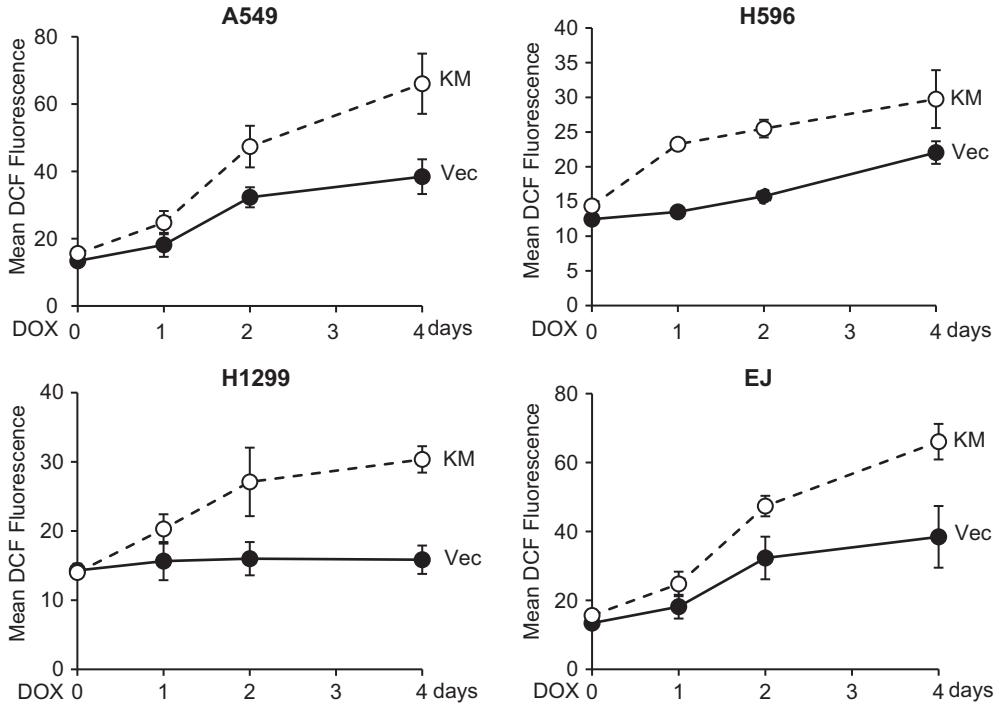

Fig. 6. Increased intracellular ROS levels after DOX treatment in Nek6KM cell lines. A549, H596, H1299, and EJ cells stably expressing empty pBabe (Vec) or pBabe-HA-Nek6 KK/MM (KM) were treated with or without 20 nM DOX, and intracellular levels of ROS were determined at the indicated timepoints using DCF-DA. The experiments were repeated three times, and the results are presented as the mean values with standard deviations.

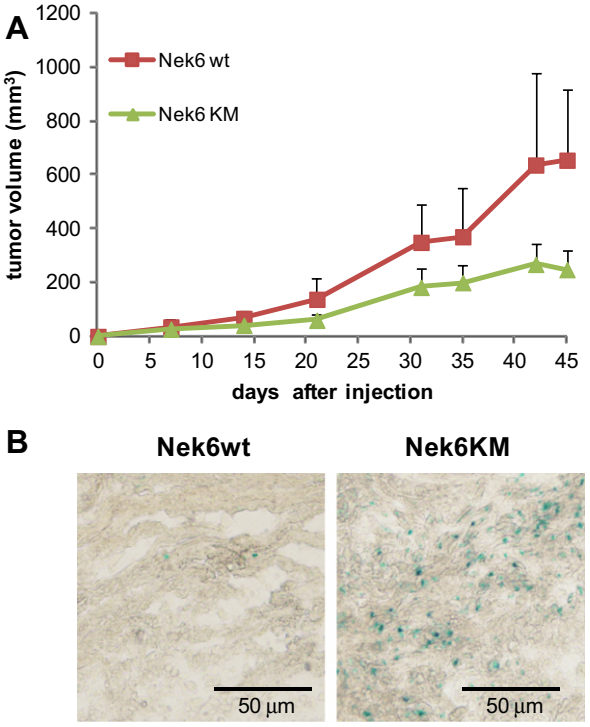

Fig. 7. Nek6KM overexpression inhibits tumor growth and promotes premature senescence *in vivo*. (A) The tumor growth in NOD/SCID mice injected subcutaneously with 2×10^6 EJ cells stably expressing either Nek6 wild type (Nek6WT) or Nek6 KK/MM (Nek6KM) was measured once per week for 45 days. The data provided represent the mean values and standard deviations calculated for each group. (B) Representative SA β-gal staining of tumor sections derived from the injection of Nek6WT or Nek6KM cells. The mice were sacrificed, and SA β-gal staining was examined in sections of fresh-frozen tumors.

precise molecular mechanism has not been fully understood. We previously proposed that Nek6 may promote tumorigenesis by inhibiting premature senescence in cancer cells [9,10] [23,24]. Thus, it is expected that the inhibition of Nek6 function may induce premature senescence in a tumor. Consistent with previous reports, we also observed that EJ human bladder cancer cells expressing Nek6KM showed significantly reduced tumor growth in a xenograft mouse model (Fig. 7A). Importantly, we found that premature senescence in tumor tissues derived from EJ-Nek6KM was markedly increased compared to tumors of EJ-Nek6wt cells (Fig. 7B). These results suggest that Nek6 promotes tumorigenesis at least in part by inhibiting the premature senescence of cancer cells.

In addition, we observed that the inhibition of Nek6 itself either by Nek6 RNAi or expression of Nek6KM did not induce premature senescence in either p53 wild-type or p53 mutant/null cancer cells. However, our results showed that the inhibition of Nek6 function sensitizes cancer cells to premature senescence in cancer lines with either p53 wild-type or p53 mutant/null genotypes (Figs. 1 and 2). These results indicate that Nek6 sensitizes cancer cells to premature senescence independently of cellular p53 status. We previously observed that the overexpression of Nek6 does not disturb p53-mediated p21 induction [9]. Thus, it is likely that Nek6 acts downstream of p53 in the premature senescence pathway. In addition, these results suggest that the strategy for targeting Nek6 could be applicable to a wide range of human cancers in both p53 wild-type and p53 mutant cancer cells.

The effects of the inhibition of Nek6 function correlated with the increase in G2/M cell cycle arrest after DOX treatment (Fig. 4). We observed that protein levels of cyclin B and cdc2, which are key regulators of G2/M cell cycle progression, were decreased at earlier timepoints in Nek6KM cells than in control cells (Fig. 5). These results suggest that the inhibition of Nek6

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function could promote G2/M cell cycle arrest by promoting decreases in cyclin B and cdc2 protein levels after DOX treatment. Consistent with this model, we previously showed that kinase activity of Nek6 is inhibited upon DNA damage, and the overexpression of Nek6 abrogates G2/M arrest in response to DNA damage [21]. These results suggest that downregulating Nek6 activity is required for G2/M cell cycle arrest upon genotoxic stress. If cell cycle arrest is a prerequisite for cellular senescence [6,7], then the inhibition of Nek6 is critical for the induction of cellular senescence. Consistent with this notion, we observed that Nek6 is downregulated during both replicative and p53-induced premature senescence prior to the induction of a cellular senescence phenotype [9]. Moreover, we also showed that the overexpression of Nek6 inhibits premature senescence, and prevents the G2/M cell cycle arrest that accompanies a reduction of decreases in cyclin B and cdc2 levels after p53 expression and DOX treatment [9,10]. Thus, these results suggest that a loss of Nek6 function sensitizes a decrease of cyclin B and cdc2 and G2/M arrest upon genotoxic stresses. However, it is not clear whether Nek6 regulates G2/M cell cycle arrest through cyclin B and cdc2 or whether Nek6 directly regulates the protein levels of cyclin B and cdc2. In addition, whether the increase in G2/M cell cycle arrest after DOX treatment indeed induces premature senescence remain to be addressed in further studies. The identification of Nek6 target substrates will be an essential step toward understanding how Nek6 mediates cell cycle regulation during premature senescence and how Nek6 prevents premature senescence. We are currently working to identify the substrates responsible for Nek6-mediated premature senescence regulation. We noted that the overexpression of Nek6KM also increased ROS induction upon DOX treatment (Fig. 6). These results suggest that Nek6 is involved in the regulation of intracellular ROS levels and cell cycle arrest after genotoxic treatment. However, whether an increase of ROS in Nek6KM cells can truly contribute to the induction of senescence and how Nek6 is involved in ROS regulation remain to be determined. Interestingly, Nek6 has recently been shown to phosphorylate the oncogenic transcription factor STAT3 [11]. Given the important role of STAT3 in cell proliferation, apoptosis, and tumorigenesis [25], STAT3 could be a potential target for the Nek6-mediated regulation of premature senescence.

In conclusion, this study reveals that the inhibition of Nek6 function induces premature senescence in human cancer cells in response to stress, such as reduced serum and genotoxic treatment. In conjunction with our previous studies, the present study provides evidence that Nek6 plays an important role in premature senescence regulation. Although the precise mechanism by which Nek6 regulates cell cycle arrest and intracellular levels of ROS during premature senescence remains to be explored, our study highlights Nek6 as a potential target for cancer treatment. Because Nek6 kinase activity seems to be essential for preventing premature senescence, the pharmacological inhibition of Nek6 kinase activity could be an especially effective strategy for inducing cytostatic effects in human cancer cells.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.02.012.

References

[1] D.A. Gewirtz, S.E. Holt, L.W. Elmore, Accelerated senescence. an emerging role in tumor cell response to chemotherapy and radiation, Biochem. Pharmacol. 76 (2008) 947–957.
[2] J.A. Ewald, J.A. Desotelle, G. Wilding, D.F. Jarrard, Therapy-induced senescence in cancer, J. Natl. Cancer Inst. 102 (2010) 1536–1546.
[3] R.S. Roberson, S.J. Kussick, E. Vallieres, S.Y. Chen, D.Y. Wu, Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers, Cancer Res. 65 (2005) 2795–2803.
[4] R.H. te Poele, A.L. Okorokov, L. Jardine, J. Cummings, S.P. Joel, DNA damage is able to induce senescence in tumor cells in vitro and in vivo, Cancer Res. 62 (2002) 1876–1883.
[5] C.A. Schmitt, J.S. Fridman, M. Yang, S. Lee, E. Baranov, R.M. Hoffman, S.W. Lowe, A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy, Cell 109 (2002) 335–346.
[6] I. Ben-Porath, R.A. Weinberg, The signals and pathways activating cellular senescence, Int. J. Biochem. Cell Biol. 37 (2005) 961–976.
[7] C.A. Schmitt, Cellular senescence and cancer treatment, Biochim. Biophys. Acta 1775 (2007) 5–20.
[8] J. Campisi, F. d'Adda di Fagagna, Cellular senescence. when bad things happen to good cells, Nat. Rev. Mol. Cell Biol. 8 (2007) 729–740.
[9] H.J. Jee, A.J. Kim, N. Song, H.J. Kim, M. Kim, H. Koh, J. Yun, Nek6 overexpression antagonizes p53-induced senescence in human cancer cells, Cell Cycle 9 (2010) 85–92.
[10] H.J. Jee, H.J. Kim, A.J. Kim, N. Song, M. Kim, J. Yun, Nek6 suppresses the premature senescence of human cancer cells induced by camptothecin and doxorubicin treatment, Biochem. Biophys. Res. Commun. 408 (2011) 669–673.
[11] Y.J. Jeon, K.Y. Lee, Y.Y. Cho, A. Pugliese, H.G. Kim, C.H. Jeong, A.M. Bode, Z. Dong, Role of NEK6 in tumor promoter-induced transformation in JB6 C141 mouse skin epidermal cells, J. Biol. Chem. 285 (2010) 28126–28133.
[12] R. Nassirpour, L. Shao, P. Flanagan, T. Abrams, B. Jallal, T. Smeal, M.J. Yin, Nek6 mediates human cancer cell transformation and is a potential cancer therapeutic target, Mol. Cancer Res. 8 (2010) 717–728.
[13] A. Takeno, I. Takemasa, Y. Doki, M. Yamasaki, H. Miyata, S. Takiguchi, Y. Fujiwara, K. Matsubara, M. Monden, Integrative approach for differentially overexpressed genes in gastric cancer by combining large-scale gene expression profiling and network analysis, Br. J. Cancer 99 (2008) 1307–1315.
[14] J. Chen, L. Li, Y. Zhang, H. Yang, Y. Wei, L. Zhang, X. Liu, L. Yu, Interaction of Pin1 with Nek6 and characterization of their expression correlation in Chinese hepatocellular carcinoma patients, Biochem. Biophys. Res. Commun. 341 (2006) 1059–1065.
[15] Y. Hashimoto, H. Akita, M. Hibino, K. Kohri, M. Nakanishi, Identification and characterization of Nek6 protein kinase, a potential human homolog of NIMA histone H3 kinase, Biochem. Biophys. Res. Commun. 293 (2002) 753–758.
[16] M.J. Yin, L. Shao, D. Voehringer, T. Smeal, B. Jallal, The serine/threonine kinase Nek6 is required for cell cycle progression through mitosis, J. Biol. Chem. 278 (2003) 52454–52460.
[17] G.P. Dimri, X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, et al., A biomarker that identifies senescent human cells in culture and in aging skin in vivo, Proc. Natl. Acad. Sci. USA 92 (1995) 9363–9367.
[18] M.A. Kim, H.J. Kim, A.L. Brown, M.Y. Lee, Y.S. Bae, J.I. Park, J.Y. Kwak, J.H. Chung, J. Yun, Identification of novel substrates for human checkpoint kinase Chk1 and Chk2 through genome-wide screening using a consensus Chk phosphorylation motif, Exp. Mol. Med. 39 (2007) 205–212.
[19] L. O'Regan, J. Blot, A.M. Fry, Mitotic regulation by NIMA-related kinases, Cell Div 2 (2007) 25.
[20] C. Belham, J. Roig, J.A. Caldwell, Y. Aoyama, B.E. Kemp, M. Comb, J. Avruch, A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases, J. Biol. Chem. 278 (2003) 34897–34909.
[21] M.Y. Lee, H.J. Kim, M.A. Kim, H.J. Jee, A.J. Kim, Y.S. Bae, J.I. Park, J.H. Chung, J. Yun, Nek6 is involved in G2/M phase cell cycle arrest through DNA damage-induced phosphorylation, Cell cycle 7 (2008) 2705–2709.
[22] L. O'Regan, A.M. Fry, The Nek6 and Nek7 protein kinases are required for robust mitotic spindle formation and cytokinesis, Mol Cell Biol 29 (2009) 3975–3990.
[23] A.M. Fry, A role for Nek6 kinase activity in preventing senescence, Cell Cycle 10 (2011) 19–20.
[24] M.J. Yin, Linking Nek6 to p53-induced senescence, Cell Cycle 10 (2011) 18.
[25] P.A. Johnston, J.R. Grandis, STAT3 signaling: anticancer strategies and challenges, Mol. Interv. 11 (2011) 18–26.