Cancer Genes and Genomics

Nek6 Mediates Human Cancer Cell Transformation and Is a Potential Cancer Therapeutic Target

Rounak Nassirpour, Lihua Shao, Peter Flanagan, Tinya Abrams, Bahija Jallal, Tod Smeal, and Min-Jean Yin

Abstract

We investigated the role of Nek6, a member of the NIMA-related serine/threonine kinase family, in tumorigenesis. Transcript, protein, and kinase activity levels of Nek6 were highly elevated in the malignant tumors and human cancer cell lines compared with normal tissue and fibroblast cells. Expression of exogenous wild-type Nek6 increased anchorage-independent growth of a variety of human cancer cell lines, whereas overexpression of the kinase-dead Nek6 and RNAi knockdown of endogenous Nek6 suppressed cancer cell transformation and induced apoptosis. Additionally, in *in vivo* xenograft nude mouse model, knockdown of Nek6 in HeLa cells resulted in reduction of tumor size relative to control siRNA tumors. Most importantly, knocking down endogenous Nek6 levels or exogenous expression of the kinase-dead form did not inhibit cell proliferation, nor did it induce apoptosis in normal fibroblast cells. Taken together, our data indicate a pivotal role for Nek6 in tumorigenesis and establish Nek6 as a potential target for treatment of a variety of human cancers. *Mol Cancer Res; 8(5); 717–28.* ©2010 AACR.

Introduction

Mitotic errors and deregulation of the cell cycle are hallmarks of human cancer, and thus, identification of physiologic targets underlying regulatory mechanisms for cell cycle regulation is critical in the development of novel and effective cancer therapies (1-3). Mitosis is mainly orchestrated by several serine/threonine kinases, of which the best-studied families are the cyclin-dependent kinases (4), the polo-like kinases (5), and the Aurora kinases (6, 7). These cell cycle regulators have been implicated in modulating tumor progression, as they are often upregulated in a variety of human cancers. Recent efforts to exploit cell cycle targets have thus focused mainly on inhibitors for these mitotic kinases (8, 9). Although some of the more recent clinical trials have resulted in partial responses in specific malignancies, unfortunately most have been unsuccessful mainly due to undesired side effects on normal cells (10). Therefore, a new generation of drugs that specifically targets cell cycle progression in cancer cells is expected to improve clinical efficacy.

More recently, members of the NIMA-related kinase (Nek) family have also been implicated in mitotic progression (11-13). Although functional characterization of these serine/threonine kinases is in its early stages, studies thus far suggest that most Neks have cell cycle–related functions. The founding member of the Nek family, NIMA (never in mitosis, gene A), identified in *Aspergillus nidulans*, is critical for cell cycle progression (14-16). NIMA is essential for mitotic entry, possibly through triggering the relocation of cyclin-dependent kinase 1/cyclin B to the nucleus (17); it is required for efficient mitosis progression by orchestrating chromatin condensation and depolymerization of microtubules (18); and its degradation is required for mitotic exit (19). Furthermore, inactivation of NIMA by expression of a temperature-sensitive mutant or by overexpression of its dominant-negative form causes cells to arrest in G_2 with uncondensed DNA and interphase microtubules (14, 20). Although none of the human Nek kinases seem to be functional homologues of NIMA (i.e., able to rescue a *nimA* mutant), they have been shown to play important roles in cell cycle progression and/or microtubule organization.

Eleven mammalian Neks have been identified to date (12, 13, 21). Nek2 exhibits the greatest homology to NIMA and is the best-characterized member of the family (22). Nek2 is overexpressed in a variety of human tumors and plays a critical role in mitotic progression by regulating centrosome duplication (23). Nek2 is degraded by anaphase-promoting complex or cyclosome (APC/C) during cell cycle progression (24). Depletion of Nek2 prevents centrosome separation, delays mitosis, and results in increased apoptosis, possibly as a result of mitotic errors, suggesting possible therapeutic potential for cancer (25). Very little is known about the remaining members of the Nek family. Functional studies indicate that Nek6, Nek7, and Nek9 form a functional complex and regulate mitotic progression (26). However, recent reports suggest that they may also have independent roles in mitosis and cytokinesis

Authors' Affiliation: Pfizer Global Research and Development, La Jolla Laboratories, San Diego, California

Corresponding Author: Min-Jean Yin, Pfizer Global Research and Development, La Jolla Laboratories, 10724 Science Center Drive, San Diego, CA 92121. Phone: 858-622-7438; Fax: 858-526-4121. E-mail: min-jean.yin@pfizer.com

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(27, 28). Nek9 has been shown to phosphorylate Nek6 and Nek7 within their activation loop (29). Functional studies of Nek9 reveal that it has major roles in organization of the mitotic spindle. Expression of inactive and truncated Nek9 mutants led to the missegregation of chromosomes, whereas injection of anti-Nek9 antibodies caused aberrant mitotic spindle formation (29).

We have previously shown that human Nek6 is required for metaphase-anaphase transition during cell cycle progression, as its expression and kinase activity are increased during mitosis (30). Furthermore, Nek6 is directly phosphorylated by Chk1 and Chk2, at least *in vitro*, suggesting that Nek6 is a direct target of the DNA damage checkpoint (31). It is also reported that Nek6 phosphorylates the kinesin Eg5 through which it regulates mitotic spindle formation (32). Although there are a few contradictory data on Nek6 function and very little is known about its downstream targets, it is generally believed that interfering with Nek6 function by either expression of a kinase-dead Nek6 or depletion of endogenous Nek6 by short interfering RNA (siRNA) causes mitotic arrest and triggers apoptosis (26, 27, 30).

Interestingly, Nek6 also seems to play a role in cancer. It was recently shown that Nek6 transcript is significantly upregulated in hepatocellular carcinoma (33). Earlier studies have also reported Nek6 transcript overexpression in breast cancer, colorectal cancer, lung cancer, and laryngeal cancer (34). Additionally, Takeno et al. (35) reported higher levels of Nek6 protein in advanced gastric cancers compared with early-stage samples.

In the current study, we investigated whether Nek6 also plays a role in tumorigenesis. We first examined Nek6 expression, at both mRNA and protein levels, as well as its kinase activity, in a collage of tumor tissues and human cancer cell lines. Results indicate that Nek6 is prevalently overexpressed in most human cancers. We then investigated the correlation between Nek6 expression and cancer cell transformation. We show that functional depletion of Nek6 by overexpressing a dominant-negative form or RNAi knockdown abolishes anchorage-independent growth in various human cancer cell lines. In addition, exogenous overexpression of Nek6 in low-expressing cancer cell lines (SW-480 and MDA-MB-231) increases anchorage-independent growth. These results suggest that there is a direct correlation between levels of Nek6 and stages of cancer progression. To examine the effects of Nek6 in *in vivo* tumor growth, we knocked down endogenous Nek6 levels in high-expressing HeLa cells. RNAi reduction of the endogenous Nek6 in HeLa cells inhibited tumor growth in a mouse xenograft model. We further show that overexpression of kinase-dead Nek6 in human cancer cells induces the apoptotic pathway and causes cell death. Contrary to its effects on cancer cells, depletion of the endogenous Nek6 in human normal fibroblast cells did not induce apoptosis. Taken together, our data provide the first direct evidence for Nek6 playing significant roles in tumorigenesis. Although expression of Nek6 is tightly controlled in normal cells, this protein is highly overexpressed in various human cancers and regulates tumor progression. Inhibition of Nek6 might therefore provide therapeutic advantages over other cell cycle regulators, as functional knockdown does not alter cell cycle regulation of normal cells.

Materials and Methods

Cancer profiling array. A PCR fragment containing the Nek6 cDNA (883-1,244 bp) was ^{32}P labeled using the Random Primed DNA Labeling kit (Roche Applied Science) and purified using a DNA purification column (Qiagen). The Cancer Profiling Array 1 (Clontech Laboratories, Inc.) was hybridized with labeled probes for 2 hours at 65°C using the ExpressHyb Mix (Clontech Laboratories). Membranes were washed in 0.2× SSC and 1% SDS. Quantitative evaluation of the array was done by phospho-image analysis using a GS250 molecular imager (Bio-Rad).

In situ *hybridization and immunohistochemistry.* A total of 169 formalin-fixed and paraffin-embedded human tumor samples were obtained from the European Institute of Oncology (Milan, Italy). Nek6 mRNA expression was assessed by *in situ* hybridization (ISH) with [^{35}S] UTP-labeled antisense and sense riboprobes. A rabbit polyclonal serum made against the NH_2 terminus of Nek6 was peptide affinity purified and used for immunohistochemistry (IHC). IHC was done by LifeSpan Biosciences service using formalin-fixed, paraffin-embedded samples in the LifeSpan tissue bank that contains tumor and matched tissue samples from the same patients.

Western blot analysis and kinase assay. Snap-frozen patient samples were provided by LifeSpan Biosciences and processed to obtain tissue lysates. Whole-cell lysates were also obtained from cell lines. Total protein (40 μg/lane) was subjected to SDS-PAGE for Western blot analysis of hemagglutinin (HA)–tagged Nek6, Nek6, β-actin, or α-tubulin. NH_2-terminal Nek6 antibody or HA antibody (12CA5; Sigma) was used to immunoprecipitate the endogenous Nek6 or HA-tagged Nek6 protein from 50 μg protein per reaction. Kinase assay was done in the presence of [^{32}P]ATP and myelin basic protein. The washed immunoprecipitate complex was subjected to SDS-PAGE and exposed to the Alpha Imager to visualize the phosphorylated Nek6 and myelin basic protein as described.

Cell culture, RNAi transfections, and soft agar assays. All cell lines were obtained from the American Type Culture Collection and cultured in media containing 10% fetal bovine serum as per the American Type Culture Collection instructions. HeLa Tet-On cells (Clontech Laboratories) were cultured according to the manufacturer's instructions. siRNA duplex transfection was done using OligofectAMINE reagent (Invitrogen) as described previously (30). A lamin A/C siRNA was used as control (5′-CUGGACUUCCAGAAGAACAdTdT). The oligonucleotides used for Nek6 were 5′-GAUCGAGCA-GUGUGACUACdTdT and 5′-GCUCGGUGA-CCUUGGUCUGdTdT. All siRNA duplexes were

purchased from Dharmacon Research. For the soft agar assay, 2,000 cells in 200 μL of culture medium were layered on the bottom agar (0.6% agar in growth media) in six-well culture plates and 0.5 mL top agar (0.3%) was added every week. Colony morphology was recorded and colony numbers were counted and calculated for each well after the 20-day study period.

Plasmid constructs and establishment of stable tumor cell lines. Two point mutations were generated in the wild-type (WT) Nek6 cDNA, K74M and K75M (KK/MM), to generate a kinase-inactive mutant. Both the WT Nek6 and KK/MM Nek6 were subcloned into the pBabe-puro retroviral vector and cotransfected with amphotropic retroviral packaging vectors into 293 cells. Viral supernatants were used to infect HeLa and MDA-MB-231 cells. Puromycin-resistant pools and individual clones were generated as described previously (30). WT and KK/MM Nek6 were also subcloned into a pRevTRE vector (Clontech Laboratories). Virus particles were collected and used to infect HeLa Tet-On cells as described in the Clontech manual.

Mouse xenograft model. Animal studies were approved by the Animal Care Committee at SUGEN (a Pfizer legacy company). Mice were maintained in temperature- and humidity-controlled rooms and given water and food throughout the experiments. Eight-week-old BALB/c *nu/nu* athymic (nude) mice were implanted by s.c. injection of 5×10^6 HeLa cells of mock, control RNAi, or Nek6 RNAi transfections in the right lower flank area. Tumor size was measured every 4 days by caliper. Tumor volume was calculated by the following formula: length × width × width × 0.5.

Fluorescence-activated cell sorting analysis and apoptosis assays. Cells were trypsinized and harvested, washed thrice with cold PBS, and fixed with cold ethanol. The fixed cells were stained with propidium iodide and subjected to flow cytometry as described (30). Sub-G_1 cells of propidium iodide staining were calculated to determine the percentage of cells undergoing apoptosis.

Detection of apoptosis/caspase-3/7 activity. Levels of caspase-3/7 activity were determined using the SensoLyte Homogeneous AMC Caspase-3/7 Assay kit (Anaspec, Inc.) according to the manufacturer's protocols. Briefly, caspase-3/7 substrate solution was added to cell extracts and incubated before measuring fluorescence on a Spectra-Max microplate reader (Molecular Devices Corp.) using excitation at 354 nm and emission detection at 442 nm. Levels of caspase-3/7 activity were reported after subtraction of fluorescence levels of wells with media only.

Examination of signaling pathways. Cells (1.2×10^6) were plated in 100-mm tissue culture dishes. After a wash with cold PBS, protein lysates were prepared with lysis buffer included in a PathScan Multi-Target Apoptosis ELISA kit (Cell Signaling) and subjected to ELISA analyses in duplicates. Experiments were repeated twice.

Lentiviral inducible knockdown in stable cell lines. Stable expression of siRNA for Nek6 was used to suppress expression in cells. Oligonucleotides for silencing Nek6

were designed based on the published sequence (30). The oligonucleotides were synthesized by Invitrogen. The annealed oligonucleotides were inserted into the doxycycline-inducible lentiviral expression vectors pPS-TRE-RFP (System Biosciences). Cells stably expressing pPS-TRE-RFP-siNek6 vector were established using the lentiviral system from System Biosciences following the manufacturer's instructions. The silencing of Nek6 in these cells was verified by quantitative reverse transcription-PCR and Western blots after the cells were induced with doxycycline (Sigma) for at least 48 hours.

Quantitative PCR. Taqman gene expression assays (Applied Biosystems, Inc.) were used to quantify the expression levels of Nek6. Total RNA was extracted using Trizol (Invitrogen) and reverse transcribed by MultiScribe (Applied Biosystems) in reaction mixture. Quantitative PCR was done in triplicate reactions containing cDNA preparation and Taqman primers in Universal Master Mix without AmpErase UNG (Applied Biosystems). The quantitative PCR was conducted at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Statistical analysis. Data are presented as mean ± SD and evaluated for statistical significance using unpaired Student's *t* test or one-way ANOVA, where $P < 0.05$ was considered significant (SigmaStat 3.0; Systat Software).

Results

Nek6 is overexpressed in patient tumor samples and human cancer cell lines. We used the Cancer Profiling Array (CPA) to examine Nek6 transcript levels in matched tumor versus normal tissues from individual patients. Nek6 transcript was found to be highly overexpressed in the majority of the cancer tissue samples (Table 1). In an effort to validate the observed elevated Nek6 mRNA levels, 169 patient tissue samples of breast, colon, and lung cancers, with detailed information of pathology history (obtained from the European Institute of Oncology), were used for ISH studies. A "positive" result was assigned if the ISH signal of Nek6 mRNA expression in the tumor tissues was more than twice that of the corresponding normal tissue. As shown in Table 2, Nek6 signals were higher in most of the patient tumor samples examined, with breast cancer tissues showing the highest elevation. These data suggest that Nek6 transcript is overexpressed in different malignancies and that it may play a general role in most cancer cells.

Next, we collaborated with LifeSpan Biosciences, Inc. to investigate whether Nek6 protein level and kinase activity are also elevated in tumors. IHC was done to compare protein expression in tumor and normal samples from a variety of organ tissues. As seen by representative images shown in Fig. 1A, Nek6 protein expression was significantly higher in tumor samples compared with normal tis-sues obtained from the same patient. To determine Nek6 kinase activity in the patient tumor samples, a limited number of colon, lung, and kidney snap-frozen

Table 1. Nek6 mRNA is overexpressed in tumors relative to normal tissues

Expression	Breast	Uterus	Colon	Stomach	Ovary	Lung	Kidney
N < T	42/53	32/44	25/38	13/28	15/16	5/21	13/20
N = T	7/53	8/44	11/38	10/28	0/16	7/21	0/20
N > T	4/53	4/44	2/38	5/28	1/16	9/21	7/20
	Rectum	Thyroid	Cervix	Prostate	Pancreas	Small intestine	
N < T	12/19	5/6	1/1	1/4	1/1	2/2	
N = T	5/19	1/6		2/4			
N > T	2/19	0/6		1/4			

NOTE: Nek6 expression was analyzed using the CPA, which contains pairs of cDNAs generated from matched normal and tumor tissue samples from individual patients. Most samples from breast, uterus, colon, stomach, ovary, lung, kidney, rectum, thyroid, cervix, prostate, pancreas, and small intestine tumor tissues showed an increase in Nek6 transcript levels.
Abbreviations: N, normal tissue; T, tumor tissue.

samples were also processed to obtain tissue lysates for use in kinase assays (Fig. 1B). Due to the nature of snap-frozen samples, equal protein loading in each lane was not achieved perfectly; however, we did observe higher Nek6 protein levels in the colon, lung, and kidney tumors compared with adjacent normal tissues. The kinase assay results showed that Nek6 kinase activity is elevated in colon and lung tumors versus their normal tissue counterpart. Contrary to most tumor tissues studied, kidney tumor samples did not show changes in Nek6 kinase activity. We cannot rule out that these results may be due to poor handling of the kidney samples during surgery, which would explain the loss of kinase activity. However, it is more likely that tumor samples from different organs may differ in their Nek6 kinase activity, and additionally, individual patients may exhibit different kinase activities. The data represented here suggest that, at least in most tumor samples examined, Nek6 kinase activity was significantly elevated compared with matched normal tissues. Taken together, the data from the CPA, ISH, IHC, and kinase assays strongly suggest that Nek6 is highly upregulated at the transcript, protein, and kinase activity levels in tumors.

We next investigated the levels of expression of Nek6 mRNA and protein levels in a collage of breast, colon, lung, liver, gastric, and cervical cancer cell lines compared with normal liver and breast RNA (obtained from Applied Biosystems). Quantitative real-time PCR data (Fig. 1C) showed dramatically elevated Nek6 transcript levels in most cancer cell lines, with the lowest Nek6 expression lines being NCI-H23 and SW-480. Interestingly, the expression levels of Nek6 seem to correlate to the tumor aggressiveness of the cell lines studied. For example, colon cancer cell lines SW-480 and SW-620 are derived from the same patient. Low Nek6 levels were observed in SW-480 cells, which are derived from the primary tumor site and exhibit less aggressive tumor progression activity compared with SW-620,

which was derived from a metastatic site and shows higher Nek6 expression. A few representative cell lines from each organ were further processed for Western blot analysis to determine if Nek6 protein levels correlate with their mRNA expression patterns. As shown in Fig. 1D, there is a fair correlation between the transcript and protein expression levels

Table 2. Nek6 ISH signals were also higher in most of the patient tumor samples examined, with breast cancer tissues showing the highest elevation

Tissue	Tumor type	Positive	Percentage
Breast	Carcinomas	25/47	53%
Colon	Carcinomas	5/13	38%
Lung	Carcinomas	48/109	44%
Breast details			
Invasive ductal carcinomas		23/41	56%
Invasive lobular carcinomas		2/4	
Lung details			
Adenocarcinomas		27/58	47%
Bronchioloalveolar carcinomas		3/4	
Large cell carcinomas		38%	18/47

NOTE: A total of 169 formalin-fixed and paraffin-embedded human tumor samples were analyzed by ISH with [^{35}S] UTP-labeled antisense and sense riboprobes of Nek6. Positive indicates ISH signals determined to be at least 2-fold higher in the tumor tissue compared with the adjacent normal sample.
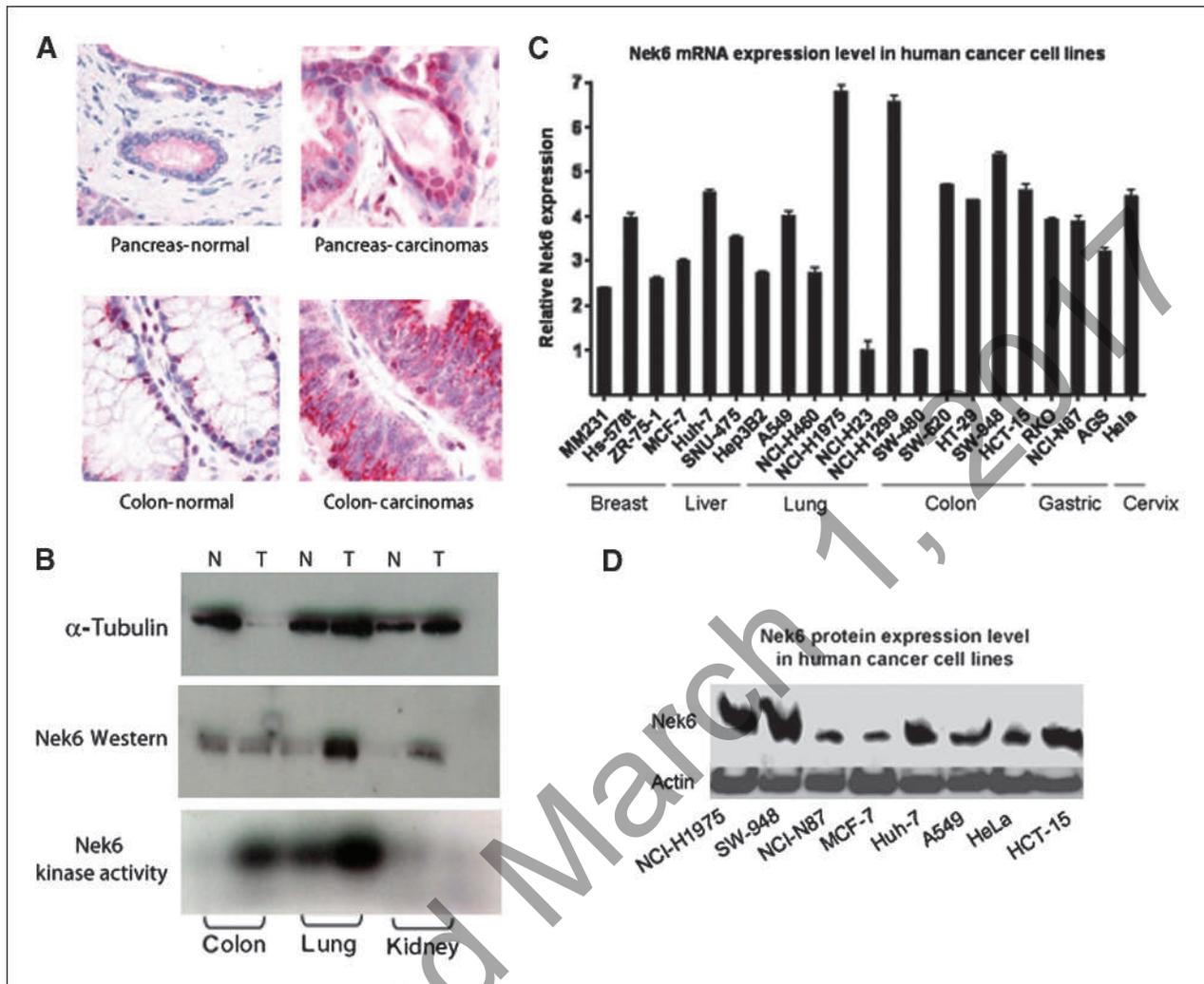
FIGURE 1. Nek6 protein level and kinase activity are upregulated in tumors and human cancer cell lines. A, IHC showed increased expression of Nek6 protein in pancreatic and colon carcinoma tissue samples compared with normal tissues obtained from the same patient. B, Nek6 protein expression and kinase activity in a limited number of colon, lung, and kidney patient samples were also elevated. Tubulin was used as the loading control. N, normal; T, tumor. C, quantitative real-time PCR analysis revealed dramatically increased Nek6 transcript levels in most human cancer cell lines. However, Nek6 expression was variable among the different cancer cell lines examined. All mRNA quantification was normalized to glyceraldehyde-3-phosphate dehydrogenase. Data are presented as mean fold change in Nek6 expression relative to normal human breast RNA. D, cell lysates from a variety of cancer cell lines were subjected to SDS-PAGE and analyzed for Nek6 protein expression. Actin was used as the loading control.

in most human cancer cell lines. We thus concluded that cancer cell lines can reliably be used for *in vitro* assays to assess the function of Nek6 in cancer progression.

Nek6 plays a crucial role in the regulation of cancer cell transformation. Soft agar assays were done to assess whether changes in Nek6 activity can affect anchorage-independent growth in cancer cell lines. The human breast cancer cell line MDA-MB-231, which expresses low/moderate Nek6 level, was used to establish clones that stably overexpress either WT Nek6 or a kinase-inactive KK/MM Nek6. Parental and vector-transfected MDA-MB-231 cells formed colonies in the soft agar assay, showing the anchorage-independent growth ability of MDA-MB-231 cells. Cells overexpressing WT Nek6 formed larger

colonies, indicating a more aggressive transformation activity induced by upregulation of Nek6, whereas cells overexpressing the kinase-inactive KK/MM Nek6 exhibited decreased size and reduced numbers of colonies in the soft agar assays (Fig. 2A and B). To confirm that this phenomenon was not limited to breast cancer, a second tumor cell line was also examined. HeLa Tet-On cells were used to produce inducible clones that overexpress either HA-tagged WT or the kinase-inactive KK/MM Nek6. Western blot analysis and kinase activity assays showed that these HeLa Tet-On cells can be induced to express the exogenous WT or KK/MM Nek6 (as determined by their HA tag) with their corresponding kinase activity (Fig. 2C). Although the Tet-inducible system was somewhat leaky in the soft agar

assay, as shown in Fig. 2E, addition of doxycycline to induce overexpression of the WT Nek6 indeed increased both the colony size and colony number in the soft agar assay, similar to the results observed for MDA-MB-231. Similarly, induction of the KK/MM clone totally abolished colony formation in these assays (Fig. 2D and E). These data suggest that there is a direct correlation between Nek6 kinase activity and tumorigenicity. Increasing the kinase activity by overexpressing Nek6 can elevate cancer cell transformation and colony formation, whereas decreasing the kinase activity by overexpressing a dominant-negative kinase-dead form of Nek6 can eliminate tumorigenicity.

Nek6 expression and activity levels directly correlate to tumorigenicity of human cancer cells. To further confirm the direct correlation between Nek6 level and tumor transformation activity, we compared anchorage-independent growth in a low Nek6–expressing and a high Nek6–expressing cell line established from the same patient. As described in the previous section, the SW-480 and SW-620 tumor cell lines have been previously characterized as primary and metastatic colon adenocarcinoma cell lines, respectively (36). Both cell lines were isolated from the same patient without any prior chemotherapy (37). Therefore, the availability of a matched pair of primary and metastatic colon adenocarcinoma cell lines allowed us to investigate the effects of various endogenous Nek6 levels in regulation of cell transformation. SW-480 cell lines form fewer and smaller colonies than SW-620 in soft agar assays. However, SW-480 cell lines stably overexpressing Nek6 showed a significant increase in colony formation, where their soft agar growth closely resembles that of the SW-620 WT cells (Fig. 3A). Similarly, overexpression of Nek6 in SW-620, a high-expressing matched cancer cell line, further increased anchorage-independent growth (Fig. 3A and B), as indicated by bigger colony morphology and higher colony numbers. Similar to the data collected for MDA-MB-231 and HeLa cells, expression of the kinase-dead Nek6 inhibited colony formation in both high- and low-expressing colon cancer cell lines (SW-620 and SW-480, respectively; Fig. 3A and B). These results suggest that there is a strong correlation between Nek6 expression/activity level and cellular transformation in cancer cells.

To establish the generality of our findings, we tested whether knockdown of Nek6 in high-expressing human cancer cell lines can decrease anchorage-independent growth. Based on our quantitative findings represented in Fig. 1, we decided to investigate the effects of stable Nek6 knockdown on colon cancer cell line HCT-15 as well as SW-620 and SW-480, hepatocarcinoma cell line Huh-7, stomach cancer cell line NCI-N87, and cervical cancer cell line HeLa. Representative images of soft agar assays of cell lines stably expressing vector and Nek6 siRNA clearly show that downregulation of endogenous Nek6 in aggressive high-expressing cancer cell lines inhibits their anchorage-independent growth (Fig. 3C and D). Results from the soft agar assays in SW-480,

SW-620, HCT-15, Huh-7, NCI-N87, MDA-MB-231, and HeLa confirm our hypothesis that Nek6 expression and activity is required in tumorigenesis and directly correlates to the tumor progression activity in human cancer cells (Figs. 2 and 3).

Nek6 is critical for in vivo tumor growth of HeLa cells in a xenograft mouse model. We next examined the consequences of Nek6 knockdown on *in vivo* tumor growth in a mouse xenograft model. Depletion of endogenous Nek6 in HeLa cells was achieved by transfection of siRNA, and Western blot analysis confirmed sufficient knockdown of Nek6 protein in the transfected cells (Fig. 4A). HeLa cells were subjected to one (Fig. 4B, group 3) or two (Fig. 4B, group 4) rounds of transfection before s.c. implantation in nude mice. Knockdown of endogenous Nek6 in HeLa cells inhibited tumor growth of the implanted HeLa cells (Fig. 4B), and this inhibition was correlated with endogenous Nek6 protein levels. These data indicated that Nek6 is required for the *in vivo* tumor growth of HeLa cells. Therefore, Nek6 can promote HeLa cell transformation and anchorage-independent growth *in vitro* (Fig. 2) and regulate tumor growth *in vivo* (Fig. 4).

Knockdown of endogenous Nek6 induces apoptosis specifically in human cancer cells and not in normal fibroblast cells. We and others have previously shown that Nek6 is required for cell cycle progression through mitosis and that loss of Nek6 function leads to defective mitosis and apoptosis (30). Programmed cell death or apoptosis of normal noncancerous cells is one of the most undesirable side effects of anticancer drugs. Thus, having shown that Nek6 is required for tumor progression in human cancer cell lines, we wanted to investigate the effects of Nek6 knockdown on normal cells. We first analyzed Nek6 expression in a number of human normal fibroblast cell lines and human primary cells (WI-38, WS1, IMR-90, MCF-10A, and MCF-12A). Although the majority showed little or no endogenous Nek6 expression (Fig. 5A), WI-38 and WS1, two human fibroblast cell lines (isolated from the lung and skin, respectively) that can only be passed <15 times in culture, did show detectable Nek6 protein expression. Therefore, WI-38 and WS1 were transfected with Nek6 RNAi to knock down endogenous Nek6 levels and analyzed for cell apoptosis (Fig. 5B). Flow cytometry on the propidium iodide–stained cells was used to determine the percentage of cells in the sub-G_1 phase undergoing apoptosis. As shown in Fig. 5C, in contrast to the results obtained in HeLa cells, knockdown of endogenous Nek6 protein in these two normal fibroblast cell lines did not induce cell death. To further understand the differential effect of Nek6 knockdown on cell death between cancer cells and normal fibroblast cell lines, we did a series of apoptosis assays to identify the apoptotic pathways triggered by Nek6 knockdown. The PathScan Multi-Target Apoptosis ELISA kit was used to examine the levels of total BAD, phosphorylated BAD, as well as cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP) to elucidate the mechanisms behind the Nek6 knockdown-induced apoptosis. As expected, knocking down endogenous
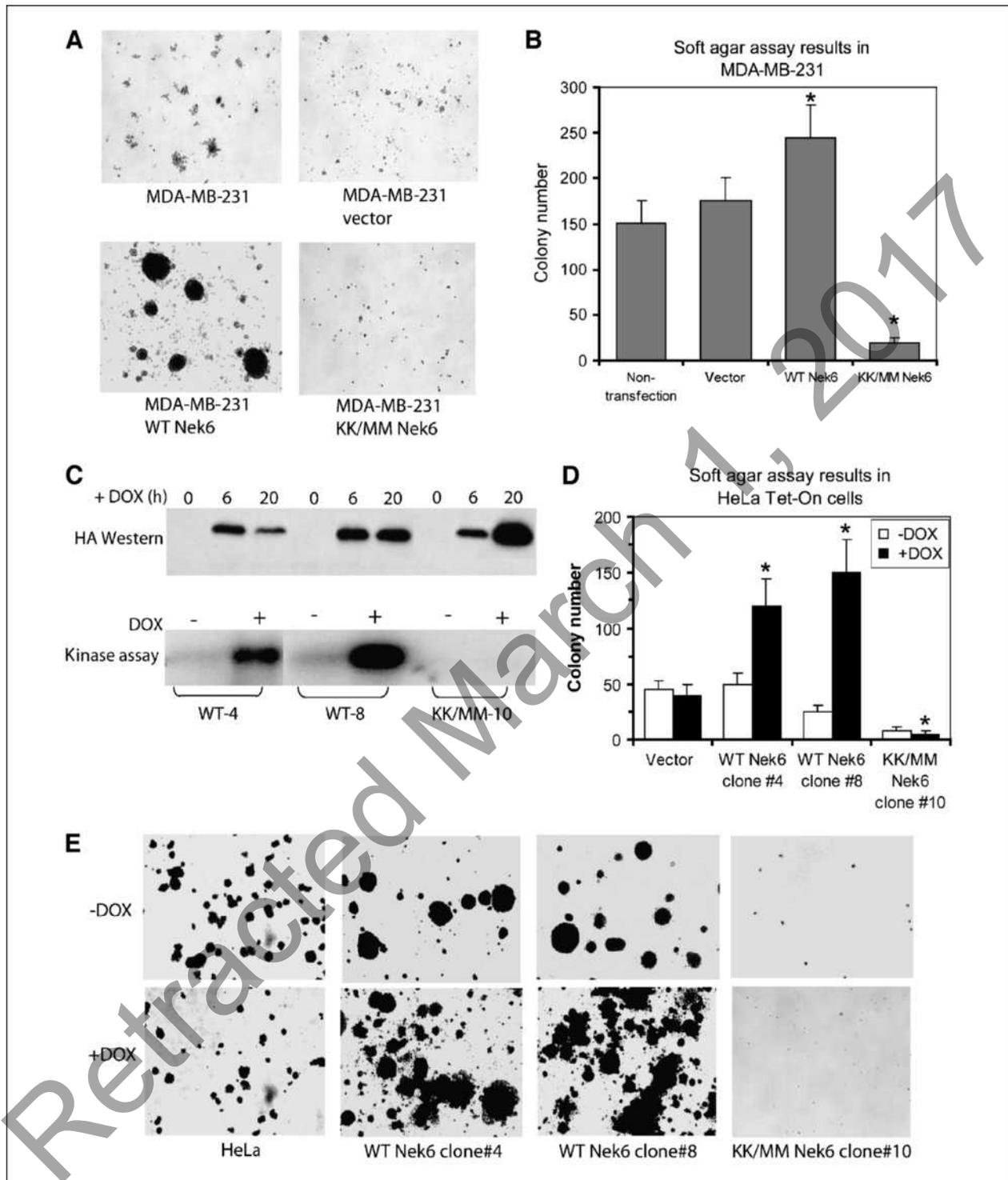
FIGURE 2. Nek6 is critical in the regulation of anchorage-independent growth of MDA-MB-231 and HeLa cells. MDA-MB-231 cells stably overexpressing either the WT or the kinase-dead (KK/MM) Nek6 were used in soft agar assays to determine changes in anchorage-independent growth. A, representative images of soft agar assays for each clone are shown. B, the soft agar assays presented in A were quantified by counting the number of colonies formed. Columns, average number of colonies; bars, SD. Each assay was repeated at least thrice. C, HA-tagged WT or the kinase-dead (KK/MM) Nek6 clones were established in the HeLa Tet-On system and expression was successfully induced at the protein level and kinase activity by the addition of doxycycline (DOX). A HA antibody was used to do the immunoprecipitation and Western blots to distinguish the exogenous expression of Nek6 protein and kinase activity from endogenous levels. D, colony numbers of soft agar assays for each clone of HeLa Tet-On cells. Columns, mean; bars, SD. E, representative images show colony formation in soft agar assays of HeLa Tet-On clones. *, P < 0.05, one-way ANOVA test.
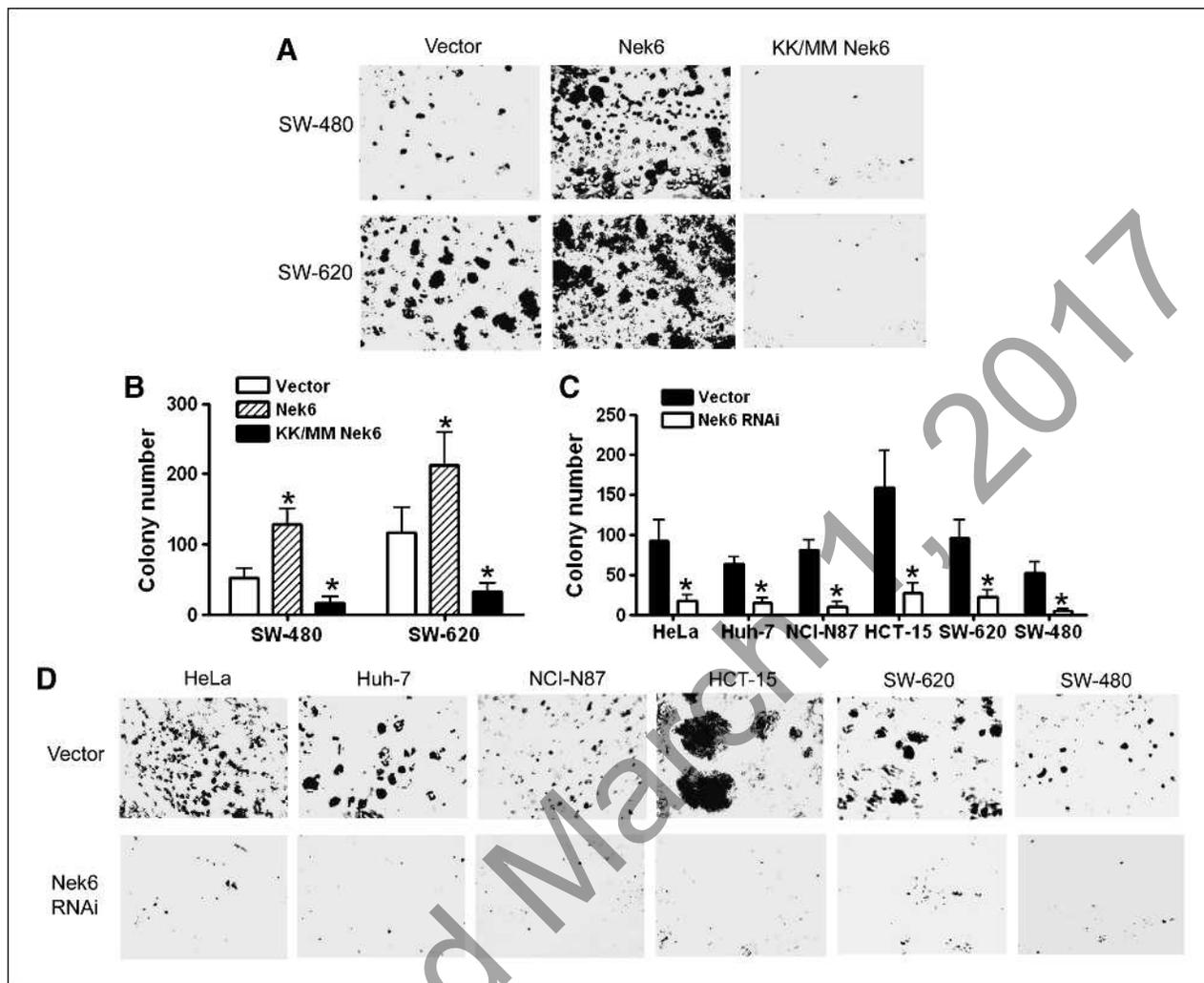
FIGURE 3. Nek6 level is directly correlated to tumor aggressiveness. A, SW-480 cells (low Nek6–expressing line) and SW-620 cells (high Nek6–expressing line) stably overexpressing vector alone, WT Nek6, and kinase-dead KK/MM Nek6 were established and used to do soft agar assays. Representative colony morphology images are shown in the top panel for SW-480 and in the bottom panel for SW-620. B, the soft agar assays presented above were quantified as previously described. Overexpression of Nek6 increased anchorage-independent growth, whereas expression of the kinase-dead Nek6 inhibited colony formation in both high- and low-expressing colon cancer cell lines. C, soft agar colony numbers from a variety of human cancer cell lines harboring stable knockdown of Nek6 were counted. Columns, mean; bars, SD. Nek6 RNAi abolished anchorage-independent growth in all cell lines studied. D, representative soft agar images of cervical HeLa, liver Huh-7, gastric NCI-N87, and colon cell lines SW-480 and SW-620. *, $P < 0.05$, one-way ANOVA test.

Nek6 levels increased total BAD expression and decreased BAD phosphorylation to induce apoptosis (Fig. 5D). Similarly, RNAi knockdown of Nek6 in MDA-MB-231 and HeLa cells increased BAX expression, as determined by Western immunoblot analysis (Fig. 5E). In addition, downregulation of Nek6 caused elevated levels of cleaved caspase-3 and cleaved PARP in MDA-MB-231 and HeLa stable cell lines compared with vector controls (Fig. 5D). However, the levels for these apoptosis indicators were not changed in WI-38 cells when Nek6 is suppressed by RNAi knockdown (Fig. 5D). These results suggest that Nek6 is specifically required in cancer cells for cell proliferation and transformation but is not required in the human normal cell lines for cell viability. Reduction of Nek6

in cancer cells induces apoptosis and leads to cell death, whereas reduction of Nek6 in normal fibroblast cells has no effects in the induction of apoptosis and cell death. To establish the generality of our findings, we also investigated the levels of caspase-3 and caspase-7 activity directly in other human cancer cell lines (Fig. 5F). Overexpression of the dominant-negative kinase-dead Nek6 mutant, or knocking down endogenous Nek6, did not alter caspase-3/7 activity in normal WI-38 cells (Fig. 5F). In contrast, functional knockdown of Nek6, by stably overexpressing the kinase dead or RNAi, significantly stimulated the caspase-3/7 activity in breast cancer cell lines MDA-MB-231 and Hs-578t, liver cancer Huh-7, and colon cancer SW-620, as well as cervical cancer HeLa cells (Fig. 5F).

Interestingly, in SW-480 cells, which express less Nek6 compared with the other cancer cell lines (Fig. 1), only overexpression of the kinase-dead mutant induced moderate caspase-3/7 activity, and knockdown of endogenous Nek6 did not induce significant increase in caspase-3/7 activity. Taken together, the data presented here strongly suggest that inhibition of Nek6 evokes proapoptotic signaling specifically in cancer cells. Thus, inhibition of Nek6 could provide a novel strategy to selectively kill cancer cells and minimize undesirable cytotoxicity in normal tissue.

Discussion

In the current study, we describe the role of Nek6 in tumorigenesis. We first show increased RNA and protein levels, as well as Nek6 kinase activity, in a variety of malignant tumors and human cancer cell lines (Fig. 1). Increased expression of Nek6 can promote cell transformation activity in a variety of human cancer cells (Figs. 2 and 3). Both soft agar assays and *in vivo* xenograft tumor growth assays presented here confirm that the expression and activity level of Nek6 are critical for tumorigenesis. Furthermore, we report that exogenous expression of Nek6 enhanced tumor progression, whereas suppression of Nek6 resulted in inhibition of anchorage-independent growth and induced apoptosis in most cancer cells (Fig. 3). Similarly, overexpression of the kinase-dead Nek6 abolished cell transformation activity in human cancer cells, suggesting that the ability of Nek6 to transform cells is dependent on its kinase activity (Figs. 2 and 3). The results presented here also suggest that expression levels of Nek6 and its kinase activity are tightly correlated with the degree of tumor aggressiveness as depicted by differences in expression levels between SW-480 and SW-620, a matched pair of primary and metastatic colon adenocarcinoma cell lines (Fig. 3). The observation that Nek6 knockdown in SW-480 did not induce significant caspase activity but blocked the colony formation ability indicates that the low-expressing SW-480 cells require Nek6 activity for tumorigenesis but not for cell viability.
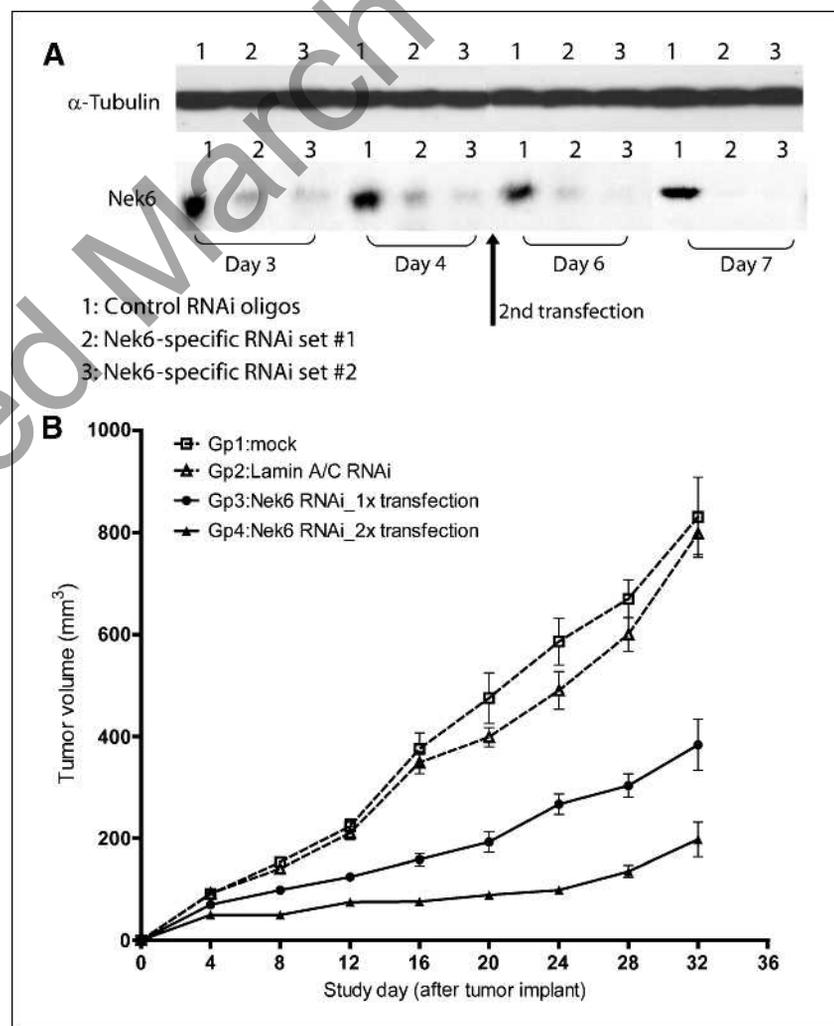

FIGURE 4. RNAi depletion of endogenous Nek6 in HeLa cells inhibited tumor growth in xenograft mouse model. A, HeLa cells were transfected with Nek6 RNAi to deplete the endogenous Nek6. Protein samples were collected at 3 to 7 d after transfection and analyzed for Nek6 expression. At 3 d after RNAi transfection, endogenous Nek6 levels are dramatically reduced. A second round of transfection almost completely abolished Nek6 protein expression. B, mice were s.c. implanted with HeLa cells harboring mock transfection, a laminin control RNAi, and cells from the first and second rounds of Nek6 RNAi transfection. Tumor volume was measured every 4 d for 36 d.
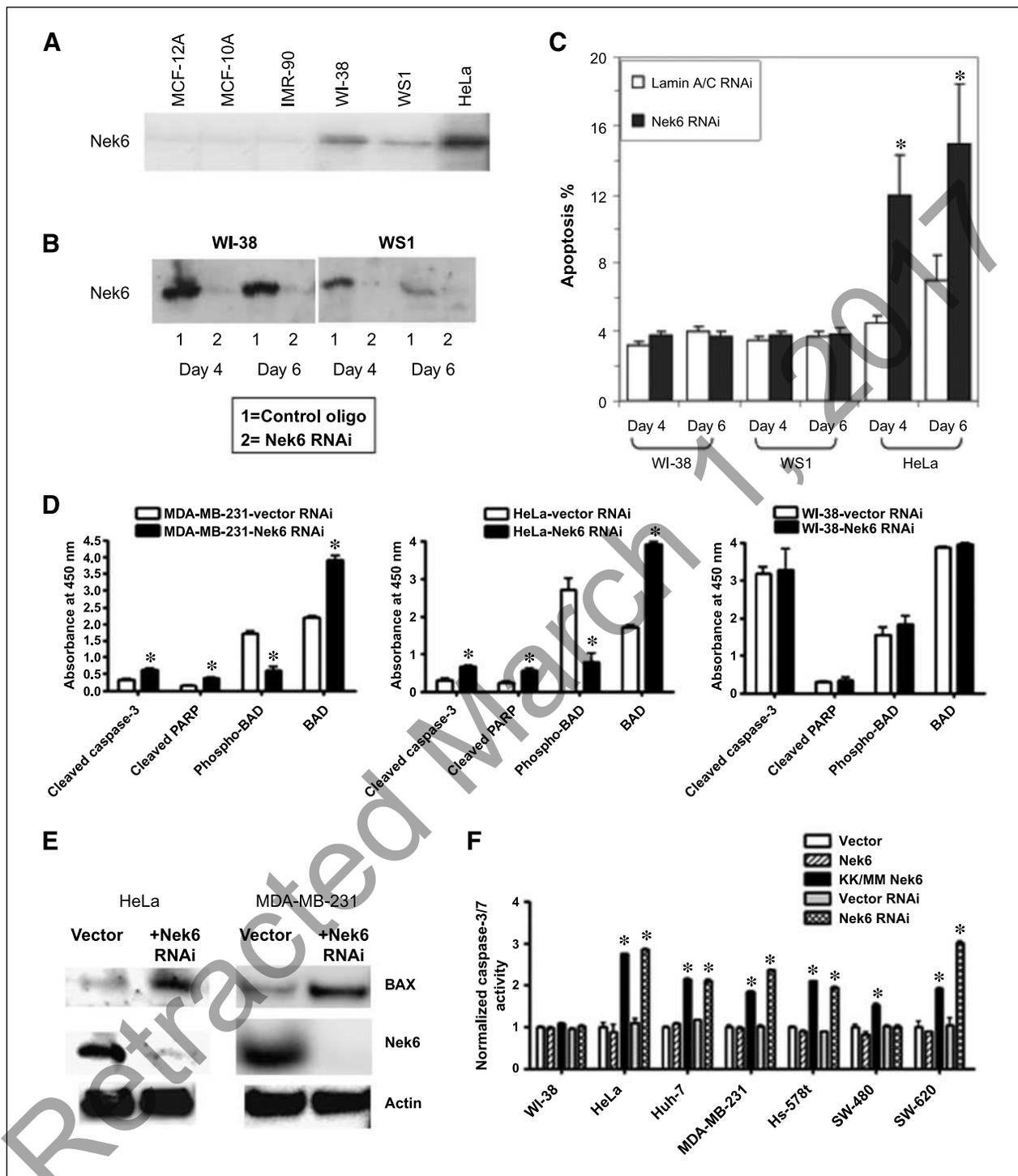
FIGURE 5. RNAi depletion of endogenous Nek6 did not induce apoptosis in normal human lung fibroblast cells. A, Western blot analysis of endogenous Nek6 expression in a variety of human primary cell lines as indicated. B, endogenous Nek6 protein levels were decreased by RNAi transfection in two human normal fibroblast cell lines, WI-38 and WS1, which expressed detectable Nek6 expression. C, flow cytometry on the propidium iodide–stained cells was used to determine the percentage of cells in the sub-G_1 phase undergoing apoptosis. D, PathScan Multi-Target Apoptosis ELISA kit used on MDA-MB-231, HeLa, and WI-38 cells reveals that Nek6 RNAi increased cleaved caspase-3, cleaved PARP, and BAD expression but decreased levels of phosphorylated BAD (Phospho-BAD). E, MDA-MB-231 and HeLa cells harboring stable RNAi knockdown of Nek6 show increased proapoptotic BAX protein expression in Western immunoblot analysis. F, caspase-3/7 activity was examined in a variety of human cancer cell lines. Overexpression of the kinase-dead Nek6 or RNAi knockdown of endogenous Nek6 increased caspase-3/7 activity. Caspase-3/7 activity was plotted as relative expression to vector alone levels. Columns, mean fold change; bars, SD. *, $P < 0.05$, one-way ANOVA test.

In comparison with SW-620 and other high-expressing lines, Nek6 is required for both the transformation and cell viability, implying that cancer cells with high overexpression of Nek6 are dependent or addicted to Nek6 for tumor transformation and cell proliferation. Most importantly, we report that although inhibition of Nek6, by either overexpression of its kinase-dead/dominant-negative form or knockdown of endogenous Nek6, activated the apoptotic pathway in cancer cells and caused cell death, it did not induce cell death in normal cells (Fig. 5). These data strongly suggest that Nek6 is critical in tumor progression and cancer cell transformation and is dispensable in normal cell survival. Therefore, Nek6 may be a potential target for cancer drug development.

Currently, there are limited reports implicating Nek family members to tumor progression. Although Nek2 and Nek8 are highly overexpressed in tumor tissues, no detailed mechanism has been described. Several labs, including ours, have reported overexpression of Nek6 in multiple cancer patient tumor samples. Here, we report for the first time that higher Nek6 levels in cancer cells generate greater kinase activity of Nek6 and subsequently lead to more aggressive transformation activity. The data presented here strongly suggest not only that Nek6 can enhance tumor progression but also that Nek6 overexpression is the driving force rather than the consequence of tumorigenesis. Knocking down Nek6 was sufficient to completely abolish cell transformation activity in a variety of aggressive cancer cell lines, underscoring the pivotal role of Nek6 activity in tumor progression. We had tested several cancer cell lines (such as MDA-MB-231) that harbor mutations of several key genes involved in tumorigenesis. These include B-Raf, K-ras, p53, p16 deletion, etc. Alteration of any one of these genes is expected to upregulate cell proliferation and induce cell cycle progression. Because knockdown of Nek6 alone by RNAi or expression of its kinase dead was enough to block colony formation, Nek6 activity seems to be not only necessary but also sufficient for cancer cells to accomplish cellular transformation. Thus, the upregulation of genes, such as B-Raf, K-ras, p53, and p16 deletion, cannot fully compensate for the loss of Nek6 function in cancer cells.

The majority of apoptotic events hinge on the balance of activity between proapoptotic and antiapoptotic members of the B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2) superfamily of proteins, which act to regulate the permeability of the mitochondrial membrane (38). Proapoptotic Bcl-2 proteins are divided into two subgroups based on the number of BH domains they contain. BH3-only proteins (such as BAD) activate the multi-BH domain proapoptotic proteins (such as BAX). On stimulation, BAX translocates to the mitochondrial membrane and permeabilizes it, releasing cytochrome c and initiating the caspase pathway (39). Activated caspases, and particularly caspase-3, are believed to then cleave proteins such as PARP and facilitate cellular disassembly and cell death (40). The proapoptotic signals are balanced by members of the antiapoptotic pathway, which prevent apoptosis and promote survival. For example, various survival factors can phosphorylate BAD and inhibit the apoptotic pathway (41). Nek6 knockdown induced apoptosis in cancer cells by increasing the expression of proapoptotic members (BAX, BAD, caspase-3/7, and PARP) and decreasing levels of phosphorylated BAD. Results from multiple assays confirm that inhibition of Nek6 induced apoptosis only in cancer cells but not in normal primary cells. Thus, Nek6 function is not important for normal cells to maintain viability. The majority of antitumor agents cause cytotoxicity in normal tissue by killing both normal and malignant cells with a narrow differential window. Because inhibition of Nek6 specifically induces cell death in tumor cells and not in normal tissues, Nek6 inhibitors could have better therapeutic window than the conventional cytotoxic antitumor agents. Given that the kinase activity of Nek6 is critical for its function to promote tumor progression, a Nek6 kinase inhibitor could be a potent and specific antitumor agent.

Conclusions

In conclusion, we have shown that knockdown of Nek6 inhibits tumorigenicity in both *in vitro* cell transformation assays and *in vivo* animal models. Furthermore, although RNAi knockdown of endogenous Nek6 induces cancer cell apoptosis, it does not cause cell death in normal fibroblasts, suggesting that Nek6 might be a potent and safe candidate for antitumor drug development.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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Retraction: Nek6 Mediates Human Cancer Cell Transformation and Is a Potential Cancer Therapeutic Target

The article titled, "Nek6 Mediates Human Cancer Cell Transformation and Is a Potential Cancer Therapeutic Target," which was published in the May 2010 issue of *Molecular Cancer Research* (1), is being retracted at the request of Pfizer.

The AACR Publications Department recently received a letter from a Pfizer representative reporting the results of an investigation of image duplication in Figs. 3A, 3D, 4A, and 5A that were also noted several months ago on the PubPeer.com website. The investigation found that all of the images in question appear to be duplications and most of the authentic, original images that should have been used in place of the duplicated images cannot be located. To ensure that the research record is correct, Pfizer is requesting Retraction of the article and has obtained consent from six authors. All authors have consented to this Retraction except for Lihua Shao who could not be located.

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Nek6 Mediates Human Cancer Cell Transformation and Is a Potential Cancer Therapeutic Target

Rounak Nassirpour, Lihua Shao, Peter Flanagan, et al.

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