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Research Paper

Knockdown of Chk1, Wee1 and Myt1 by RNA Interference Abrogates G_2 Checkpoint and Induces Apoptosis

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KEY WORDS

Wee1, Chk1, Myt1, siRNA, G_2 checkpoint, apoptosis, p53, and DNA damage

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ABSTRACT

Mammalian cells undergo cell cycle arrest in response to DNA damage due to the existence of multiple checkpoint response mechanisms. One such checkpoint pathway operating at the G_1 phase is frequently lost in cancer cells due to mutation of the p53 tumor suppressor gene. However, cancer cells often arrest at the G_2 phase upon DNA damage, due to activation of another checkpoint pathway that prevents the activation of Cdc2 kinase. The kinases, Chk1, Wee1, and Myt1 are key regulators of this G_2 checkpoint, which act directly or indirectly to inhibit Cdc2 activity. Here we show that RNA interference (RNAi)-mediated downregulation of Wee1 kinase abrogated an Adriamycin®-induced G_2 checkpoint in human cervical carcinoma Hela cells that are defective in G_1 checkpoint response. Wee1 downregulation sensitized HeLa cells to Adriamycin®-induced apoptosis. Downregulation of Chk1 kinase in Hela cells also caused a significant amount of cell death independent of DNA damage. In contrast, Myt1 downregulation abrogated Adriamycin®-induced G_2 arrest but did not cause substantial apoptosis. Reduction in Wee1, Chk1, or Myt1 levels did not sensitize normal human mammary epithelial cells (HMEC) cells to Adriamycin®-induced apoptosis unlike the situation in Hela cells. Our study reveals distinct roles for Chk1, Wee1, and Myt1 in G_2 checkpoint regulation. The data reported here support the attractiveness of Wee1 and Chk1 as molecular targets for abrogating the G_2 DNA damage checkpoint arrest, a situation that may selectively sensitize p53-deficient tumor cells to radiation or chemotherapy treatment.

INTRODUCTION

The cell division cycle is a tightly regulated set of events that ensures proper duplication of DNA and segregation of genetic materials. Progression of the cell cycle is controlled by a series of cyclin-dependent kinases (CDKs), which in turn are tightly regulated by several mechanisms. First, CDK (CDK1, 2, 4, 6) activity requires binding to a regulatory subunit, cyclin (cyclin A, B, D, E, H). Cyclins are expressed only at specific points during the cell cycle. Second, CDK activity is directly regulated through phosphorylation-mediated mechanisms. Third, CDKs are negatively regulated through specific interaction with proteins such as p14 (INK4a), p15 (INK4b), $p21^{CIP1}$, and $p27^{Kip2}$; collectively know as CDK inhibitors (CDIs). Thus, CDK activity, in response to specific signals, is regulated by modulation of cyclin levels, CDK phosphorylation, and CDI levels.[1-3]

When normal cells are subjected to DNA damage, cell cycle checkpoint pathways are activated that can arrest cells in both the G_1 and G_2 phases. Checkpoint arrest allows cells to repair damaged DNA in order to ensure maintenance of genomic integrity. Without these checkpoints, cells could accumulate undesired mutations, which could eventually lead to unregulated cell growth, cancer, or death.[4] Indeed, most cancer cells are defective at the G_1 checkpoint control due to nonfunctional p53/Rb pathways.[1] In addition to its role in checkpoint control, p53 also regulates programmed cell death. p53 can initiate apoptosis by transcriptionally activating proapoptotic Bcl-2 family members and repressing antiapoptotic Bcl-2 proteins. p53 deficient tumor cells therefore are more resistant to apoptosis than normal cells. One potential therapeutic approach is to exploit this G_1 checkpoint deficiency by abrogating the functional G_2 checkpoint in tumor cells to enhance their apoptotic response to DNA damage. Combination of DNA damage and G_2 checkpoint abrogators is likely to be selective for tumor cells, since DNA damage in the absence of a G_2 checkpoint could be more harmful to G_1 checkpoint-deficient cancer cells than to normal cells, which still have a functional G_1 checkpoint response. Indeed, small molecular weight G_2 checkpoint abrogators, such as caffeine and UCN-01, have shown promising results in sensitizing p53-deficient cells to apoptosis.[5,6]

The CDK responsible for the G_2/M transition is Cdc2.[7] In addition to regulation through its association with cyclin B, both activating and inactivating phosphorylations are required to modulate Cdc2 activity. In mammalian cells, the activating phosphorylation of Cdc2 at threonine 161 (T161) in the T-loop is catalyzed by Cdc2 activating kinase, (CAK; Cdc7/cyclinH) and CAK activity is quite constant throughout the cell cycle.[8] The inhibitory phosphorylations of threonine 14 (T14) and tyrosine 15 (Y15) at the ATP-binding pocket are mediated by Wee1 and Myt1 kinases.[9-13] Wee1 is the major kinase phosphorylating the Y15 site. While Myt1 preferentially phosphorylates the T14 site, it can also phosphorylate the Y15 site. At the onset of mitosis, inhibitory phosphates at T14 and Y15 sites of Cdc2 are removed by the dual-specific phosphatase Cdc25C[14-16] triggering Cdc2 activation and progression of mitotic events.

Most cancer cells have lost their G_1 checkpoint and therefore only arrest at the G_2 phase of the cell cycle when subjected to DNA damage. This G_2 arrest correlates with an increase of Cdc2 inhibitory phosphorylation at the T14 and Y15 sites.[17] Upon DNA damage, the PI3K-like protein kinases, ataxia-telangiectasia-mutated (ATM) and the ATM- and Rad3-related protein kinase (ATR) are activated.[18-22] Activated ATM/ATR kinases subsequently phosphorylate and activate Chk1/Chk2 kinases.[23-26] Both Chk1 and Chk2 are known to phosphorylate Cdc25C on serine 216 (S216) and this phosphorylation makes Cdc25C functionally inactive.[23,27] In addition, Chk1 has also been reported to phosphorylate and increase the kinase activity of Wee1.[28-31] Thus either Cdc25C inactivation and/or Wee1/Myt1 activation could contribute to G_2 cell cycle arrest in response to DNA damage. Mutations of both the T14 and Y15 sites of Cdc2 to alanine and phenylalanine respectively, lead to an activated form of Cdc2 that cannot be inhibited by phosphorylation. Expression of this constitutively active Cdc2 mutant causes premature mitosis even in the presence of damaged DNA.[32-34] Inhibition of Wee1, Myt1 and Chk1 can directly or indirectly reduce phosphorylation of Cdc2 on the T14 and Y15 sites and cause premature mitosis due to abrogation of the G_2 checkpoint. Therefore, Wee1, Myt1 and Chk1 could be envisioned as potential target(s) for cancer therapeutic intervention. It is noteworthy that small molecular G_2 checkpoint abrogators, such as UCN-01 and caffeine, are now known to inhibit certain checkpoint regulatory kinases, although they are not truly selective for these kinase targets. For example, UCN-01 was first identified as protein kinase C (PKC) inhibitor but later found to inhibit both Chk1 and Chk2.[35,36] Caffeine, on the other hand, can inhibit ATM/ATR, which are upstream regulators of Chk1 and Chk2 kinases.[37,38]

Here, we have used an RNA interference (RNAi)-mediated gene knockdown approach to investigate the comparative roles of Wee1, Chk1, and Myt1 kinases in the G_2 checkpoint pathway in Hela versus normal human mammary epithelial cells (HMEC), which lack or express functional p53 alleles, respectively. Our data suggest that downregulation of Wee1 sensitizes Hela cells to DNA damaging-induced apoptosis. Downregulation of Chk1 is lethal in Hela cells independent of DNA damage but not in HMEC cells. While downregulation of Myt1 was found to abrogate the Adriamycin® induced G_2 checkpoint, no appreciable apoptosis was observed as occurred in the case of Chk1 or Wee1 downregulation.

MATERIALS AND METHODS

Cell Culture and siRNA Transfection. Hela cells were grown in DMEM medium supplemented with 10% FBS and 20 μg/ml gentamicin. Human mammary epithelial cells (HMEC) cells were purchased from Clonetics (CC-0228) and grown in MEG medium as recommended. Transfections were carried out by plating 10,000-20,000 cells per well on 12-well plates or 100,000 cells per 10-cm dish. Invitrogen's oligofectamine reagent was used for transfection of siRNA duplexes. For each transfection, 0.1 nmol of each siRNA and 2 μl Oligofectamine (12-well plates) and 0.6 nmol of each siRNA and 9 μl Oligofectamine (10-cm dishes) were used following protocols suggested by the manufacturer.

SiRNA Oligos and RT-PCR. The siRNA duplex oligos were purchased from Dharmacon. The sense strand sequence of the oligos employed in the study are as follows: Chk1 oligo#1, aagcagucgcagugaagauug; Chk1 oligo#2, aaccagaugcucagagauucu; Wee1 oligo#1, aaugauuccuguggugaagac; Wee1 oligo#2, aacguauuggaaugauuccug; Wee1 oligo#3, aacuccgggguaguucucucu; Myt1 oligo#1, aaccuggauucucccucaaga; Myt1 oligo#2, aagcguuccaugucaccauuc; control oligo, aaagcccagccuucgaucaac. The control oligo was designed using a random sequence bearing no significant homology to any published gene or EST sequence as tested by BLAST analysis. Hela cells were harvested two days after siRNA transfection and total RNA was prepared with Invitrogen's Trazol method (Invitrogen, Cat#15596-026). Semi-quantitative RT-PCRs were performed using Ambion's QuantumRNA kit (Cat# 1716) with 18S as internal standards. PCR primers for Chk1 were: forward-gatatgaagcgtgccgtagac, reverse-ggctgagaactggagtacttc; for Wee1 were: forward-gaagcagagttgaaggatctc, reverse-cttcctcttgtcacagtgttc; and for Myt1 were: forward-gtccttcttccagcagagctt, reverse-cagcatcatgacaaggacaga. The primers were custom synthesized at GibcoBRL.

Antibodies and Immunoblots. A polyclonal antibody against Cdc2 (sc-747) and monoclonal antibodies against Chk1 (sc-8408), Wee1 (sc-5285), and vimentin (sc-6260) were purchased from Santa Cruz. A phospho-specific antibody against Cdc2Yp15 and antibodies against Myt1 were obtained from Onyx Pharmaceuticals.[13,31,39] For immunoblot analysis, attached Hela cells were harvested by addition of 10 mM EDTA in PBS for 5 min. Both floating and attached cells were combined and centrifuged at 2,000 rpm for 5 min to collect the cell pellet. For HMEC cells, 0.25-mg/ml trypsin (Clonetics, cc-5012) was used to remove attached cells from the cell culture dish. Both floating and attached cells were combined in the same manner as described above for Hela cells. Cell pellets were incubated in 50-to100 μl ice-cold lysis buffer [50 mM Hepes, pH7.4, 250 mM NaCl, 0.1% NP-40, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM EDTA, 0.1 mM Na_3VO_4, 40 μl/ml complete proteinase cocktail (Roche, Cat# 1697498)] for 30 min on ice. Cell lysates were centrifuged at 10,000 rpm for 5 min to collect the cell supernatants.

Clonogenic Cell Growth. Hela cells (10,000 cells/well) were plated on 12-well plates and transfected as described above. One day after transfection, cells were trypsinized and 1/20 of the total cell population was plated into individual wells of a 6-well plate and grown for 10 days in DMEM with 10% FBS and 20 μg/ml gentamicin. Cells were then stained with 0.25% commassie blue in 10% acetic acid and 40% methanol for 20 min. After staining, plates were washed three times with water and air-dried.

Cell Counting. Cells were transfected with the indicated siRNAs. Two days after transfection, cells were treated with 250 nM Adriamycin®. Cells were grown for an additional period of two days (Hela cells) or 10 days (HMEC cells) after addition of Adriamycin®. Both floating and attached cells were then collected separately and counted on a Coulter Counter.

Apoptosis Assays. Apoptotic DNA fragmentation was quantitated by an ELISA assay. Hela cells were first plated on 12-well plates and transfected with indicated siRNAs as described above. Transfected cells were trypsinized the next day and replated after diluting 4-fold into 96-well plates. Two days post-transfection, i.e, 1 day after plating on 96-well plates, the cells were treated with 250 nM Adriamycin® in dimethyl sulfoxide (DMSO) or mock-treated with DMSO alone. Eighteen hours after DNA damage, cells were analyzed for apoptosis using the cell death detection ELISA plus (Roche, Cat# 1 774 425). Cells were also analyzed by a flow cytometry-based assay using an annexin V-PE apoptosis detection kit (BD PharMingen, Cat# 559763). This assay detects cells in the early stages of apoptosis through specific binding of labeled annexin V protein to the externalized phosphatidylserine molecules

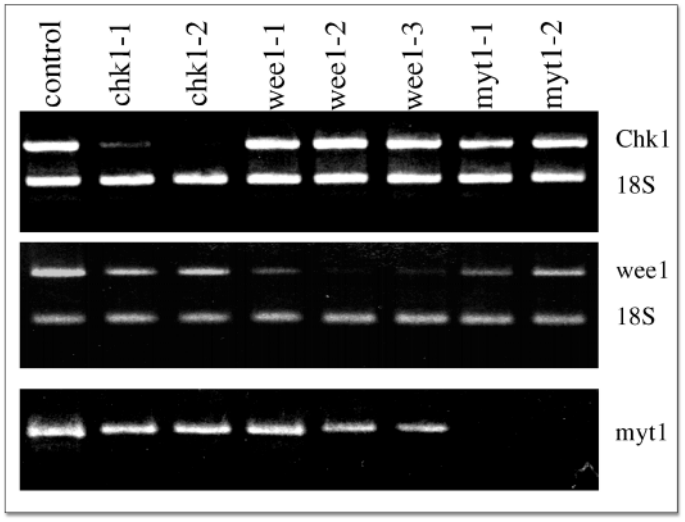

Figure 1. Detection of human Wee1, Chk1, and Myt1 RNA expression in siRNA-treated Hela cells by semi-quantitative RT-PCR. Hela cells were transfected with control or the indicated siRNA in 12-well plates. Two days after transfection, cells were harvested, and total RNA was prepared with GibcoBRL's trazol method. Semi-quantitative RT-PCR was then performed to measure the amount of target downregulation with 18S as internal control.

on the outer leaflet of the plasma membrane in unfixed apoptotic cells. For this assay, Hela cells in 100-mm dishes were transfected with the indicated siRNA duplexes and two days after transfection, cells were treated with Adriamycin® for 18 hrs. Cells were harvested in 10 mM EDTA in PBS for 5 min. Both floating and attached cells were combined prior to cell staining with the annexin V-PE kit as per manufacturer's protocol.

Cell Cycle Analysis. Hela cells were plated, transfected, and treated as for the annexin V-PE apoptosis assay. Adherent cells were collected from tissue culture dishes by treatment with 10 mM EDTA in PBS for 5 min. Cell pellets were resuspended in 0.5 ml PBS with 0.1% FBS and dropwise added to 75% ice-cold ethanol overnight. The fixed cells were then centrifuged at 2,000 rpm for 5 min, washed once in PBS containing 0.1% FBS, and then treated with 0.5 ml PBS containing 0.2 mg/ml RNAse A and 50 µg/ml propidium iodide (PI) for 30 min at 37°C. Cells were passed through a 5 ml strainer-capped tubes and cell cycle distribution was analyzed as previously described[31] with the modification of using a BD LSR flowcytometer.

RESULTS

Downregulation of Wee1, Chk1 and Myt1 by siRNA. In order to knockdown Wee1 expression, three pairs of siRNA based on the human Wee1 sequence were designed and synthesized. The siRNAs were first individually transfected into HeLa cells using Invitrogen's oligofactamine reagent. As shown in Figure 1, all three Wee1 siRNAs significantly reduced Wee1 mRNA as measured by semi-quantitative RT-PCR. Similar experiments with Myt1 and Chk1 specific siRNA also yielded suppression of the respective mRNA levels. Of the two Chk1 siRNAs tested, the Chk1#2 oligo proved to be more effective than the Chk1#1oligo in reducing Chk1 mRNA levels (Fig. 1). Chk1#2 siRNA was used for all subsequent experiments. The two Myt1 siRNAs appeared equally effective in eliminating Myt1 specific mRNA. In fact, no Myt1 mRNA was detected in cells transfected with either of the specific siRNA duplexes (Fig. 1) and therefore we arbitrarily chose the Myt1#2 siRNA for subsequent experiments.

The effects of siRNA treatment on respective protein levels were evaluated by immunoblots. Although the reduction at the mRNA level was significant with each individual Wee1 siRNA (Fig. 1), the reductions in Wee1 protein levels were incomplete (data not shown). In an attempt to achieve a more complete reduction of Wee1 protein level, we tested the combination of all

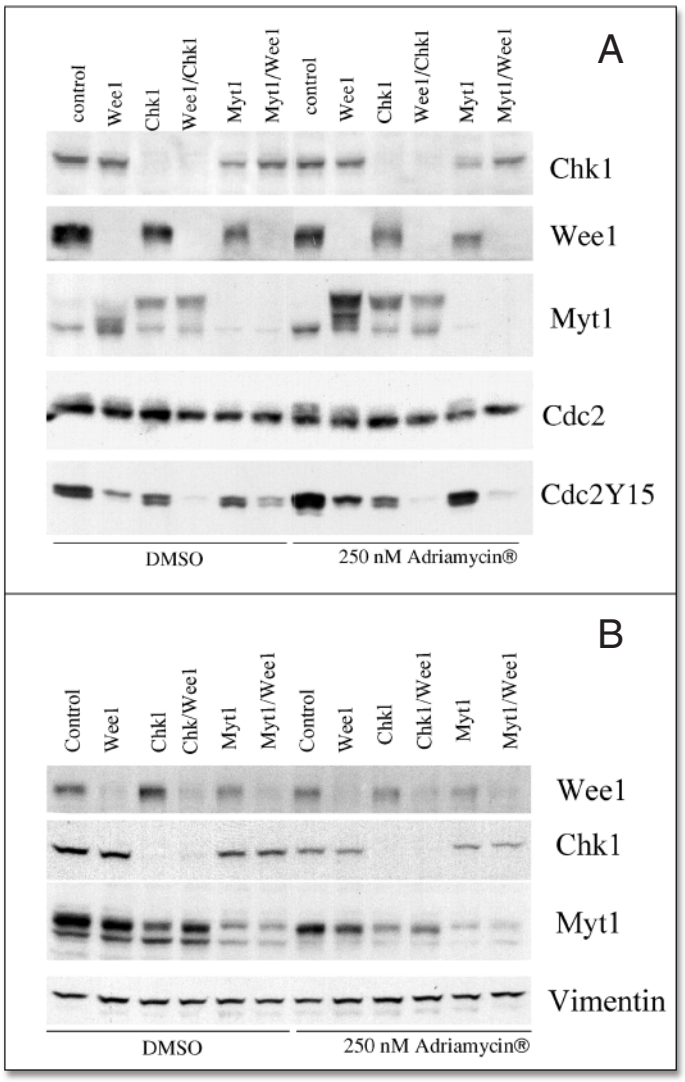

Figure 2. Effect of DNA damage and Wee1, Chk1, or Myt1 downregulation on Cdc2-Y15 phosphorylation. Cells were transfected with the indicated siRNA using oligofectamine reagent as described in the material and method section. Two days after transfection, cells were treated with 250 nM Adriamycin® overnight. The cells were then harvested for immunoblotting with the indicated antibodies. (A) Hela cells (B) HMEC cells.

three Wee1 siRNAs. Immunobloting showed that Wee1 protein was consistently reduced by over 90% when a combination of three Wee1 siRNAs were transfected together (Fig. 2A). Therefore, a pool of these three siRNAs was used for subsequent experiments. As shown in Figure 2, expression of the Chk1, Wee1, and Myt1 proteins were specifically reduced by their corresponding siRNA duplexes. Among the seven siRNAs tested in the above experiment, greater than 90% downregulation of the target protein was achieved by all siRNAs either singly or in combination. Similar results were also obtained with primary human mammary epithelial cells (Fig. 2B). Reduction of Wee1, Myt1, and Chk1 protein lasted for a minimum of 5 days post-transfection in Hela cells (data not shown).

Knockdown of Wee1, Myt1 and Chk1 Reduced Cdc2-Y15 Phosphorylation in Hela Cells. We observed that treatment with Chk1 siRNA and to a lesser extent with Wee1 siRNA caused a dramatic mobility shift of Myt1 (Fig. 2A). Slower migration of Myt1 on SDS-PAGE was reported to be associated with mitosis.[13] These observations indicate that treatments of Wee1 and Chk1 siRNA altered the cell cycle distribution of Hela cells. Since Wee1 and Myt1 directly phosphorylate Cdc2 on Y15 and

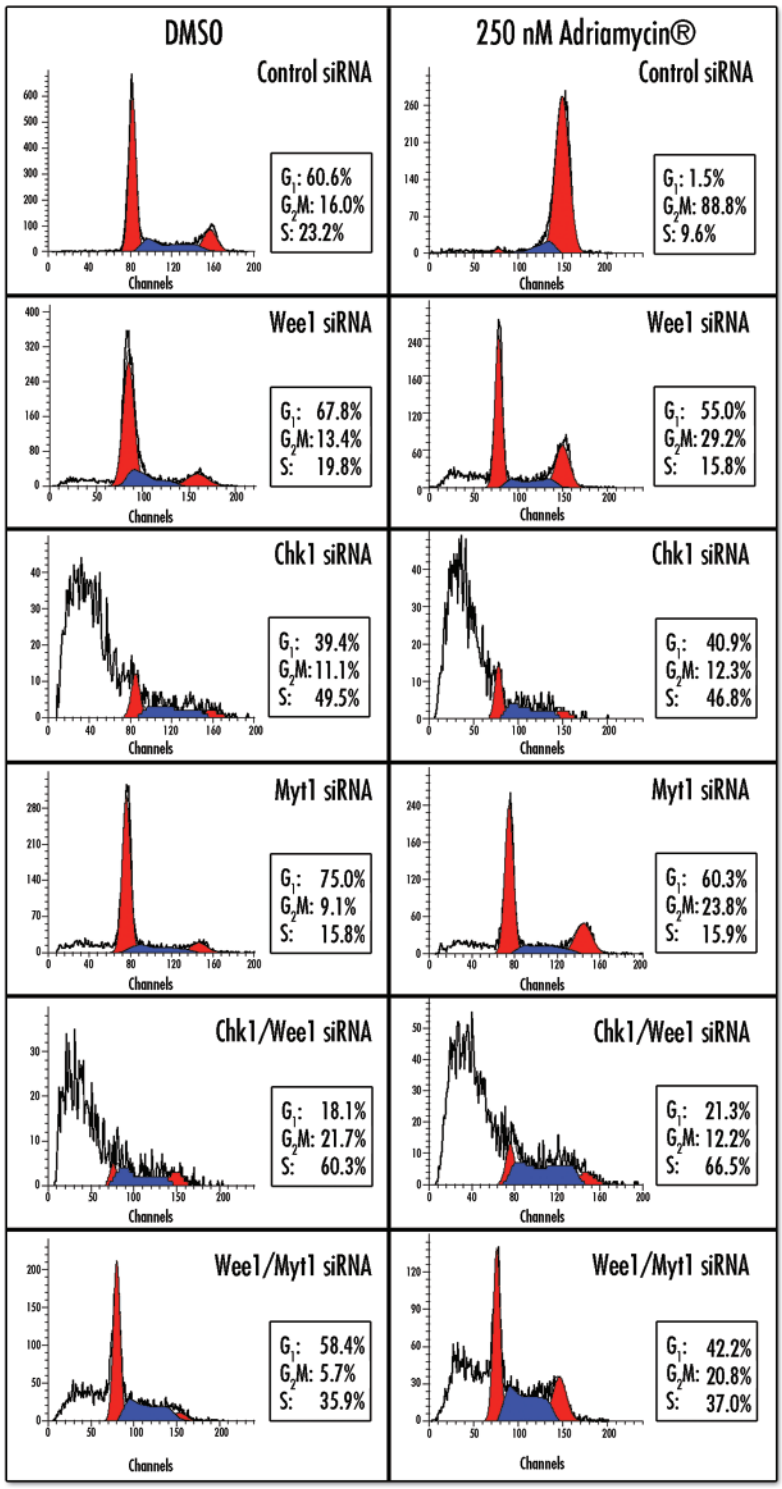

Figure 3. Effects of Wee1, Chk1, and Myt1 siRNAs on Hela cell cycle distribution and the preG_1 peak in the presence or absence of Adriamycin® treatment. Hela cells were plated in 10 cm dishs. Two days after transfection, the cells were treated with 250 nM Adriamycin® or a corresponding volume of DMSO. The cells were then harvested for cell cycle analysis 18 hrs after Adriamycin® treatment.

T14 sites and Chk1 indirectly affects Cdc2 phosphorylation, the status of Cdc2 phosphorylation in response to the knockdown of Wee1, Myt1, and Chk1 was evaluated. The Cdc2-Y15 phosphorylation was reduced by Wee1, Myt1, or Chk1 knockdown in Hela cells, while Wee1 knockdown was by far the most efficient in producing this effect (Fig. 2A). Furthermore, a combination of Wee1 and Chk1 siRNA completely eliminated the Cdc2-Y15 phosphorylation, but a combination of Wee1 and Myt1 did not generate a synergistic decrease of the Cdc2-Y15 phosphorylation. In contrast, Myt1 downregulation significantly reduced Cdc2-T14 phosphorylation (data not shown). These results are consistent with the current model that Wee1 (directly) and Chk1 (indirectly, via its action on Cdc25), modulate the phosphorylation of Cdc2-Y15. In contrast, Myt1 mainly regulates the phosphorylation of Cdc2-T14 and has a less important role in the phosphorylation of Cdc2-Y15.

Upon DNA damage induced by treatment of HeLa cells with 250 nM Adriamycin®, the Cdc2-Y15 phosphorylation increased in control siRNA-transfected cells as anticipated due to the G_2 arrest (Fig. 2A). Chk1 knockdown blocked the Adriamycin®-induced increase of Cdc2-Y15 phosphorylation although the basal Cdc2- Y15 phosphoryaltion levels were modestly decreased by Chk1 knockdown. Simultaneous knockdown of Wee1 and Chk1 expression completely eliminated both the basal and the Adriamycin®-induced phosphorylation of Cdc2-Y15 (Fig. 2A). These results demonstrate that Chk1 plays an essential role in response of Hela cells to DNA damage.

Interestingly, knockdown of Wee1 expression partially blocked the increase of Cdc2-Y15 phosphorylation due to Adriamycin® treatment although the basal levels of Cdc2-Y15 phosphorylation were significantly lower when compared to control cells (Fig. 2A). Similarly, Myt1 knockdown did not block the increase of Cdc2-Y15 phosphorylation in response to Adriamycin®, although it lowered the basal phosphorylation of Cdc2-Y15 to an extent similar to that observed with Chk1 knockdown. A double knockdown of Wee1 and Myt1 expression completely prevented the increase of Cdc2-Y15 phosphorylation by Adriamycin®. These data suggest that Wee1 and Myt1 have a partially redundant role in the cellular response to Adriamycin®. Our results clearly indicate distinct roles for Chk1, Wee1, and Myt1 in regulation of Cdc2-Y15 phosphoryaltion in response to the DNA damaging agent Adriamycin®.

Our attempts to study the phosphorylation status of Cdc2-Y15 in HMEC cells yielded inconclusive results due to weak signals on Cdc2-Y15 immunoblots (Fig. 2B; data not shown). Collectively, our data demonstrate that downregulation of Wee1, Chk1, and Myt1 can reduce Cdc2-Y15 phosphorylation to various degrees while the total Cdc2 expression is not changed by any RNAi in Hela cells. Downregulation of Wee1, the major kinase that phosphorylates Cdc2 on Y15, produces the most significant reduction of Cdc2-Y15 phosphorylation.

Downregulation of Wee1, Chk1, and Myt1 Abrogates the G_2 Checkpoint in Hela Cells. Since Wee1, Chk1, and Myt1 all play roles at the G_2/M transition, the effects of their downregulation on cell cycle distribution were analyzed by flow cytometry. As shown in Figure 3, both Wee1 and Myt1 siRNA abrogated Adriamycin®-induced G_2 arrest. In control siRNA treated cells, as expected the population of G_1 cells decreased from 60.6% to 1.5% upon Adriamycin® treatment. However, in Wee1 and Myt1 siRNA treated cells, the G_1 population remained at 55% and 60%, respectively, even in the presence of Adriamycin®. The effect of Chk1 on cell cycle distribution was difficult to evaluate since Chk1 siRNA treatment resulted in a large population of cells with less than G_1 DNA content (Fig. 3).

It is worth noting that Wee1 siRNA treatment increased the amount of cells with DNA content less than G_1, the apoptotic cell population, in the presence of Adriamycin®. Furthermore, the combination of Wee1 and Myt1 siRNA increased the frequency of apoptosis under basal conditions and further increased the fraction of apoptotic cells in response to Adriamycin® treatment. These flow cytometry analysis data are generally in agreement

Table 1 **EFFECT OF siRNA TREATMENT ON CELL ATTACHMENT**

Treatment	Control	Wee1	Chk1	Chk1/Wee1	Myt1	Myt1/Wee1
DMSO	94.3	90.6	32.4	40.9	82.3	73.3
Adriamycin	86.6	50.1	16.7	21.4	56.5	43.3
DMSO	86	81.9	81	79	78.9	73.2
Adriamycin	63.3	67.4	64.7	64.1	56	62.4

Hela or HMEC cells were transfected with the indicated siRNA as described in Materials and Methods. Two days after transfection, cells were treated with 250 nM Adriamycin® or DMSO. Two days after Adriamycin® treatment, both floating and attached Hela cells were harvested separately and counted with a Coulter counter. Total cells were calculated from the combination of floating cells and attached cells. The percentage of attached cells was expressed as the attached cells divided by the total cells. HMEC cells were harvested ten days after Adriamycin® treatment and counted the same as for Hela cells.

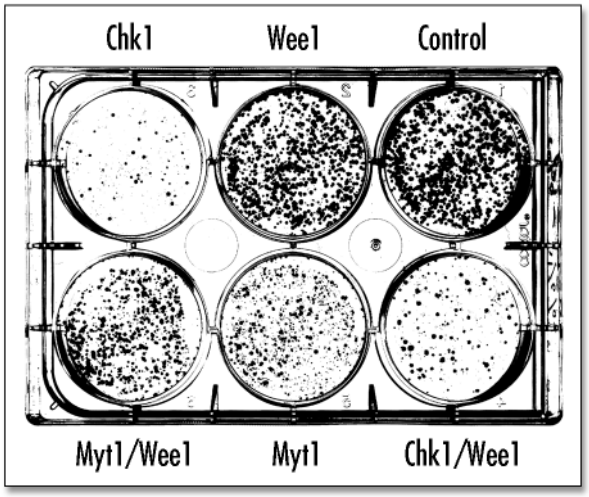

Figure 4. Effect of siRNA treatment on Hela cell colony formation. Hela cells were transfected with the indicated siRNA in 12-well plates. One day after transfection, cells were trypsinized and both floating and attached cells were combined. One twentieth of original cells were replated on 6-well plate. Cells were then grown for another 10 days before fixing and staining.

with results obtained by other apoptotic assays in Wee1 or Myt1 siRNA treated cells (Fig. 5). Chk1 siRNA transfected cells displayed a high percent of cells with DNA content less than G_1. These data are consistent with the notion that Chk1 is required for Hela cell survival. The size of the pre-G_1 peak increased for Wee1 transfected cells after DNA damage but not to an extent corresponding to the percentage of apoptosis shown in Figure 5C. This could be due to incomplete DNA degradation at an early stage of apoptosis, such that the DNA content of the apoptotic cells was not significantly less than the G_1 population.[40]

Abrogation of the G_2 checkpoint by knockdown of Wee1 and Myt1 is consistent with decreased phosphorylation of Cdc2-Y15 in the presence of Adriamycin® (Fig. 2A). However, we were surprised to find that Myt1 downregulation abrogated the G_2 checkpoint to the same extent as Wee1 siRNA, although Cdc2-Y15 phosphorylation is clearly higher in the Myt1 siRNA treated cells compared to that observed in the Wee1 siRNA treated cells. One possible explanation for this finding is that Myt1 siRNA activates Cdc2 to the same extent as the Wee1 siRNA because phosphorylation of T14 in Cdc2 is also blocked by Myt1, but not by Wee1 siRNA. Our data clearly establish that both Wee1 and Myt1 siRNA can abrogate the G_2 checkpoint in response to DNA damage in Hela cells.

Effects of Chk1 and Wee1 Downregulation on Apoptosis. The effect of siRNA on cell viability was investigated. We observed that Chk1 siRNA treatment significantly increased the number of floating cells (a 70% increase over the control) four days after transfection in Hela cells (Table 1). The floating cells were detected as early as two days after Chk1 siRNA treatment (data not shown). In contrast, floating cells were not detected in Chk1 siRNA treated HMEC cells although the Chk1 protein was effectively downregulated (Fig. 2B). The HMEC cells were monitored up to 12 days with no significant increase in floating cells. Wee1 siRNA produced little effect (5% floating cells over the control) while Myt1 siRNA also had a limited effect in Hela cells (12% over the control) (Table 1). We tested Adriamycin® at a concentration which did not induce massive cell death in Hela cells (8% over the control, Table 1). Adriamycin® further increased the incidence of floating cells in Chk1 siRNA treated cells. Interestingly Adriamycin® dramatically increased the incidence of floating cells in both Wee1 siRNA and Myt1 siRNA treated cells (40% and 26%, respectively over the control) (Table 1). These results demonstrate that Wee1 siRNA produces the most dramatic sensitization of Hela cells to Adriamycin® induced cell death. Similar experiments were performed with HMEC cells. Downregulation of Wee1, Chk1, and Myt1 by siRNA did not significantly sensitize HMEC cells to Adriamycin® treatment (Table 1).

To determine the long-term effects of suppression of G_2 regulators on cell growth, transfected Hela cells were grown in 6-well plates for 10 days as described by the clonogenic assay procedure in Materials and Methods. As shown in Figure 4, cells treated with Chk1 siRNA formed significantly fewer colonies than cells transfected with control siRNA. Myt1 siRNA treatment also resulted in fewer colonies compared to the control, but allowed more colony growth than Chk1 siRNA treatment. In contrast, transfection of Wee1 siRNA had a minor effect on colony formation. The colony-formation data correlate well with previously described data summarized in Table 1, further supporting the induction of cell death by Chk1 siRNA.

To investigate whether the observed cell death was due to apoptosis, two types of assays were conducted. In an ELISA assay designed to detect apoptotic DNA fragmentation, Wee1 or Myt1 suppression alone did not cause a significant incidence of apoptosis, whereas Chk1 downregulation triggered massive apoptosis (Fig. 5A). Wee1 downregulation significantly sensitized Hela cells to apoptosis in the presence of Adriamycin® (Fig. 5A and B). The above results were corroborated by a second assay based on annexin V-binding to membrane phosphatidylserines designed to detect early apoptotic cells. This experiment confirmed that Chk1 siRNA alone, in the absense of DNA damaging agent, induced apoptosis while Wee1 siRNA significantly sensitized Hela cells to apoptosis, but only in response to Adriamycin®. The extent of apoptosis in the Adriamycin® treated cell population cannot be accurately calculated due to a fluorescence signal shift in annexin-negative cells after Adriamycin® treatment. However, by comparison of control and Wee1 siRNA transfected cells in the absence or presence of Adriamycin® as reference, the percentage of apoptotic cells was estimated. Adriamycin® treatment increased the incidence of apoptosis in Wee1 siRNA transfected cells from 17% to 50%, compared to negligible increases in cells transfected with either Chk1 or Myt1 (68% to 73% and 24% to 26% respectively) (Fig. 5C).

DISCUSSION

The aim of this study was to compare the roles of the three G_2 checkpoint regulators, Wee1, Chk1, and Myt1, in regulation of the G_2 checkpoint by using an RNA interference-mediated gene knockdown approach. We were able to significantly reduce the expression of Wee1-, Chk1-, and Myt1- kinases as judged by semi-quantitative RT-PCR and immunoblots. Our data demonstrate that downregulation of Wee1 sensitizes Hela cells but not normal HMEC cells to apoptosis in response to Adriamycin®. Chk1 downregulation alone is lethal in Hela cells but not in normal HMEC cells in the absence of DNA damage. Myt1 knockdown can abrogate Adriamycin®-induced G_2 arrest, but does not lead to cell death.

Wee1 was first identified in *S. pombe* from a genetic screen for wee mutants that undergo mitosis and cell division at a reduced size compared with wild type.[41,42] It encodes a protein kinase that phosphorylates

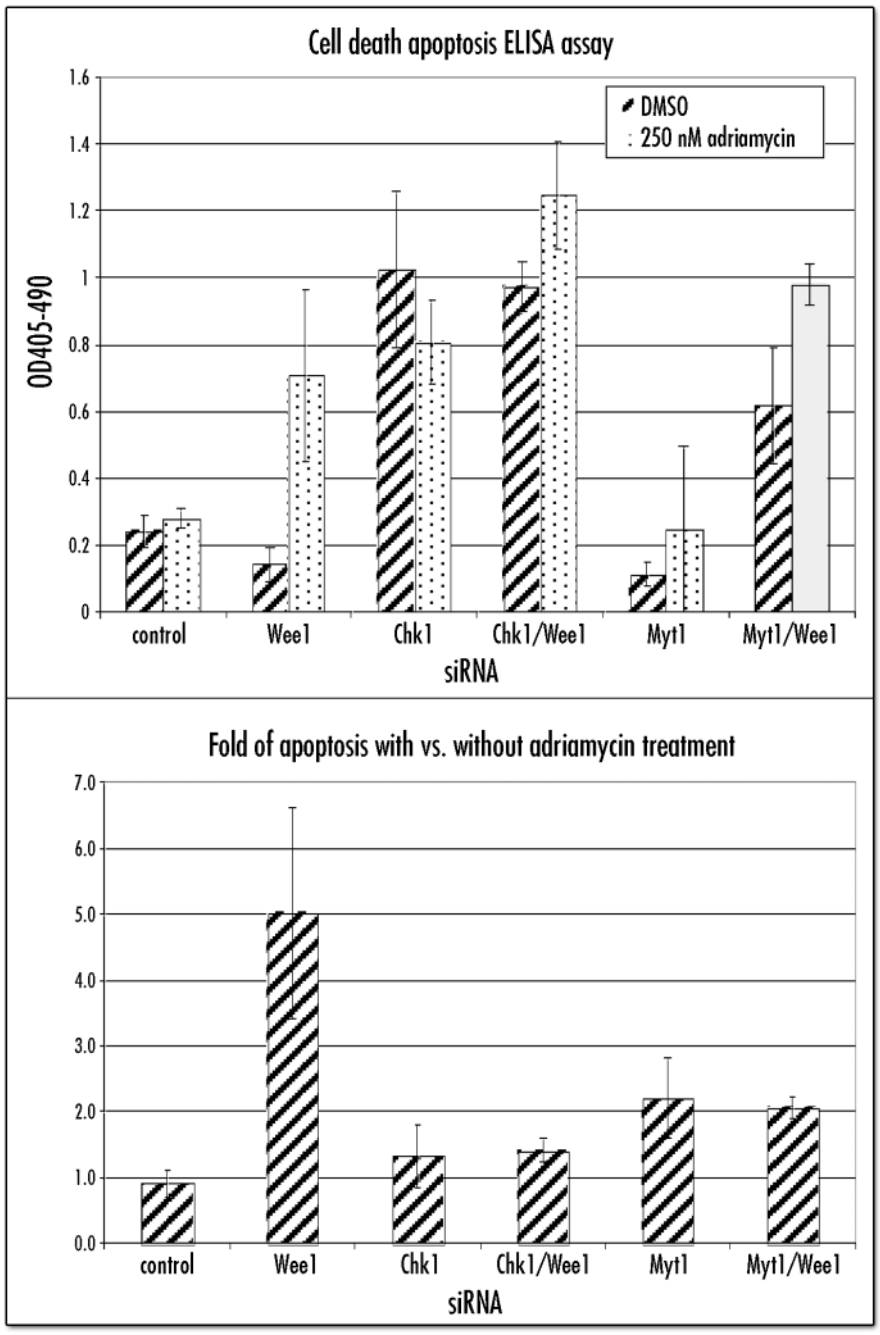

Figure 5A and B. Wee1 downregulation sensitizes Adriamycin® induced apoptosis in Hela cells. (A) Apoptosis detection by measurment of DNA fragmentation in 96-well plates. Each OD reading is an average of duplicate 96-well plate ELISA assays from two siRNA transfections. (B) Fold of apoptosis with vs. without 250 nM Adriamycin® treatment. The fold change is an average of two independent experiments as described in (A).

and inhibits Cdc2.[43] Wee1 can be phosphorylated by Chk1 kinase[28] and both Wee1 and Cdc25 have been implicated in DNA damage-induced G_2 checkpoint control.[29] Human Wee1 was first identified by complementation of fission yeast wee1/mik1 deficient cells.[44] Consistent with its role as initially defined in yeast, Wee1 overexpression causes G_2 arrest and mutation of Cdc2Y15 to F in yeast and T14 to A and Y15 to F in mammalian Cdc2 cause premature mitosis. When cells are subjected to DNA damage, Chk1 can phosphorylate Wee1 and phosphorylated Wee1 interacts with 14-3-3.[30,31,45] This interaction can increase Wee1 protein stability and kinase activity, thereby keeping Cdc2 in its inactive state.

Wee1 has also been implicated in apoptosis. It has been reported that Wee1 overexpression can suppress granzyme-induced apoptosis.[46] Wee1 can also protect cells from HIV Vpr-induced apoptosis.[47] Furthermore, Wee1 protein is cleaved in Fas ligand induced apoptosis[48,49] and the Wee1 protein is reduced in HIV Vpr-induced apoptosis,[47] implying a role of this kinase in apoptosis. Depletion of Wee1 by siRNA in Hela cells alone has been reported to cause apoptosis (the apoptotic cells population increased from 18% to 30% three days after siRNA treatment).[47] Our data are consistent with the published results, as we observed an increase in the apoptotic fraction from 6% to 17% under similar conditions. However, we noted that the sensitizing effect of Wee1 downregulation in response to Adriamycin® is much more dramatic. Clearly, we have demonstrated that Wee1 siRNA abrogates the G_2 checkpoint and sensitizes Hela cells to apoptosis in response to DNA damaging induced by Adriamycin®.

Chk1 siRNA treatment alone without DNA damage causes apoptosis in Hela cells but not in HMEC cells. This observation indicates that cancer cells and normal cells may have differential sensitivity to Chk1 downregulation. Therefore, downregulation of Chk1 may selectively induce apoptosis in cancer cells. Alternatively, Chk2 or C-TAK1[50] may exert a partially redundant function in HMEC but not in Hela cells. *CHK1* is required for DNA damage checkpoint but is not an essential gene for normal cell growth in yeast.[51,52] However, previous studies in higher eukaryotes also support an essential function of Chk1 in cell proliferation or viability. First, knockout of the *drosophila* and mouse homologues were embryonic lethal.[24,53] Second, Chk1$^{-/-}$ ES cells could not be generated, suggesting that Chk1 is required for cell proliferation.[24,54] Furthermore, downregulation of Chk1 using anti-sense and ribozyme approaches also caused significant cell death in the absence of DNA damage in mammalian cells.[55] However, other studies indicate that Chk1 inhibition might not be lethal in mammalian cells. It was shown that Chk1 downregulation abrogated radiation-induced S and G_2 checkpoints[56,57] but had no significant effect on cell death. Another study carried out with Chk1 and Chk2 peptide inhibitors derived from the Chk1/Chk2 phosphorylation sequence in Cdc25,[58] showed no increase of cell death although the peptide inhibitors abrogated DNA damage-induced G_2 checkpoint.

There are several possible reasons to explain the apparent discrepancies on whether Chk1 is an essential gene in higher eukaryotes. One possible difference is the completeness of Chk1 downregulation.

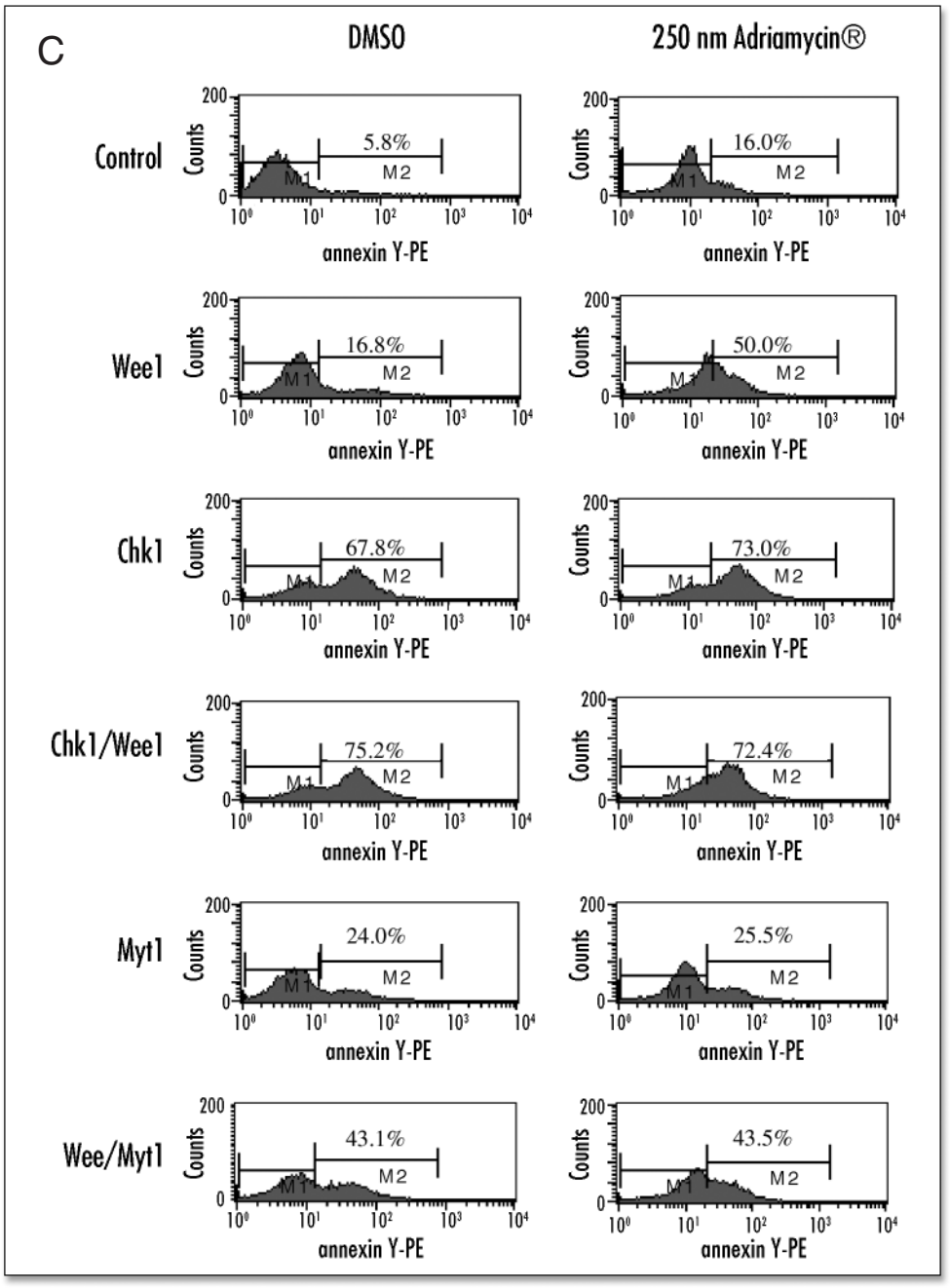

Figure 5C. Apoptosis measured by flowcytometry for annexin V-PE staining.

The Chk1 siRNAs utilized in the published studies are targeted to regions different from our Chk1 siRNAs.[56,57] The extent of Chk1 downregulation appears complete in our study and in the study reported by Zhao et al[57] but not in the report by Sorensen et al.[56] A second possible difference could be due to the time point selected for analysis. While Sorensen et al. harvested cells 30 to 36 hrs after transfection, we normally harvested cells 64 to 68 hrs after transfection. In our experiments, floating cells, presumably dead cells, are not visible at 28 hrs but appear at 44 hrs after siRNA transfection (data not shown). Furthermore, the Cdc25C peptide inhibitors may only inhibit the phosphorylation of Cdc25C by Chk1 and Chk2. Those peptides might not efficiently inhibit Cdc25A because they are based on the Cdc25C sequence surrounding the phosphorylation site S216, which has only 40% sequence homology with the Cdc25A S123 that is the phosphorylation site recognized by Chk1.

If Chk1 is essential for Hela cells, the next key question is why Chk1-deficiency in HMEC cells did not cause cell death. One possible explanation lies with the p53 status of these cells. Hela cells do not have functional p53 and DNA damage only causes G_2 arrest. HMEC cells, on the other hand, have wild-type p53 and can arrest at both the G_1 and G_2 phases of the cell cycle after DNA damage. Chk1 siRNA treatment of Hela cells renders a loss of both G_1 and G_2 checkpoints whereas HMEC cells may retain a normal G_1 checkpoint in response to the same treatment. It has been reported that Chk1-deficiency alone can increase Cdc25A to super-physiological levels.[56,57] The high level of Cdc25A may contribute to the G_2 checkpoint abrogation in Chk1 siRNA treated cells. Therefore, Chk1 siRNA selectively kills tumor cells. Chk1 downregulation abrogates G_2 checkpoint in tumor cells while damaged DNA can be repaired in normal cells because of the existence of a G_1 checkpoint. Another explanation for the difference in response between Hela and HMEC cells is that Hela cells grow much faster than HMEC cells. The rapid cell cycling of Hela cells might render them more sensitive to Chk1 siRNA downregulation compared to HMEC cells.

In mammalian cells both T14 and Y15 are phosphorylated. The T14 phosphorylation is catalyzed by the membrane-associated kinase, Myt1.[13] The role of this kinase in the G_2/M transition is not very clear. Downregulation of Myt1 in mammalian cells with ribozyme and anti-sense was not successful in the past[59] and unpublished data). *Drosophila* Myt1 downregulation has been achieved using a siRNA approach.[59] In that study, the authors found that dMyt1 reduction increased the rate of cell proliferation and decreased Cdc2T14 phosphorylation. They also reported that a reduction of Cdc2Y15 phosphorylation is only observed by downregulation of both dMyt1 and dWee1, but not by dWee1 downregulation alone. Our data show that the human Myt1 kinase is successfully downregulated using a siRNA approach. The human Myt1 reduction alone caused cell death as shown by less colony formation (Fig. 4) and a higher incidence of apoptosis cells (Fig. 5C) in the absence of DNA damage. The extent of apoptosis, however, is much less than that induced by Chk1 siRNA alone. Adriamycin® treatment of cells transfected with Myt1 siRNA, increases their apoptotic frequency, but not to the extent observed in Wee1 siRNA treated cells (Fig. 5A and B). Collectively, the data generated in our study point to distinct cellular functions of Wee1, Myt1, and Chk1.

In summary, among the three potential G_2 checkpoint abrogation targets tested in this report, Wee1 is likely to be the most promising kinase of pharmacological intervention based on multiple observations. First, Wee1 downregulation alone did not cause significant cell death compared to Chk1 and Myt1. Secondly, Wee1 siRNA treatment dramatically sensitized cancer cells but not normal cells to apoptosis in response to Adriamycin®. Therefore, the combination of a Wee1 inhibitor with a DNA damaging agent may serve to selectively kill cancer cells. Since downregulation of Chk1 alone killed Hela cells, but not HMEC cells, Chk1 may also represent a viable target. Studies aimed at functional knock down of these targets in tumor-bearing animals are warranted to compare the relative merits of these potential anti-cancer drug targets.

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