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NEK9 depletion induces catastrophic mitosis by impairment of mitotic checkpoint control and spindle dynamics

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ABSTRACT

NEK9 is known to play a role in spindle assembly and in the control of centrosome separation, but the consequences of NEK9 targeting in cancer cells remain to be elucidated. In this study, we used siRNA to investigate the consequences of targeting NEK9 in glioblastoma and kidney cancer cells as a first step in assessing its potential as an anti-cancer therapeutic target. Live cell imaging revealed that NEK9 depletion of U1242 glioblastoma and Caki2 kidney carcinoma cells resulted in failure of cytokinesis. Interestingly, NEK9-depleted Caki2 cells overrode mitosis under incorrect chromosome alignment and were converted to a micronucleated phenotype, leading to cell death. Whereas, the RPE1 normal epithelium cell line was refractory to abnormal mitosis upon NEK9 knockdown. Nocodazole-induced mitotic arrest was compromised after NEK9 depletion, indicating that NEK9 has an important role in mitotic checkpoint system. Taken together, we propose that NEK9 inhibition represents a novel anti-cancer strategy by induction of mitotic catastrophe via impairment of spindle dynamics, cytokinesis and mitotic checkpoint control.

1. Introduction

The NIMA family of protein kinases is named after the Aspergillus nidulans protein kinase encoded by the nimA (never in mitosis A) gene [1]. NIMA is required for entry into mitosis and is involved in the control of chromatin condensation, spindle and nuclear envelope organization, and cytokinesis [2,3]. Eleven protein kinases, NEK1 to NEK11, with a catalytic domain related to NIMA have been identified in the human genome [4]. Nek9 is a 107-kDa polypeptide whose N-terminal catalytic domain is followed by a domain homologous to regulator of chromatin condensation (RCC1). NEK9 C-terminal coiled coil motif binds to DYNLL/LC8 and this interaction is regulated by NEK9 activity through the autophosphorylation [5]. Microinjection of anti-NEK9 antibodies in prophase results in spindle abnormalities and/or chromosomal misalignment [6]. NEK9 co-immunoprecipitates gamma-tubulin and the activated NEK9 localizes to the centrosomes and spindle poles during early mitosis, indicating that active NEK9 has important functions at the microtubular organizing center during cell division [7]. Nek6 and Nek7 can bind strongly to RCC1 domain of NEK9 and are phosphorylated and activated by NEK9 [8] and depletion of either NEK6 or NEK7 leads to defective mitotic progression [9]. It is clear that NEK6, NEK7 and NEK9 contribute to the establishment of the microtubule-based mitotic spindle [10].

Besides spindle tubule formation, Betran et al. reported that NEK9 activation by Polo-like kinase 1 (PLK1) contributed to the phosphorylation of the mitotic kinesin Eg5 is necessary for subsequent centrosome separation and timely mitosis [11]. In addition, NEK9 phosphorylates NEDD1 which recruits gamma-tubulin to centrosome to ensure the formation of two dense microtubule asters in cells entering mitosis [12]. These data indicate that NEK9 has important function not only in spindle formation but also in centrosome maturation and separation.

Spindle assembly checkpoint (SAC) is a fail-safe mechanism that monitors the fidelity of chromosome segregation in space and time. Until all chromosomes are properly aligned at the spindle equator, the mitotic checkpoint inhibits the anaphase-promoting complex/cyclosome (APC/C) that prevents cells from entering anaphase. If the kinetochore-microtubule attachment is not correct, SAC generates the wait-anaphase signal that propagates throughout the cell to inhibit the APC/C [13]. In various human cancers, mitotic checkpoint function is partially compromised, and altered expression or mutations in mitotic checkpoint genes have been shown to be related to chromosome instability (CIN) and aneuploidy. Nevertheless, no evidence for checkpoint malfunctions as a direct cause of CIN in tumor cells has been found [14–16]. However, complete inactivation of the mitotic checkpoint results in gross chromosomal missegregation and is not compatible with cell viability [17–20]. This has led to the suggestion that

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inhibition of the mitotic checkpoint could have therapeutic potential in cancer treatment. Moreover, tumor cells that have acquired a decreased checkpoint activity could be more sensitive to mitotic checkpoint inhibition when compared to healthy, checkpoint proficient cells [21].

Here, we explored the potential of NEK9 as an anti-cancer therapeutic target. We found that NEK9 plays an important role in the spindle checkpoint. NEK9 inhibition induced apoptosis through abnormal mitosis in tumor cells, whereas the hTERT-RPE1 (REP1) normal human retinal pigment epithelium cell line was refractory to abnormal mitosis.

2. Materials and methods

2.1. Human cell lines

Caki2 kidney carcinoma cells was obtained from the American Type Culture Collection and U1242 glioblastoma cells was obtained from Sugen Inc. were cultivated in RPMI1640 supplemented with 10% FCS and glutamine (Life Technologies). U1242 and Caki2 cells which were transfected with a human histone H2B/green fluorescence protein (GFP) fusion gene (H2B-GFP) were used to analyze mitotic processes. RPE1 cells which stably express H2B-GFP was a kind gift by Taylor [14]. Cell proliferation was measured by using CellTiter-Glo (Promega) according to the manufacturer's instructions.

2.2. RNA interference

Two independent siRNAs against NEK9 were used in all experiments. These are referred to as NEK9#115-siRNA (Ambion, Silencer Validated siRNA (#1115)) and NEK9#72-siRNA (Ambion, Silencer Select siRNA (#s40772)). Non-targeting RNA duplexes (Negative control siRNA (Ambion, #4390843)) were used as a negative control in all experiments. siRNAs were transfected using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions (final concentration of siRNA was 3.75 nM).

2.3. Quantitative PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA using AMV reverse transcriptase (Roche) with random hexamers. For real-time PCR, Assays-

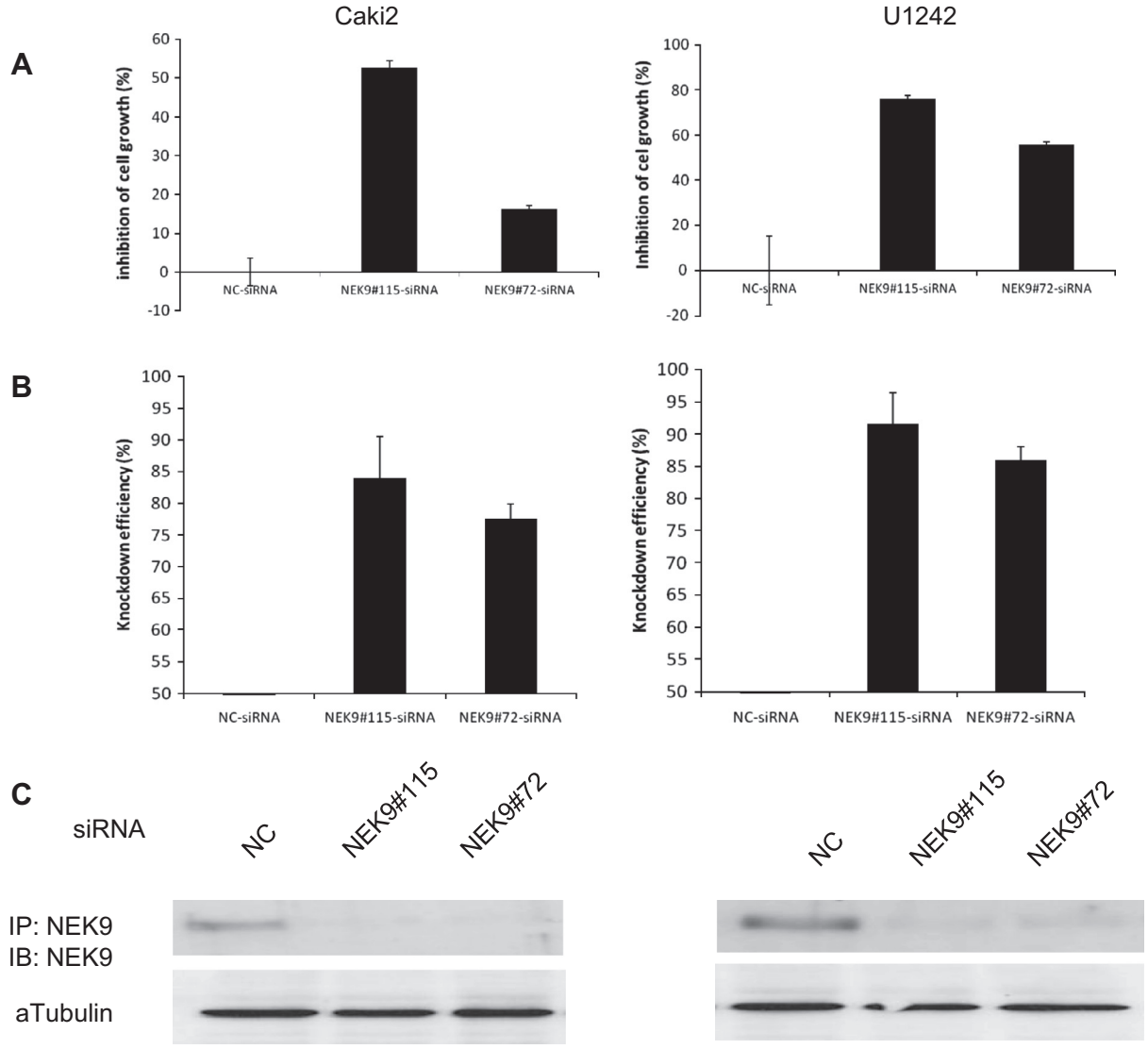

Fig. 1. Depletion of NEK9 induced growth inhibition in cancer cells. Inhibition of cell growth was measured 4 days post-transfection of siRNA. Growth inhibition of negative control siRNA (NC-siRNA) transfectants was normalized to zero (A). After siRNA transfection, cells were incubated for 4 days. Knockdown efficiency of NEK9 mRNA, which was normalized to GAPDH mRNA, was calculated by comparison to NC-siRNA transfectants (B). Knockdown efficiency was also confirmed by western blotting after immunoprecipitation (C).

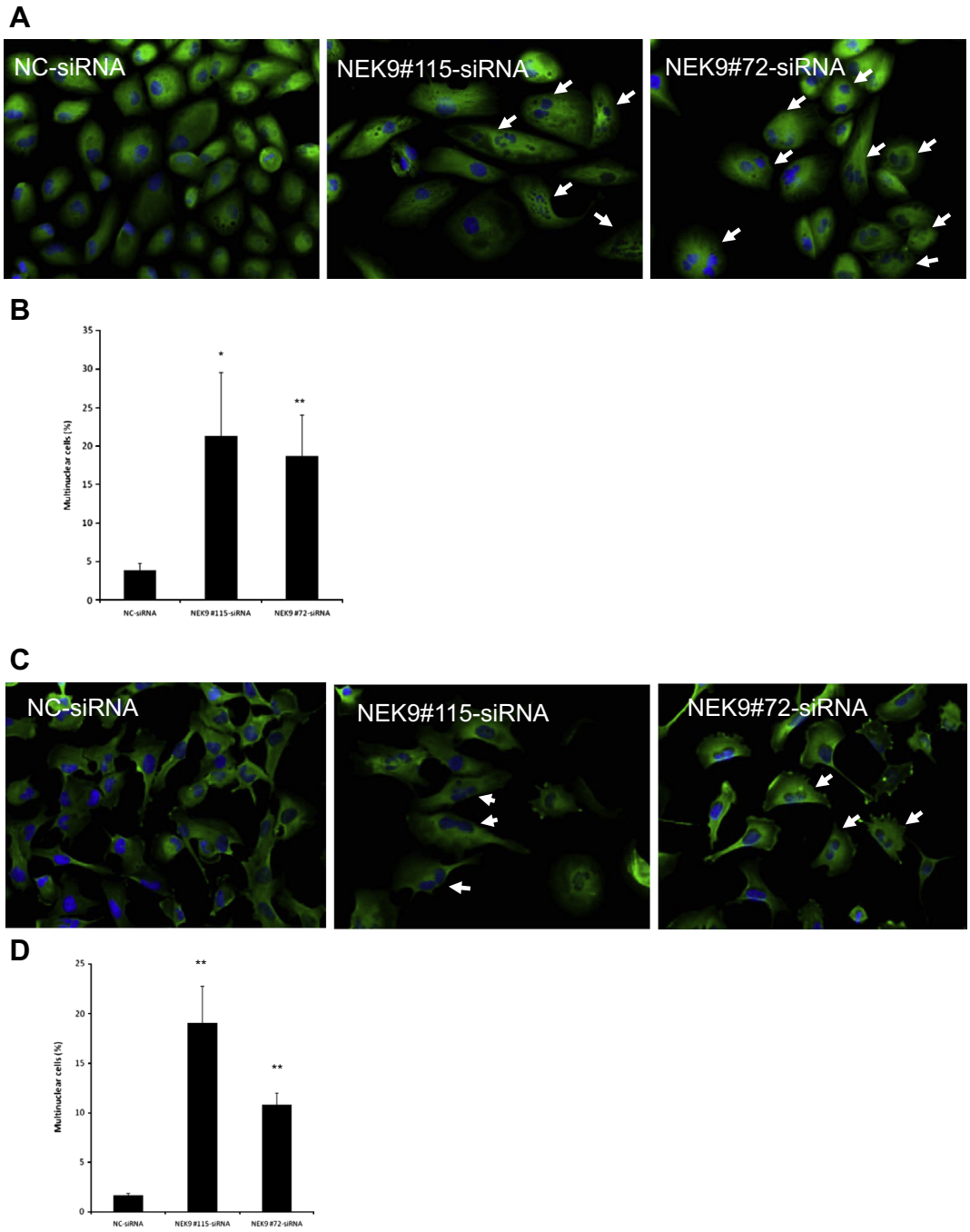

Fig. 2. Depletion of NEK9 generated multi-nuclear cells. Cells were incubated for 4 days after siRNA transfection and stained with alpha-tubulin (green) and DAPI (blue) in Caki2 (A) and U1242 (C) cells. White arrows indicate multi-nuclear cells. The numbers of multi-nuclear cells were counted (B and D) in 5 microscopic fields of view. The results are the mean of three experiments. Statistical analysis was performed using Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$. (For interpretation of color in this figure legend, the reader is referred to the web version of this book. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on-Demand Pre-Developed TaqMan Assay Reagents (NEK9 (Applied Biosystems, Hs00263874_m1) and GAPDH (Applied Biosystems, 402869)) and Universal PCR Master Mix (Applied Biosystems) were used for preparation of each reaction mixture. The reactions were run in a StepOnePlus instrument (Applied Biosystems).

2.4. Western blotting and immunoprecipitation

Cells were lysed in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 μg/ml Aprotinin, 1 mM PMSF, 10 mM NaF, 2.5 mM Na_3VO_4) on ice for 5 min and the lysates were homogenized in a Qiashredder column (Qiagen) and subsequent centrifuged at 13,000 rpm at 4 °C for 15 min.

For immunoprecipitation (IP), samples were incubated with NEK9 antibody (Santa Cruz (N-20)) at 4 °C overnight. After 1 h incubation with protein G beads (GE healthcare), samples were washed with lysis buffer three times.

For western blotting, cell lyses and IP samples were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The

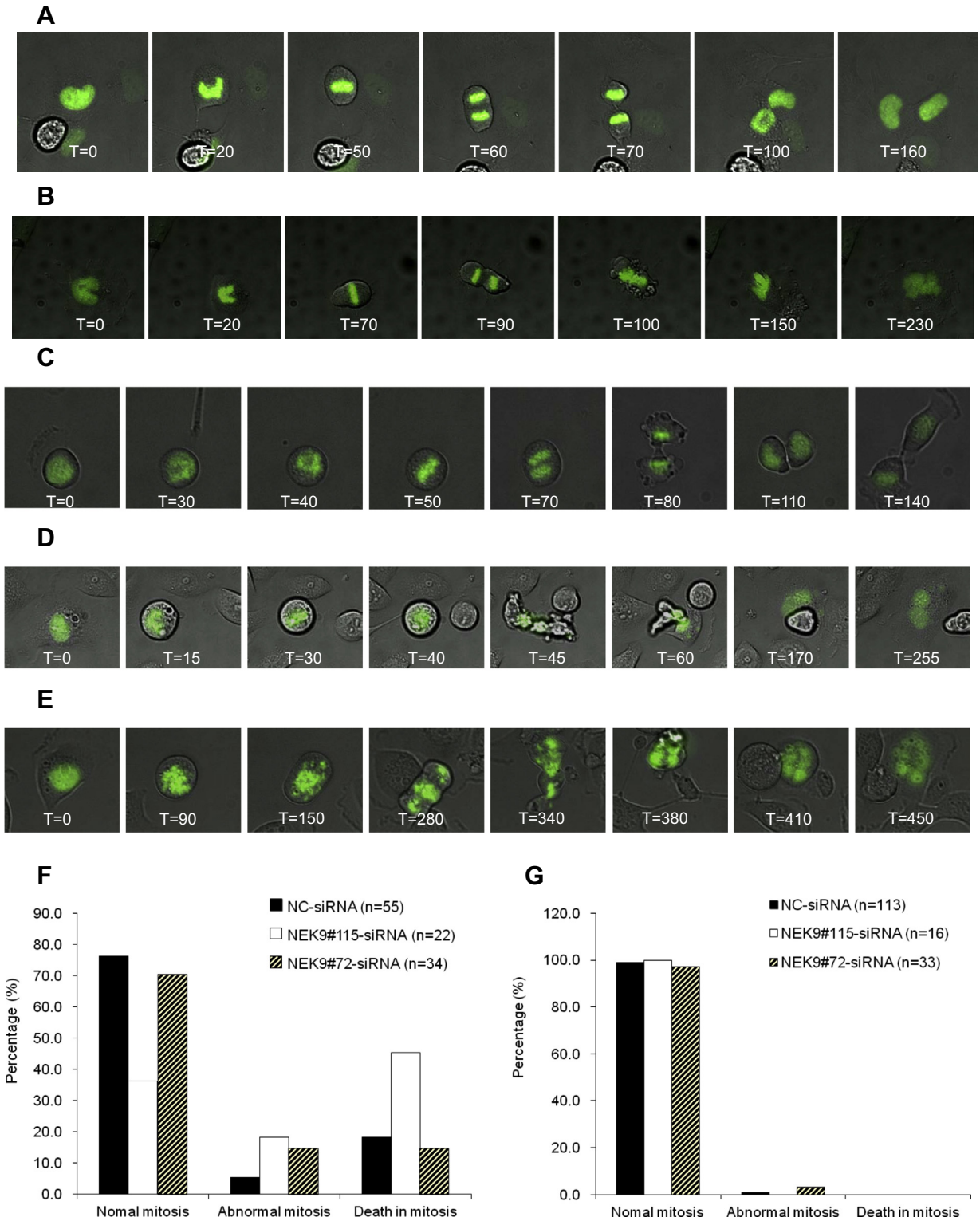

Fig. 3. Depletion of NEK9 induced abnormal mitosis in cancer cells, but not in normal cells. U1242 (A and B) and Caki2 (C, D and E) cells expressing a histone H2B-GFP gene fusion were analyzed by time-lapse microscopy. Representative pictures were shown in A–D; normal mitosis of NC-siRNA transfectants in U1242 (A) and Caki2 (C), cytokinesis failure of NEK9-siRNA transfectants in U1242 (B) and Caki2 (D) and exited mitosis without chromosome separation of NEK9-siRNA transfectants in Caki2 (E). The *T* value at the bottom indicates time in minutes. The progressions of mitosis in U1242 (F) and RPE1 (G) were observed by a time-lapse microscope and categorized into normal, abnormal and death in mitosis. *n* = the number of observed mitotic cells. Representative cell images in NC-siRNA and NEK9-siRNA transfectants are shown (H).

membranes were incubated with primary antibody. Primary antibodies against alpha-tubulin from Sigma, NEK9 from Novus Biological were used at a dilution of 1:1000. Secondary horseradish peroxidase-conjugated anti-mouse IgG (Sigma), anti-rabbit IgG (BioRad) and anti-goat (Jackson ImmunoResearch Laboratories) antibodies were used at a dilution of 1:10,000. Signals were developed using the ECL chemiluminescence detection system (GE healthcare).

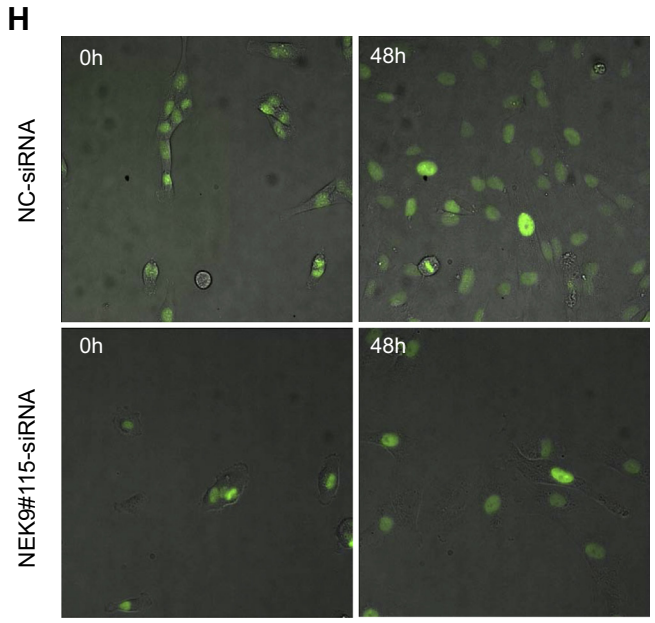

Fig. 3. (*continued*)

2.5. Immunohistochemistry

Cells were seeded onto coverslips and fixed with a fixation solution (2% paraformaldehyde, 125 mM Sucrose in PBS) at 4 °C for 10 min. Cells were permeabilized with 0.1% Triton X in PBS for 10 min, followed by a 10 min incubation with 100 mM Glycine. After blocking with 5% fetal bovine serum (FBS) in PBS for 1 h, cells were incubated with anti-alpha-Tubulin antibody (Sigma) in 5% FBS in PBS at room temperature for 1 h. After washing cells with PBS, cells were stained with anti-mouse Alexaflour546 for 1 h. After washing cells with PBS, cells stained with Hoechst33342 were mounted with Fluoromount-G (Southernbiotech).

2.6. Flow cytometry (FCM) analysis

Both attached and floating cells were collected and fixed in 70% ethanol for 30 min. Cells were subsequently stained with PI buffer (0.1% Triton X-100, 100 μg/ml RNase A and 20 μM propidium iodide in PBS) and analyzed using FACSCalibur (Becton Dickinson).

2.7. Live cell imaging

Cells were seeded in a 4-well Cellview glass bottom dish (Greiner) at 1.3×10^4 cells per well in a volume of 500 μl. After 3 h, imaging was performed on an Applied precision DeltaVision RT system (Issaquah) equipped with an Olympus IX-71 inverted base microscope, an Olympus UPlanFluorit 20x/NA0.5 objective and a Photometrics CoolSNAP HQ 12 bit monochrome camera (Tucson). Images were acquired every 10 min for 48 h and were subsequently processed using softWoRx software.

3. Results

3.1. NEK9 knockdown inhibits cell proliferation and gives rise to multinuclear Caki2 and U1242 cells

To investigate whether NEK9 inhibition could impair cell proliferation, we performed growth assay with Caki2 and U1242 cell lines by using two independent NEK9 siRNAs (Fig. 1A–C). We observed mRNA degradation by NEK9#115-siRNA which always had a stronger effect than NEK9#72-siRNA (Fig. 1B). Coincident with the knockdown efficiency, all tested biological properties induced by NEK9#115-siRNA in this study were more pronounced than those induced by NEK9#72-siRNA. Immunohistochemical analysis revealed that the fraction of multinuclear cells was increased in Caki2 (Fig. 2A and B) and U1242 cells (Fig. 2C and D). Moreover, we observed micronucleated cells in Caki2 cells, which contained many nuclei (Fig. 2A).

3.2. NEK9 depletion induces cytokinesis failure in U1242 cells

To analyze the development of abnormal nuclear morphology, we performed live cell imaging after NEK9 knockdown in U1242 cells (Fig. 3A and B). Mitosis cells were categorized into three groups; normal mitosis, abnormal mitosis (cytokinesis failure or chromosomes were not aligned in the metaphase plate) and death in mitosis (cells showed blebbing and nuclear fragmentation before attachment onto plate) (Fig. 3F). In cytokinesis failure mitosis, chromosomes were aligned in the metaphase plate, followed by chromosome separation in anaphase. However, furrow ingression was repressed in cytokinesis, resulting in fused daughter cells (Fig. 3B). This data suggests one of the reasons to generate multinuclear cells is that NEK9 knockdown compromised cytokinesis.

3.3. NEK9 depletion compromises the spindle checkpoint system in Caki2 cells but not in RPE1 cells

In NEK9-siRNA transfected Caki2 cells, we observed that 33.3% of mitotic cells were unable to undergo normal mitosis (NC-siRNA (1/16 (6.25%)), NEK9#115-siRNA (5/15 (33.3%)), NEK9#72-siRNA (4/12 (33.3%)) (Fig. 3C, D and E). In addition to cytokinesis failure like U1242 cells (Fig. 3D), we observed more abnormal mitotic cells whose chromosomes could not be aligned correctly at the metaphase plate (Fig. 3E). After remaining in mitosis for several hours, cells exited from mitosis without cytokinesis, generating micronucleated cells. This data suggests that spindle tubules in NEK9 depletion cells were imprecisely connected to chromosomes and mis-regulated. Moreover, the spindle assembly checkpoint (SAC) complex in NEK9-siRNA transfectants seemed not to function correctly.

Next, to explore the effect of NEK9 knockdown on normal cells, we analyzed RPE1 cells (human retinal pigment epithelium). Similar to cancer cells, NEK9 depletion inhibited cellular growth of RPE1 cells (Fig 3H). Interestingly, the nuclear shape in the NEK9-siRNA transfectants looked like normal cells (Fig. 3H), although the number of nuclei was much less than in the control group. Moreover, live cell imaging analysis revealed that normal cells were refractory to abnormal mitosis (Fig. 3G). The insensitivity of RPE1 cells to NEK9 knockdown is not a consequence of insufficient mRNA deletion, as NEK#115 and NEK9#72-siRNA showed 89.75% and 89.66% knockdown, respectively. These data suggested that normal cells were less dependent on NEK9 at mitosis and NEK9 inhibitors might be able to kill cancer cells selectively.

3.4. NEK9 depletion compromises mitotic arrest and generates micronucleated cells

It is well established that if all chromosomes are not connected with microtubules precisely, the SAC complex is activated and postpones the onset of anaphase to ensure segregation of the correct number of chromosomes into daughter cells [22]. However, live cell imaging data of Caki2 cells revealed that cells exited from mitotic phase after NEK9 knockdown, even when chromosomes were not aligned in the metaphase plate (Fig. 3C). This observation suggests that NEK9 inhibition could

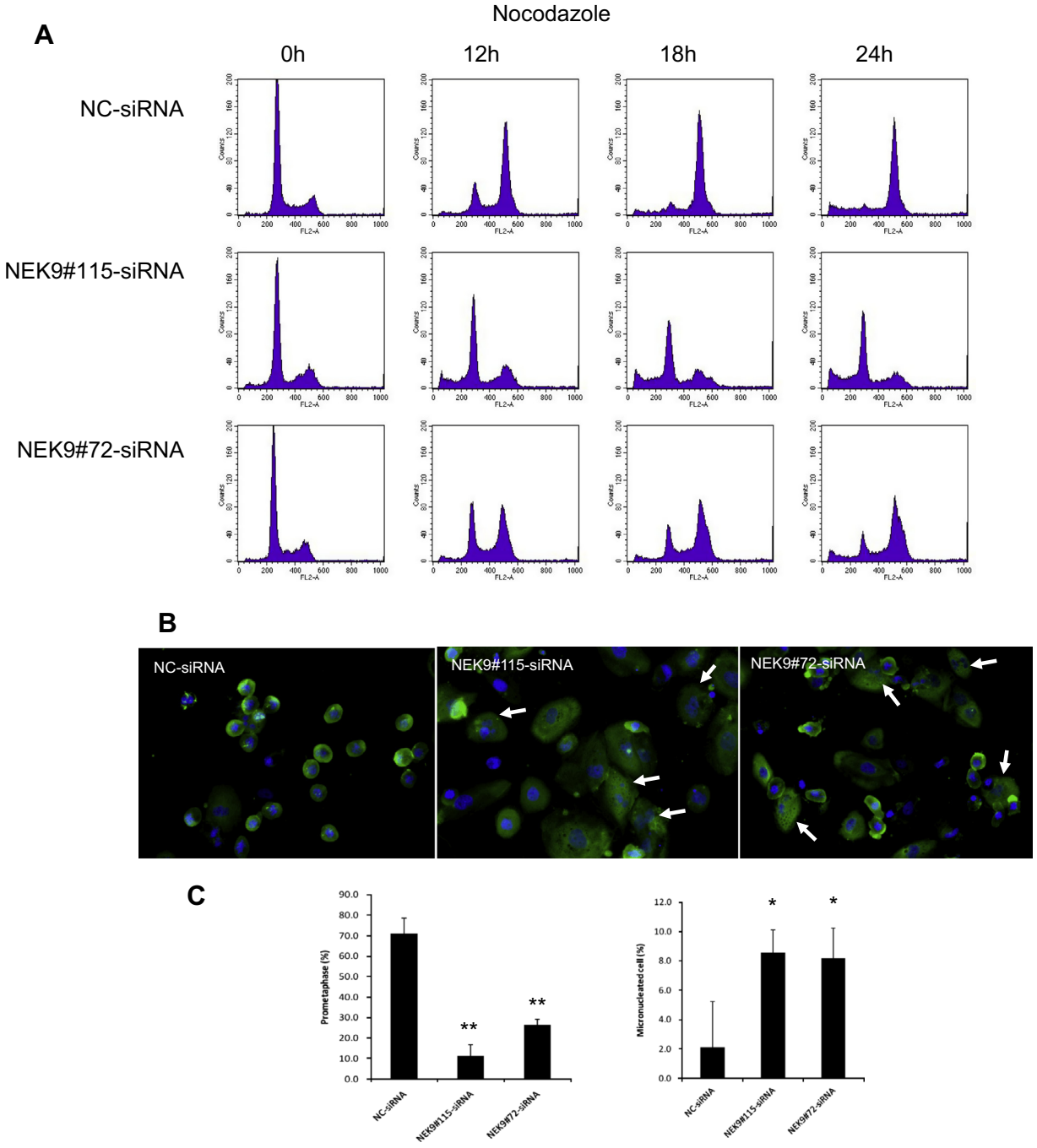

Fig. 4. NEK9 depletion compromised mitotic arrest induced by nocodazole. Cells were transfected with each siRNA and incubated for 48 h. Following nocodazole treatment, cells were harvested for flow cytometry analysis at the indicated time (A), whereas cells were fixed after 16 h nocodazole treatment and immunohistochemical analysis was performed (B). White arrows indicate multinuclear cells. The number of prometaphase and micronucleated cells were counted (C). Prometaphase cells were defined by presence of condensed chromatin and a round-shaped cellular morphology. Confocal microscope images show prometaphase (D), micronucleated cells (E and F) and apoptotic cells (G and H). Statistical analysis was performed using Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

compromise the functioning of the SAC and allow cells to undergo abnormal mitosis.

First, to investigate whether NEK9 is implicated in the mitotic checkpoint system, we analyzed the cell cycle after nocodazol treatment, which inhibits the metaphase-to-anaphase transition by preventing microtubule polymerization. FCM analysis showed that NC-siRNA transfectants were arrested at G2/M phase by nocodazole treatment (Fig. 4A). By contrast, NEK9 transfectants did not stop cell cycle like NC-siRNA transfectants (Fig. 4A). This data suggests that NEK9-depletion compromised SAC function and forced cells to exit from mitosis.

Next, we performed immunohistochemical stain analysis to observe morphological change. In NC-siRNA treated group, a lot of typical prometaphase cells with round shape and condensed chromatin in the center were observed (Fig. 4B). On the other hand, the NEK9-siRNA transfectants contained less prometaphase cells and more adherent cells, which have numerous micronuclei (Fig. 4B and C). The morphology of micronucleation was clearly different from that of chromatin condensation triggered by apoptosis (Fig. 4E–H). The structure of each small nucleus in micronucleated cells was apparent (Fig. 4E and F), whereas that in the apoptotic cells was ambiguous (Fig. 4G and H).

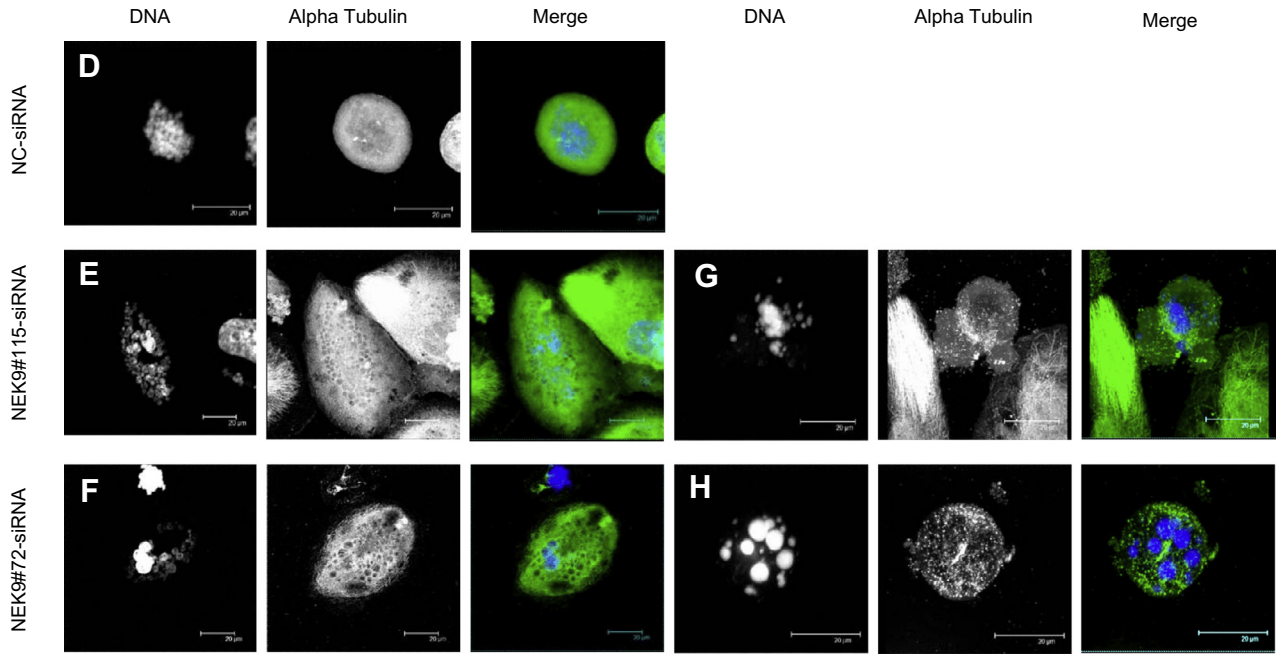

Fig. 4. (*continued*)

4. Discussion

In this study, we revealed NEK9 knockdown induced cytokinesis failure and incorrect chromosome separation. It is assumed that Nek6 and Nek7 are activated via the C-terminal domain of NEK9 in mitosis [23]. Mouse embryonic fibroblast (MEF) cells derived from Nek7-deficient mice showed cytokinesis failure [24]. Depletion of Nek6 by siRNA also resulted in a significant increase in cells with two or more nuclei [25]. Our observations were consistent with these reports and indicated that the Nek6/7/9 pathway is involved in the cytokinesis process.

In addition, we found that NEK9 depletion overrode mitotic arrest by nocodazole treatment, indicating that NEK9 function could be included in SAC system. We explored interacting proteins with NEK9 in prometaphase to identify responsible proteins for SAC impairment, we failed to find out know mitotic checkpoint component (data not shown). To address molecular mechanism how NEK9 affect spindle checkpoint system and identify the causative protein for SAC impairment should be interesting.

It has been reported that overexpression of a kinase dead NEK9 mutant compromised spindle dynamics and microinjection of anti-NEK9 antibodies disrupted spindle formation [6]. At prometaphase and metaphase, NEK9 localize at centrosome [7]. Tan and Lee reported that NEK9 depletion by siRNA resulted in slower cellular growth due to prolonged G1 and S phase [26]. It has been suggested that NEK9 is required for normal cell cycle progression. However, Pelka et al. reported that siRNA-mediated knockdown of NEK9 does not impede cell cycle progression [27]. This discrepancy might be explained by two possibilities. Firstly, cell cycle analysis based only on the amount of DNA is not an accurate method of discrimination between diploid G2/M and tetraploid G1 populations. Live cell imaging is essential to analyze the detailed abnormalities in mitosis [14]. A second possible explanation is due to insufficient knockdown. High knockdown efficiency seems to be necessary to induce apoptosis, probably because small amounts of NEK9 protein were functionally sufficient to rescue severe abnormalities. Indeed, our data also indicated that approximately 80% knockdown efficiency was not enough to generate clear cellular growth retardation.

We propose NEK9 as a new target for anti-cancer therapy development by interfering with normal mitosis and spindle checkpoint control, leading to mitotic catastrophe. Mitotic catastrophe is often characterized by the formation of giant micronucleated cells, which reflects the abnormal segregation of chromosomes induced in a p53-independent manner [28–30]. It is considered that mitotic catastrophe is one of the protective mechanisms reducing cancer incidence, thus applying mitotic catastrophe to cancer treatment appears to be a promising strategy [29,31].

It was reported that depletion of either Mad2 or BubR1 was able to induce cell death via abnormal mitosis [17,18]. Mad2 loss effectively inactivates the checkpoint while inducing a checkpoint stress owing to defective microtubule formation, resulting in highly efficient tumor cell killing. Janssen et al. reported that inactivation of an essential checkpoint protein, Mps1, induced cell death accompanied by a chromosome segregation error [32]. Partial reduction of Mps1 or BubR1, which have dual roles in checkpoint activation and chromosome alignment resulted in sensitivity to taxol. This sensitization was observed only in tumor cells, not in normal cells [21]. Our data show that NEK9 also has dual function as a regulator of spindle formation and in the mitotic checkpoint, similar with Mad2, Mps1 and BubR1. This observation suggests to us that inhibition of NEK9 could be a promising approach for cancer therapy.

Remarkably, NEK9 depletion did not induce abnormal mitosis in normal cells although cellular growth was impaired. It is supposed that the existence of multiple backup systems for the spindle checkpoint pathway in normal cells make them resistant to abnormal mitosis. These redundant pathways seem to be lost or compromised in cancer cell lines underlining the potential of spindle checkpoint interfering drugs as an attractive new approach in anti-cancer therapy.

It is considered that the cell cycle of most cancer cells contains defects in the checkpoint system, which allows cancer cells to proliferate infinitely. To selectively target the abnormal cell cycle machinery in cancer cells is a rational approach for anticancer therapy. In this study, we propose the inhibition of NEK9 as a novel anticancer therapeutic strategy by inducing mitotic catastrophe via impairment of spindle dynamics, cytokinesis and the mitotic checkpoint. A greater understanding of the mitotic progress in normal and cancer cells will improve strategic targeting of cancer cells by NEK9 inhibitors.

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