

Human Chk1 Expression Is Dispensable for Somatic Cell Death and Critical for Sustaining G₂ DNA Damage Checkpoint

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Abstract

Mammalian Chk1 is an essential kinase for embryonic development and plays an important role in the cellular response to DNA damage. However, it remains unclear whether inhibition of Chk1 induces apoptosis in somatic cells. The uncertainty has become a critical issue for rationale design of Chk1 mechanism-based anticancer drugs. Here we show that Chk1 small interfering RNA (siRNA) effectively eliminates Chk1 protein expression without altering the cell cycle profile or inducing apoptosis in various human cancer cell lines under normal conditions. In the presence of DNA-damaging agents, however, Chk1 siRNA alone is sufficient to abrogate the DNA damage-induced G₂ checkpoint and significantly enhance apoptosis. Cell cycle kinetic profiles show that abrogation of G₂ arrest is mediated through shortening of the checkpoint. We also demonstrate that Chk1 siRNA enhances DNA damage-induced apoptosis in p53-deficient cancer cell lines and augments the growth inhibition conferred by DNA-damaging agents. These findings imply that Chk1 inhibitors will have low cytotoxicity on their own and can enhance the efficacy of DNA-damaging drugs.

Introduction

Genetic instability is a hallmark of virtually all tumors and an avenue for tumor initiation, progression, and the development of drug resistance (1). Most of the tumor cells have a defect in the G₁-S checkpoint that provides them a survival advantage. However, this defect also causes the tumor cells to be more dependent on the G₂ checkpoint when the cells encounter stimuli that threaten the genomic integrity. Abrogation of the G₂ checkpoint in the presence of DNA-damaging agents can lead to mitotic catastrophe in the tumor cells.

Chk1 is a protein kinase that maintains the G₂ checkpoint when the cells are under DNA damage conditions (2). Inhibition of Chk1 provides an attractive opportunity for gene-targeted intervention that will abrogate the checkpoint and selectively enhance toxicity of the genotoxic drugs in the cancer cells (3, 4).

Several groups have developed Chk1 inhibitors such as UCN-01 and SB-218078 and demonstrated that inhibition of Chk1 abrogates the G₂ DNA damage checkpoint (5, 6). However, the specificity of the inhibitors inevitably places uncertainties in the interpretation of the results. In addition to inhibition of Chk1, the inhibitors also inhibit other kinases including Chk2 that may also play a role in the DNA damage checkpoints (7, 8).² Thus, whether inhibition of Chk1 alone is sufficient to disrupt the G₂ checkpoint remains questionable. The Chk1 inhibitor exhibits toxicity on its own in short-term cellular assays (9). It is unclear whether the cytotoxic effect is due to a Chk1-based mechanism.

Studies in Chk1 recombinant gene knockout showed that Chk1-null cells failed to maintain the DNA damage checkpoint. At the same time, these studies showed that knocking out the Chk1 gene in mice was embryonically lethal (10), and a conditional knockout of Chk1 induced apoptosis in embryonic stem cells in one cycle (2). These studies confirm that Chk1 is essential to cell cycle progression and that elimination of Chk1 will be fatal in embryonic development.

Chk1 siRNA³ provides a target-specific entity to examine the cellular activities of Chk1. We investigate the role of Chk1 siRNA in apoptosis, cell cycle progression, and the response to DNA-damaging agents in somatic cells. Chk1 siRNA efficiently knocks down the expression of Chk1 proteins and causes little increase of apoptosis and inhibition of growth in the absence of DNA-damaging agents. In the presence of DNA-damaging agents, Chk1 siRNA significantly abrogates the G₂ checkpoint of the cell cycle and enhances DNA damage-induced apoptosis. Abrogation of DNA damage-induced G₂ arrest occurs through shortening of the G₂ checkpoint. These results not only show that inhibition of Chk1 alone is sufficient to abrogate the DNA damage-induced G₂ checkpoint but also demonstrate that inhibition of Chk1 does not cause somatic cell death. These observations strengthen the notion that Chk1 is a valid target for design of drugs that can enhance the efficacy of chemotherapeutic drugs in cancer patients without cytotoxicity on their own.

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² Unpublished observations.

³ The abbreviations used are: siRNA, small interfering RNA; FACS, fluorescence-activated cell-sorting; BrdUrd, bromodeoxyuridine; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

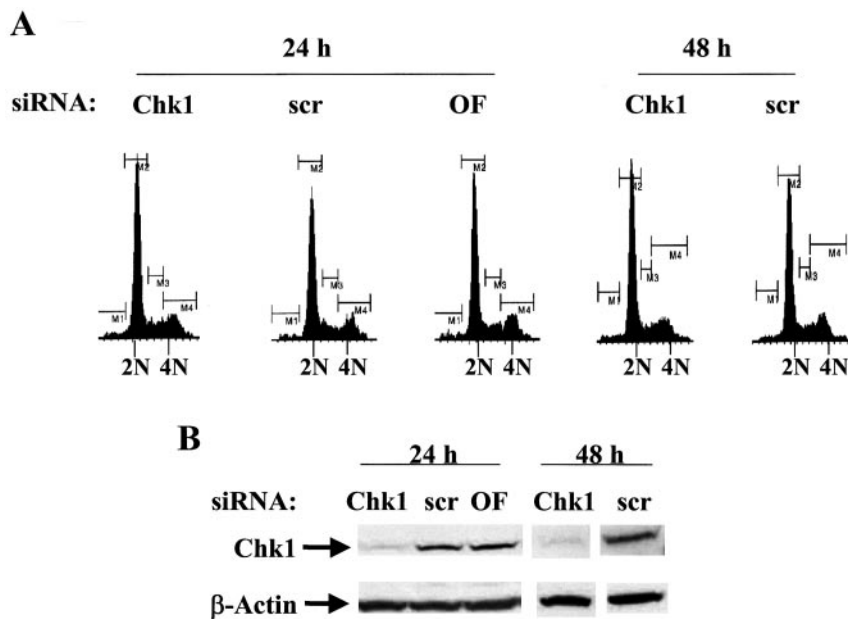


Fig. 1. Chk1 siRNA and cell cycle profiles in the absence of DNA damage. H1299 cells were transfected with Chk1 siRNA (*Chk1*), the scramble control (*scr*), or Oligofectamine alone (*OF*). After 24 and 48 h, half of the samples were subjected to FACS analysis, and the other half were subjected to Western blot analysis. **A**, FACS analysis results for the 24- and 48-h samples were grouped in four populations including apoptotic (M_1), G_0 - G_1 (M_2), S (M_3), and G_2 -M (M_4) cells. **B**, Western blot results show the levels of Chk1 protein and β -actin as control.

Materials and Methods

Cell Culture. Human lung cancer cell line H1299 and cervical cancer cell line HeLa S3 were obtained from American Type Culture Collection (Manassas, VA). H1299 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 0.45% glucose at 37°C in a 5% CO_2 incubator. HeLa S3 cells were grown in F12 Ham's Nutrient Mixture with 10% fetal bovine serum.

Transfection and Drug Treatment. Human Chk1 siRNA and scrambled control siRNA were designed according to the procedure described by the vendor (Dharmacon Research). Chk1 siRNA oligonucleotide contains the following sequence: aactgaagaagcagtcgcagt. The scrambled siRNA derived from Chk1 siRNA contains the following sequence: aacaagtgaagcagtcgcagt. H1299 and HeLa S3 cells were seeded at 2.5×10^5 cells/well into a 6-well plate with 2 ml of medium in each well. The next day, the cells were transfected with siRNA oligonucleotides using Oligofectamine reagents (Invitrogen, Carlsbad, CA) according to the vendor's protocol. DNA-damaging agent was added to the cells 6 h after transfection.

Western Blot Analysis. Western blot analysis was performed essentially the same way as described previously (11). Briefly, protein samples were separated in Bio-Rad ready gel (Bio-Rad, Hercules, CA) under denaturing condition and blotted to nitrocellulose membrane using a semi-dry blotting technique according to the manufacturer's protocol (Bio-Rad). The membrane was blocked with 5% nonfat dry milk and probed with Chk1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescence detection was performed using enhanced chemiluminescence reagents according to the vendor's protocols (Amersham Pharmacia Biotech, Piscataway, NJ).

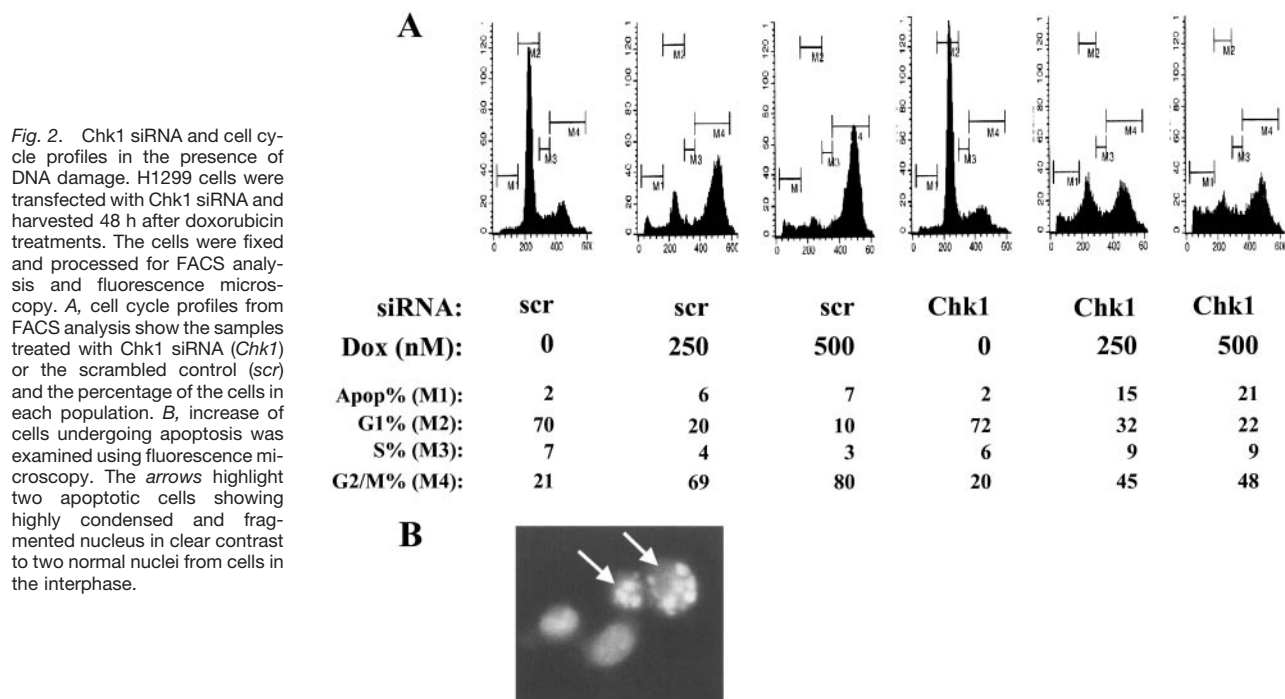
Cell Cycle Analysis. The medium in the cell culture and PBS were collected to include the floating cells. The adher-

ent cells were then trypsinized and added to the tubes. The cells were washed in PBS and fixed in 70% ethanol followed by treatment with RNase A at 37°C for 30 min. Finally, the cells were stained with propidium iodide, and analyzed by FACS analysis (Becton Dickinson, San Jose, CA). A total of 10,000 cells were counted for each sample. Thymidine and Taxol were used as control for positioning S phase and G_2 -M phase, respectively.

For the time course study, H1299 cells were transfected with siRNA on day 1. The next day, cells were pulse-labeled with 10 μM BrdUrd for 60 min and treated with or without doxorubicin in fresh medium for the indicated time periods. Detection of BrdUrd label and DNA contents was performed using BrdUrd kit (BD PharMingen, San Jose, CA) according to the manufacturer's manual.

Detection of Apoptosis. Two methods were used in this report to identify apoptotic cell death. In fluorescence microscopy, propidium iodide-stained samples were spotted to a slide, air dried, sealed under a piece of cover glass, and examined under a Zeiss AxioVission 200 microscope (Carl Zeiss, Inc., Thornwood, NY). Cells with apoptotic morphology display shrinkage, membrane blebbing, and highly condensed and fragmented nuclear structure. In the caspase-3 assay, HeLa S3 cells were seeded in 96-well plates and transfected with siRNA followed by treatment with drugs. Caspase activity was detected using *N*-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin) substrate as described previously (12). Fluorescence measurement was performed using CytoFluor Series 400 from PerSeptive Biosystems (Framingham, MA). Changes of fluorescence units/hour were used to determine the amount of caspase activity.

MTS Assay. HeLa S3 cells were seeded in 96-well plates and transfected with Chk1 siRNA or control siRNA. Eight h after transfection, the cells were treated with or without



doxorubicin for 48 h. After treatment, MTS reagents that measure the amount of live cells (Promega, Madison, WI) were added to the cells and allowed to develop for 20 min to 2 h. Colorimetric measurement was taken at 490 nm on Spectra MAX 190 from Molecular Device (Sunnyvale, CA).

Results

Chk1 Has Little Effect on Apoptosis and the Cell Cycle in the Absence of DNA-damaging Agents. To determine whether down-regulation of Chk1 expression would affect apoptosis and cell cycle in somatic cells, we transfected Chk1 siRNA into H1299 cells for 24 and 48 h before they were harvested and processed for Western blot and FACS analysis. FACS analysis (Fig. 1A) showed that down-regulation of the Chk1 protein (Fig. 1B) produced the same cell cycle profile as the control siRNA samples, with no increase of apoptotic cells up to 48 h in three independent experiments. Extension to 72 h (data not shown) showed the same profiles as those at the 24 and 48 h time points. These observations indicate that Chk1 is dispensable for cell survival and the control of regular cell cycle progression in somatic cells.

Chk1 siRNA Abrogates the G₂ Checkpoint and Enhances Apoptosis Induced by DNA-damaging Agents. To determine whether Chk1 siRNA, which has no apparent toxicity in the absence of DNA-damaging agents, can enhance the efficacy of doxorubicin, we transfected H1299 cells with Chk1 siRNA and then treated them with doxorubicin for 48 h before they were harvested for FACS analysis. Only scr-transfected samples were used as control because they were the same as mock-transfected samples (data not shown). Doxorubicin at 250 and 500 nM resulted in a dose-

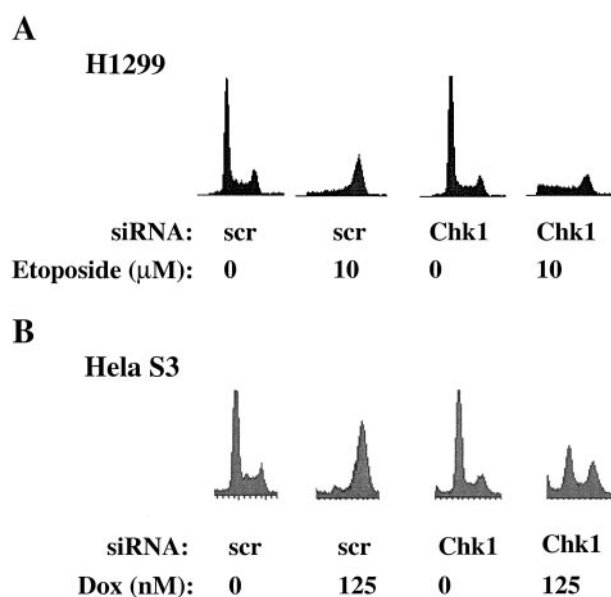


Fig. 3. Chk1 siRNA with etoposide and HeLa S3 cells. *A*, FACS analysis shows the profiles for H1299 cells after 48-h treatment with etoposide. *B*, cell cycle profiles show HeLa S3 cells transfected with Chk1 or scr siRNA and treated with doxorubicin for 48 h.

dependent increase of G₂ arrest with a minimal amount of apoptosis in H1299 cells (Fig. 2A). In the presence of Chk1 siRNA, doxorubicin treatment failed to arrest the cells at the G₂ checkpoint and resulted in more than double the amount of apoptotic cells. Microscopic examination confirmed that the sub-G₀-G₁ population detected in the FACS samples

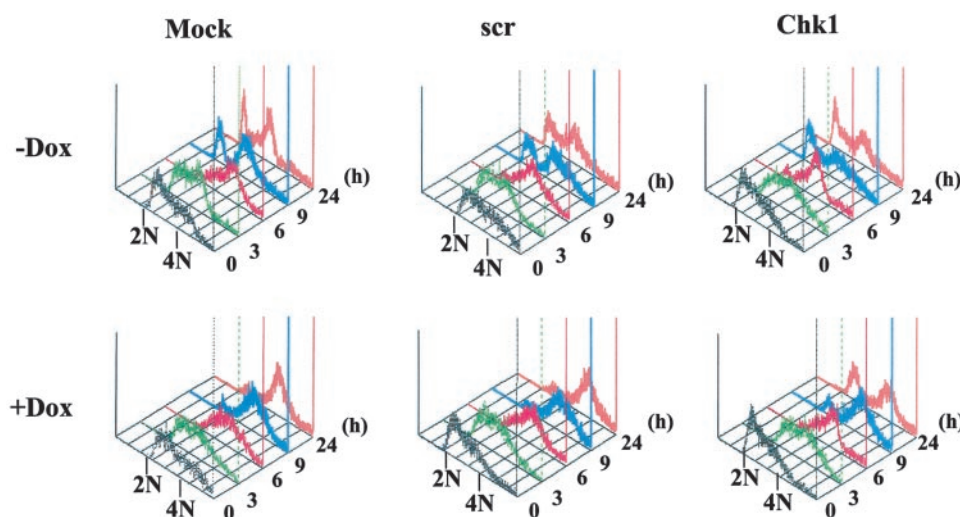


Fig. 4. Chk1 siRNA and cell cycle kinetics. H1299 cells were transfected with siRNA and pulse-labeled with BrdUrd followed by treatment of doxorubicin. The cells were analyzed by FACS analysis at 0, 3, 6, 9, and 24 h time points. DNA content of BrdUrd-positive cells was analyzed and is indicated as 2N and 4N for each treatment. Cells transfected with Oligofectamine are shown under *Mock*. *scr* refers to cells transfected with scrambled siRNA. *Chk1* indicates cells transfected with Chk1 siRNA. The samples were treated with either vehicle ($-Dox$) or 500 nM doxorubicin ($+Dox$).

was due to the increase of apoptotic cells (Fig. 2B). These results showed that down-regulation of Chk1 expression was sufficient to augment the cytotoxic effect induced by doxorubicin.

The Essential Role of Chk1 Extends to Other DNA-damaging Agents and Different Cell Types. In addition to doxorubicin, we tested whether Chk1 siRNA also augments other DNA-damaging agents such as etoposide. A titration curve was used to select the concentration of etoposide at 10 μM that produces about 80% of G_2 cells with minimal apoptosis (results not shown). As shown in Fig. 3A, Chk1 siRNA abrogated the G_2 checkpoint and enhanced cell death induced by etoposide. This result suggested that the role of Chk1 might apply to a broad range of DNA-damaging agents.

To investigate the role of Chk1 in different cell types, we transfected Chk1 siRNA into another cell line, HeLa S3, and treated the cells with doxorubicin. As shown in Fig. 3B, Chk1 siRNA abrogated the G_2 checkpoint and enhanced apoptosis in the cells treated with doxorubicin. Chk1 siRNA did not increase apoptosis or alter the cell cycle in the somatic cells in the absence of the DNA-damaging agent.

Chk1 siRNA Shortens the G_2 Arrest. To examine the requirement of Chk1 for the observed G_2 arrest, we transfected H1299 cells with Chk1 siRNA and pulse-labeled the cells with BrdUrd. The cells were then treated with and without doxorubicin and harvested for FACS analysis at various time points. In the absence of doxorubicin, BrdUrd-labeled cells progressed to G_2 -M in 6 h and completed G_1 entry in 9 h (Fig. 4). Chk1 siRNA-treated samples showed the same kinetic profiles as the control samples without delay of cell cycle progression. These data further confirmed that Chk1 played very little, if any, role in the control of regular cell cycle in somatic cells.

In the presence of doxorubicin, accumulation of G_2 cells occurred 6 h after BrdUrd labeling, and cells remained in G_2 in the control samples throughout the 24 h period (Fig. 4). Cells treated with Chk1 siRNA also accumulated in G_2 in 6 h. However, the cells started to exit G_2 -M phase in 9 h and

produced a strong G_2 abrogation profile in 24 h. These data confirmed that Chk1 played a key role in the maintenance of the G_2 checkpoint under DNA stress conditions. Treatment of cells with Chk1 siRNA had a dominant effect, and inhibition of Chk1 alone was sufficient to induce exit of the cells from DNA damage-induced G_2 arrest.

Chk1 siRNA Augments Apoptosis and Inhibits Cell Growth in the Presence of Doxorubicin. To determine whether abrogation of the G_2 checkpoint and apoptosis could result in the enhancement of DNA damage-induced inhibition of cancer cell growth, we transfected HeLa S3 cells with Chk1 siRNA and treated the cells with or without doxorubicin. A typical titration curve in MTS assay showed that doxorubicin at 125 nM had about 5% inhibition of HeLa cell proliferation (data not shown). This concentration was used for the MTS and caspase assays. In the absence of the drug, Chk1 siRNA had very little inhibitory effect on cell growth (Fig. 5A) or activation of caspase activity (Fig. 5B). These results demonstrated that Chk1 was not important for cell growth and survival in the absence of the DNA-damaging agent. In the presence of the drug, Chk1 siRNA strongly augmented the inhibitory effect of doxorubicin and the activation of caspase activity. These results support the idea that down-regulation of Chk1 expression renders G_1 -defective cancer cells vulnerable to DNA-damaging agents.

Discussion

Chk1 is potentially a very good anticancer target, providing that inhibition of Chk1 does not increase somatic cell death and control of the cell cycle. Otherwise, inhibition of Chk1 alone may lead to cellular toxicity and provide little cancer cell selectivity. Data on embryonic lethality in Chk1-null mice (10), apoptosis in Chk1-deficient embryonic stem cells (2), and the lack of germ-line mutation of Chk1 in humans (13) have firmly established the crucial role of Chk1 in mammalian development. In somatic cells, on the other hand, the role of Chk1 in survival has been uncertain. Early Chk1 inhibitors such as UCN-01 (5) and SB-218078 (6) induced apoptosis in

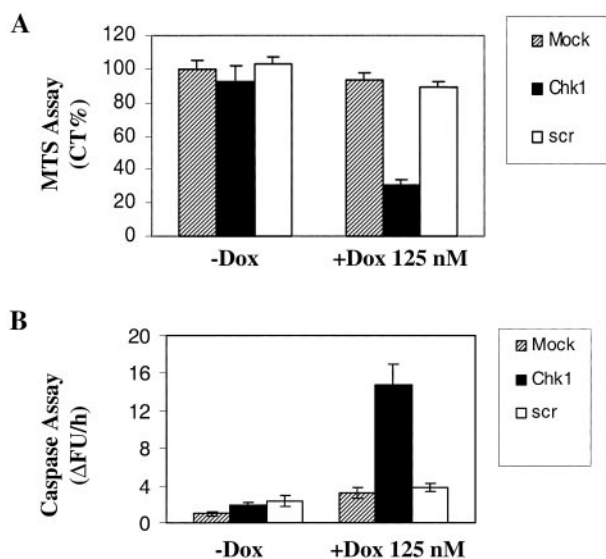


Fig. 5. Effects of Chk1 siRNA on cell growth and apoptosis. HeLa S3 cells were transfected with Oligofectamine (hatched bars), control siRNA (white bars), or Chk1 siRNA (black bars) and treated with vehicle (–Dox) or doxorubicin (+Dox). **A**, MTS measurements are shown as a percentage of the mock control (CT%) in the absence of doxorubicin. **B**, caspase activity assay is shown as changes of fluorescence units/hour (Δ FU/h) to determine the occurrence of apoptosis in the treated samples.

somatic cells. These observations raised the possibility that Chk1 might also control somatic cell survival. However, the specificity of these compounds deeply complicated the interpretation of the results. A previous report showed that Chk1 antisense induced significant apoptosis in H1299 cells, suggesting that Chk1 played a role in survival (14). The experiments using siRNA in the same cell line have not been able to substantiate the finding. It is known that some antisense DNA may have nonspecific effects independent of the target sequence. The siRNA data here clearly demonstrate that the major role of Chk1 is to control DNA damage-induced checkpoints in somatic cells. Regular cell cycle progression and survival are independent of Chk1.

One likely concern is that the siRNA data only define the outcome from short-term experiments. In this context, it is noteworthy that apoptosis induced by the Chk1 inhibitors was detected in short-term assays. Similar conditions were used to detect apoptosis in the Chk1 antisense studies. Moreover, programmed cell death occurred in one cycle in embryonic stem cells with conditional knockout of Chk1 (2). The siRNA results here are consistent with several previous reports using different approaches. Studies using Chk1 substrate peptide showed that inhibition of Chk1 caused little apoptosis (15). In a long-term Chk1 inhibitory environment, a group reported that stable clones of HeLa cells expressing Chk1 dominant negative proteins survived well in the absence of DNA-damaging agents (4). After the first submission of this manuscript, a group using somatic gene targeting reported data on a Chk1-null chicken lymphoblast cell line and reached the same conclusions as our observations in the short-term Chk1 knockout experiments (16). These results show that, unlike in embryonic development, Chk1 is dispensable for somatic cell survival.

Another issue regarding Chk1 as a target is whether inhibition of Chk1 is sufficient to abrogate DNA damage-induced checkpoints in the presence of other potentially redundant cell cycle regulators such as Chk2. Gene targeting experiments have established Chk1 as the dominant regulator for the checkpoints in embryonic cells (2, 10). In somatic cells, various reports using other Chk1 pathway-interfering methods also suggested the dominant role of Chk1 in the control of the checkpoints (3, 4, 6, 14, 17, 18). The findings from this study using Chk1-specific siRNA reinforce the concept that inhibition of Chk1 is sufficient to abrogate the DNA damage checkpoints. The siRNA data bear a close resemblance to Chk1 inhibitors and, consequently, have significant implication in rationale design of Chk1 mechanism-based anticancer drugs. A specific Chk1 inhibitor, therefore, is expected to show little effect on cell cycle progression and survival in the absence of DNA-damaging agents. In the presence of DNA-damaging agents, Chk1 inhibitors will abrogate the checkpoints and augment apoptosis.

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