



Nek6 suppresses the premature senescence of human cancer cells induced by camptothecin and doxorubicin treatment

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ARTICLE INFO

Article history:

Received 16 April 2011

Available online 23 April 2011

Keywords:

Nek6
Premature senescence
Tumorigenesis
Camptothecin
Doxorubicin

ABSTRACT

Cellular senescence plays an important role in tumor suppression. The mitotic kinase Nek6 has recently been shown to be overexpressed in various cancers and has been implicated in tumorigenesis. Previously, we reported that the down-regulation of Nek6 expression was required for p53-induced senescence. In this study, we examined the effect of Nek6 overexpression on the premature senescence of cancer cells induced by the anticancer drugs camptothecin (CPT) and doxorubicin (DOX). We found that CPT- and DOX-induced morphology changes and increases in senescence-associated β -galactosidase staining were significantly inhibited in EJ human bladder cancer cells and H1299 human lung cancer cells overexpressing HA-Nek6. DOX-induced G2/M cell cycle arrest and the reduction in cyclin B and cdc2 levels after DOX treatment were significantly reduced by Nek6 overexpression. In addition, an increase in the intracellular levels of ROS in response to DOX was also inhibited in cells overexpressing Nek6. These results suggest that the increased expression of Nek6 renders cancer cells resistant to premature senescence, and targeting Nek6 could be an efficient strategy for cancer treatment.

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1. Introduction

The proliferation of normal cells is restricted after a limited number of cell divisions after cells enter an irreversible arrest stage, known as replicative senescence. Generally, the progressive shortening of telomeres plays a critical role in the induction of replicative senescence [1,2]. Recently, another type of senescence, termed premature senescence or accelerated senescence, has been reported. Premature senescence is induced as a result of various cellular stresses, such as oncogene activation, oxidative stress, and DNA damage, and is independent of telomere shortening [3,4]. Premature senescence is accompanied by physiological and phenotypic changes similar to replicative senescence, including growth arrest, enlarged and flattened morphology, and the appearance of senescence-associated β -galactosidase activity (SA β -gal) at pH 6.0 [3,4]. Premature senescence is thought to act as an important internal barrier against tumorigenesis by eliminating premalignant cells [5]. Bypassing the senescence process is therefore an essential prerequisite for cancer development.

A number of previous studies have shown that various cancer cells undergo premature senescence after treatment with ionizing radiation and anticancer drugs, such as camptothecin (CPT) and doxorubicin (DOX), suggesting that premature senescence is a

major cellular response of cancer cells to anticancer drugs [6]. The premature senescence of cancer cells has also been reported *in vivo*. In breast cancer and lung cancer patients, premature senescence was observed in tumor samples obtained from patients treated with neoadjuvant chemotherapy, whereas tumors from untreated patients did not show premature senescence [7,8]. Schmitt et al. [9] reported that the tumor response following anticancer drug chemotherapy correlated to the amount of premature senescence in a transgenic murine lymphoma model. These results indicate that premature senescence is an important determinant for cancer chemotherapy. Therefore, the identification of the regulatory proteins of premature senescence is important to understanding how cancer cells acquire resistance to anticancer drug chemotherapy and to develop efficient strategies for cancer treatment.

Nek6 is a serine/threonine kinase belonging to the Nek (NIMA-related kinase) family, which plays an important role in mitotic cell cycle progression [10]. In addition to its role in normal cell cycle progression, we have identified Nek6 as a direct target of the DNA damage checkpoint. We previously showed that the downregulation of Nek6 activity was essential for G2/M cell cycle arrest after DNA damage [11]. The upregulation of Nek6 protein expression and kinase activity has recently been reported in a variety of malignant cancers, such as breast, colon, lung, and gastric cancers [12–14]. Furthermore, it has been shown that the overexpression of Nek6 increases the anchorage-independent growth of cancer cells and that the knockdown of

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Nek6 results in the reduction of tumors in the nude mouse xenograft model [12,13]. These studies collectively suggest that Nek6 plays a role in tumorigenesis, but the underlying mechanism is not fully understood.

Recently, we have shown that Nek6 expression levels are decreased during premature senescence, and the ectopic expression of Nek6 inhibits p53-induced premature senescence of human cancer cells [15]. In this study, we examined the effect of Nek6 overexpression on the anticancer drug-induced premature senescence of cancer cells. We found that Nek6 overexpression significantly reduced the phenotypic changes characteristic of cellular senescence after CPT and DOX treatment in EJ and H1299 cells, suggesting that Nek6 also suppresses the premature senescence of human cancer cells induced by anticancer drugs.

2. Materials and methods

2.1. Cell lines and treatments

EJ-vector control cells (EJ-vec), EJ-Nek6 cells, H1299-vector control cells (H1299-vec), and H1299-Nek6 cells were generated using a retrovirus control vector or a retrovirus encoding wild-type HA-tagged Nek6 as previously described [15]. EJ and H1299 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO).

Camptothecin (CPT) and doxorubicin (DOX) were purchased from Sigma. To induce premature senescence, cells were treated with 30 nM of CPT or DOX for 16 h and incubated with drug-free culture medium for the indicated time.

2.2. Senescence associated (SA)- β -galactosidase staining

Cells were fixed in 0.25% glutaraldehyde, and SA- β -galactosidase staining was performed at pH 6.0 as described previously [16]. After staining, cells were imaged with a microscope using a CCD camera. 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 are presented as mean values with standard deviations.

2.3. Cell cycle analysis

To determine the cell cycle distribution, 1×10^6 cells were seeded in a 100-mm dish. After DOX treatment, cells were trypsinized at the indicated time points and fixed in 70% ethanol. Subsequently, 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.

2.4. Western blot analysis and antibodies

Cells were lysed in RIPA buffer and subjected to western blot analysis as described previously [17]. Antibodies for cyclin B and cdc2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Actin levels were monitored as an internal loading control using anti-actin antibodies (Sigma).

2.5. Measurement of ROS level

To measure the intracellular levels of ROS, cells were stained with 50 μ M of 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma) 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 are presented as mean values with standard deviations.

3. Results

3.1. Nek6 overexpression overrides CPT and DOX-induced premature senescence

To address the effect of Nek6 overexpression on the anticancer drug-induced premature senescence of cancer cells, we compared the premature senescence of EJ human bladder cancer cells and H1299 human lung cancer cells, which express moderate levels of endogenous Nek6, to previously generated EJ and H1299 cells overexpressing HA-tagged Nek6 cells (EJ-Nek6 and H1299-Nek6) [15] after camptothecin (CPT) or doxorubicin (DOX) treatment. When treated with 30 nM CPT or DOX, both EJ- and H1299-vector control cells became flat and showed enlarged morphology, which is a characteristic phenotypic change in premature senescence [3,4]. Moreover, senescence-associated SA β -galactosidase (SA β -gal) staining was greatly increased. These morphological changes and the increase in SA β -gal stained cells confirmed that CPT and DOX treatment efficiently induced the premature senescence of EJ human bladder carcinoma cells and H1299 human lung cancer cells.

However, neither EJ-Nek6 nor H1299-Nek6 cells showed these morphological changes, and most of the cells maintained normal morphology following CPT and DOX treatment (Fig. 1A and B, left panel). Moreover, the CPT- and DOX-induced increase in the proportion of SA β -gal stained cells was significantly reduced in both EJ-Nek6 and H1299-Nek6 cells (Fig. 1A and B, right panel). These results suggest that Nek6 overexpression suppresses the anticancer drug-induced premature senescence in human cancer cells.

3.2. Overexpression of Nek6 inhibits DOX-induced cell cycle arrest

Cell cycle arrest and the loss of cell proliferation potential are two of the most obvious characteristics during the onset of premature senescence [5]. To investigate the mechanism through which Nek6 overexpression affects anticancer drug-induced premature senescence, we examined changes in cell cycle distribution after DOX treatment in both Nek6-overexpressing cells and vector control cells of EJ and H1299. Consistent with a previous study [18,19], EJ- and H1299-vector control cells were mainly arrested in G2/M cell cycle phase after DOX treatment. The proportion of cells in G2/M phase was increased from 12.6% to 46.7% in EJ-vector control cells and from 12.3% to 37.4% in H1299-vector control cells 4 days after DOX treatment (Fig. 2A and B). In contrast, the percentage of cells in S phase was decreased after DOX treatment. However, EJ- and H1299-Nek6 cells did not show an increase in the proportion of cells in G2/M phase after DOX treatment and maintained the proportion of cells in S phase 4 days after DOX treatment (Fig. 2A and B). These results suggest that Nek6 overexpression in cancer cells overrides DOX-induced G2/M cell cycle arrest and maintains the proliferative potential, even after DOX-treatment.

3.3. DOX-induced decrease of cyclin B and cdc2 is suppressed by Nek6

Cyclin B and cdc2/cdk1 are essential regulators of cell cycle G2/M phase progression and have been shown to be key regulators of premature senescence after genotoxic treatment [20]. To investigate the mechanism by which Nek6 overexpression inhibits DOX-induced cell cycle arrest, we examined the protein levels of cyclin B and cdc2 after DOX treatment using western blot analysis. In EJ- and H1299-vector control cells, the cyclin B level was profoundly decreased 2 days after DOX treatment, and the cdc2

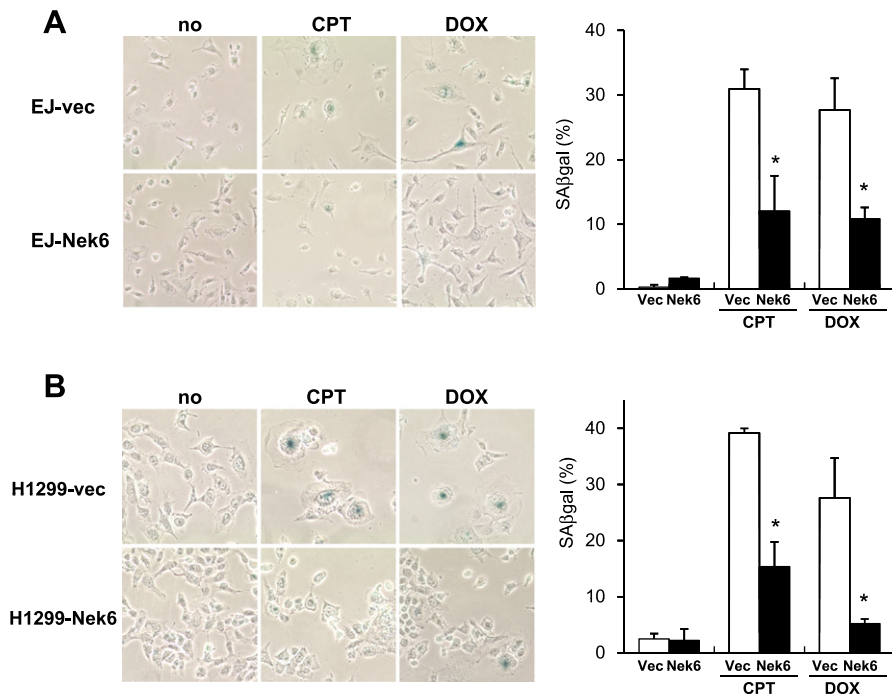


Fig. 1. Nek6 overexpression inhibits CPT- and DOX-induced senescence in EJ and H1299 cells. (A) EJ-vector control (EJ-vec) and EJ-Nek6 cells were treated with 30 nM CPT or DOX for 16 h. After 6 days of culture in drug-free medium, cells were stained with freshly prepared SA β -gal staining solution. Cell images were captured under a microscope. The data shown are representative of three independent experiments (left panel). SA β -gal-positive values counted in three independent experiments are presented as mean values with standard deviations as described in materials and methods (right panel). * $p < 0.05$ using Student's *t*-test. (B) H1299-vector control (H1299-vec) and H1299-Nek6 cells were treated with CPT or DOX, and SA β -gal staining was performed as in (A).

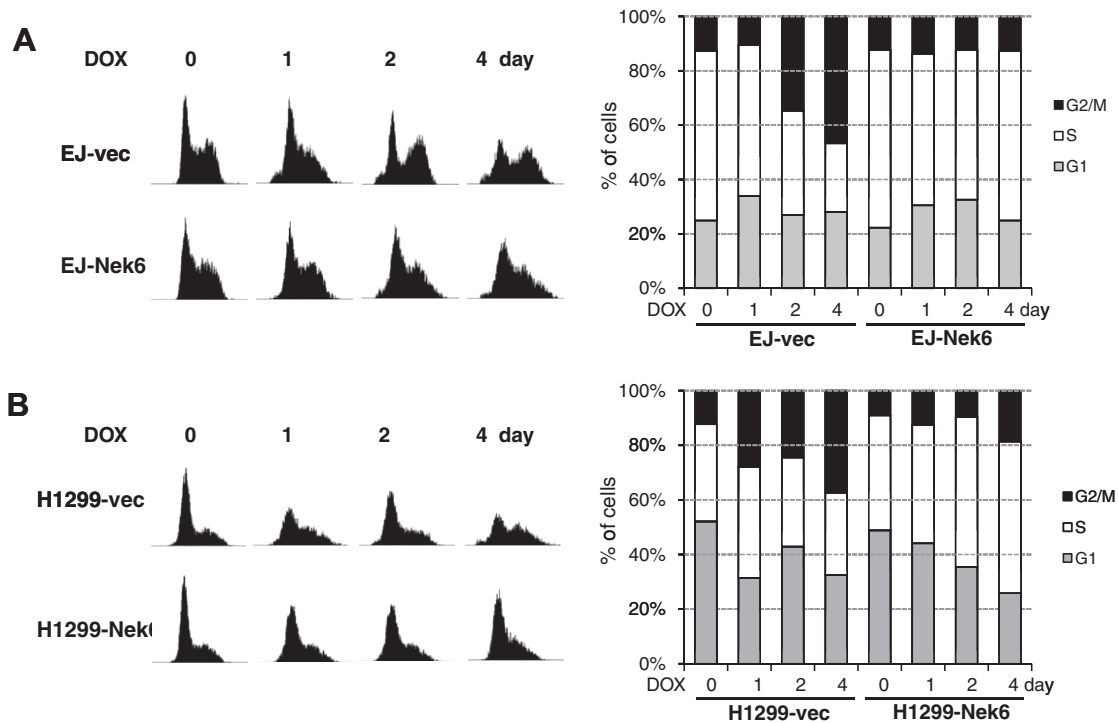


Fig. 2. Nek6 inhibits DOX-induced cell cycle arrest in EJ and H1299 cells. (A) EJ-vector control (EJ-vec) and EJ-Nek6 cells were treated with or without 30 nM DOX and harvested at the indicated time points. Cells were fixed, stained with PI, and then analyzed using flow cytometry as described in materials and methods. The data shown are representative of three independent experiments (left panel). Cell cycle distributions obtained in three independent experiments are presented as mean values. (B) H1299-vector control (H1299-vec) and H1299-Nek6 cells were treated with or without 30 nM DOX, and cell cycle distributions were determined as in (A).

level was also decreased at 4 days (Fig. 3A and B). However, EJ-Nek6 and H1299-Nek6 did not show a clear decrease in cyclin B and cdc2 protein levels 4 days after DOX treatment (Fig. 3A

and B). These results suggest that Nek6 inhibits DOX-induced G2/M cell cycle arrest by suppressing the decrease in cyclin B and cdc2 protein levels.

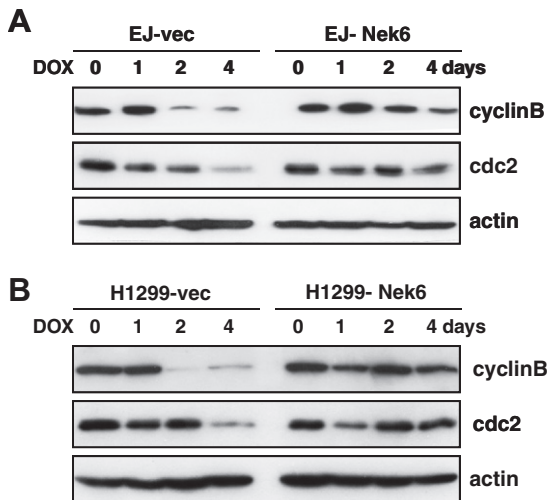


Fig. 3. Nek6 inhibits the DOX-induced decrease in cyclin B and cdc2 levels. (A) EJ-vector control (EJ-vec) and EJ-Nek6 cells were treated with or without 30 nM of DOX and harvested at the indicated time points. Cell lysates were subjected to western blotting using the indicated antibodies. (B) H1299-vector control (H1299-vec) and H1299-Nek6 cells were treated with or without 30 nM DOX, and western blotting was performed as in (A).

3.4. Nek6 inhibits the increase in intracellular ROS after DOX treatment

It has been shown that intracellular reactive oxygen species (ROS) play an important role in the induction of premature senescence in cancer cells after various genotoxic treatments [3]. To examine whether Nek6 overexpression also affected the induction of ROS levels, we measured intracellular ROS levels after DOX treatment using the redox-sensitive fluorophore 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). As shown in Fig. 4, whereas the level of ROS was increased approximately 2.9-fold 4 days after DOX treatment in EJ-vector control cells, ROS levels in EJ-Nek6 cells were not significantly changed after DOX treatment. Similarly, ROS induction was observed in H1299-vector cells after DOX treatment (2.7-fold increase at 4 days) but was not detected in H1299-Nek6 cells (Fig. 4B). These results suggest that Nek6 suppresses the DOX-induced increase in intracellular ROS in human cancer cells.

4. Discussion

In this report, we describe our findings that Nek6 overexpression suppresses camptothecin (CPT)- and doxorubicin (DOX)-

induced senescence in EJ human bladder cancer cells and H1299 human lung cancer cells. First, whereas EJ- and H1299-vector control cells exhibited an enlarged and flattened morphology and approximately 27–39% of senescence-associated β -galactosidase (SA β -gal) staining after CPT and DOX treatment, EJ-Nek6 and H1299-Nek6 cells maintained normal morphology, and SA β -gal staining was significantly decreased (2.5–5.4-fold decrease compared to control cells; Fig. 1A and B). Second, DOX-induced G2/M cell cycle arrest was inhibited by Nek6 overexpression in both EJ and H1299 cells (Fig. 2A and B). Finally, the increase in intracellular levels of ROS after DOX treatment was inhibited in EJ and H1299 cells overexpressing Nek6. These results suggest that Nek6 overrides the anticancer drug-induced premature senescence of human cancer cells.

Chemotherapy remains the primary treatment for human cancers. The treatment of tumors with chemotherapeutic drugs primarily relies on the induction of either apoptosis or the permanent loss of proliferative activity (premature senescence) [20]. Recent studies have shown that antitumor therapeutic drug-induced premature senescence is one of the main mechanisms to suppress tumor growth and eliminate solid tumors [6,20]. Consistent with this notion, te Poele et al. [8] reported that senescence was observed in 41% of breast cancer patients after chemotherapy. Schmitt et al. [9] demonstrated that tumor prognosis following anticancer drug chemotherapy correlated to the amount of senescence in a transgenic murine lymphoma model and that p53 or p16 mutations reduced drug-induced senescence and the tumor response. These results indicate that premature senescence contributes to therapeutic outcomes *in vivo*. Thus, an understanding of how cancer cells bypass premature senescence is important to develop efficient cancer treatment. Notably, the increase in Nek6 expression has been reported in various human cancers [12–14]. The data presented here suggest that Nek6 overexpression could confer resistance against drug-induced premature senescence to tumor cells. Previously, we have reported that Nek6 overexpression also suppresses the p53-induced senescence of cancer cells [15]. Therefore, our current results in combination with our previous study suggest that Nek6 plays a critical role in tumorigenesis by, at least in part, inhibiting premature senescence.

We showed here that the DOX-induced decrease in cyclin B and cdc2 levels was inhibited in both EJ and H1299 cells overexpressing Nek6 (Fig. 3). Both cyclin B and cdc2 are critical regulators for G2/M cell cycle progression and are key regulators of premature senescence [6,20]. We have previously shown that the overexpression of Nek6 overrides DNA damage-induced G2/M arrest [11]. Thus, our results suggest that Nek6 may inhibit CPT- and DOX-induced G2/M cell cycle arrest by preventing a decrease in the protein levels of cyclin B and cdc2. Notably, we also found that the

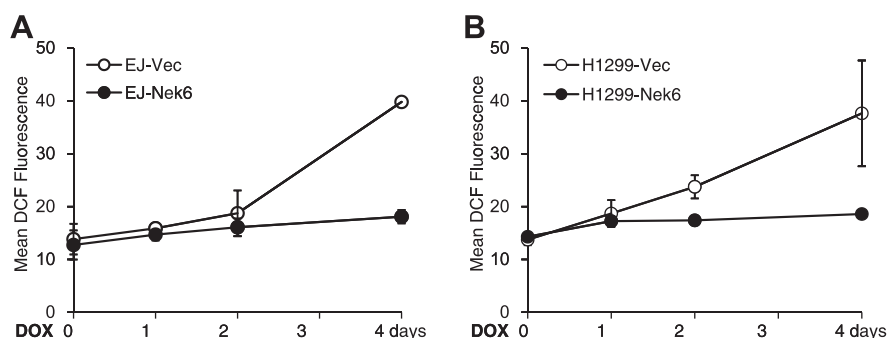


Fig. 4. The effect of Nek6 overexpression on intracellular ROS levels after DOX treatment. (A) EJ-vector control (EJ-vec) and EJ-Nek6 cells were treated with or without 30 nM DOX, and intracellular levels of ROS were determined at the indicated time points as described in materials and methods. Experiments were repeated three times, and the results are presented as mean values with standard deviations. (B) H1299-vector control (H1299-vec) and H1299-Nek6 cells were treated with or without 30 nM DOX, and intracellular levels of ROS were determined as in (A).

increase in intracellular ROS levels after DOX treatment was inhibited by Nek6 overexpression (Fig. 4). Cell cycle arrest and the induction of intracellular ROS levels are important pathways to induce cellular senescence [3]. However, it is not clear whether Nek6 directly regulates intracellular ROS levels or if the inhibition of ROS increase is an indirect result of preventing cell cycle arrest. Notably, Nek6 overexpression also resulted in the reduction of both cell cycle arrest and an increase in ROS in p53-induced senescence [15], suggesting that Nek6 suppresses p53- and anticancer drug-induced premature senescence through a common regulatory mechanism. Additional studies focused on the identification of the physiological target of Nek6 would be helpful to understand how Nek6 suppresses premature senescence.

Taken together, our results reveal that the increased expression of Nek6 confers resistance to anticancer drug-induced premature senescence to cancer cells. Future studies, including an investigation of the precise mechanism by which Nek6 suppresses premature senescence and whether the inhibition of Nek6 makes cancer cells more sensitive to premature senescence, will help clarify the role of Nek6 in tumorigenesis and develop efficient strategies for cancer treatments targeting Nek6.

Acknowledgments

This work was supported by a Grant of the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A090331).

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