# Rapid functional screening of effective siRNAs against Plk1 and its growth inhibitory effects in laryngeal carcinoma cells

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**Plk 1 is overexpressed in many human malignancies including laryngeal carcinoma. However, its therapeutic potential has been never examined in laryngeal carcinoma. In the present study, a simple cellular morphology-based strategy was firstly proposed for rapidly screening the effective siRNAs against Plk1. Furthermore, we investigated the effects of Plk1 depletion via a novel identified effective siRNA against Plk1, Plk1 siRNA-607, on human laryngeal carcinoma Hep-2 cells. The results indicated that Plk1 siRNA-607 transfection resulted in a significant inhibition in Plk1 expression in cells, and subsequently caused a dramatic mitotic cell cycle arrest followed by massive apoptotic cell death, and eventually resulted in a significant decrease in growth and viability of the laryngeal carcinoma cells. Taken together, our present study not only suggests a simple strategy for rapidly screening effective siRNAs against Plk1 but also implicates that Plk1 may serve as a potential therapeutic target in human laryngeal carcinoma. [BMB reports 2010; 43(12): 818-823]**

## INTRODUCTION

The polo-like kinases (Plks) are a family of serine/threonine kinases and highly conserved from yeasts to mammals (1-5). Plk1 is the first and the best-characterized member of human Plks. Numerous studies have shown that Plk1 is over-expressed in a broad range of human tumors, and has prognostic value for predicting outcomes in patients with various types of cancers including laryngeal carcinoma (3, 4, 6, 7). The constitutive expression of Plk1 causes the transformation of NIH 3T3 fibroblasts (8). Moreover, a recent study has shown that Plk1 is also involved in invasion process through extracellular

matrix (9). Therefore, Plk1 has emerged as a novel attractive target for anti-cancer drug development (3-5). Currently, several pharmacological small-molecule inhibitors have been developed and showed promising results in clinical trials (3, 4). Additionally, several studies have also shown that depletion of Plk1 by RNA silencing inhibits cellular growth and induces apoptosis in some cancer cells but not in normal cells (10-12).

The RNA interference (RNAi) approach has been widely used for drug development with promising results (13, 14). For RNAi-based therapeutics, one of the major challenge is to screen and select effective specific siRNAs against a given target gene. The conventional method is to use the tedious real time RT-PCR and/or immunoblotting analysis to screen and identify effective siRNAs. Notably, however, numerous studies have shown that Plk1 plays critical roles during mitosis (3). We and others have demonstrated that depletion of Plk1 via RNAi silencing caused a significant mitotic arrest accompanied with remarkable abnormal cellular morphological changes such as typical round shape and complete detachment from the culture plates (10, 12, 15). Therefore, we speculate that Plk1-depletion-induced morphological changes might be conversely used for rapid screening effective specific siRNAs against Plk1.

Laryngeal carcinoma is a common head and neck malignancy with high incidence as it accounts for approximately 2.4% of new malignancies worldwide every year (16, 17). Despite extensive application of many different treatment modalities, the prognosis for patients with laryngeal carcinoma especially at late stage remains poor. The overall 5-year survival rate is about 73-92% for early disease stages and 50-64% for advanced disease stages (17). Thus, more efforts are needed to develop novel approaches and strategies for the treatment of this disease.

In the present study, we firstly proposed and demonstrated a simple cellular morphology-based strategy for rapid screening effective siRNAs against Plk1. In addition, we also investigated the growth inhibitory effects of Plk1 depletion in human laryngeal carcinoma cell line, Hep-2. Our results not only demonstrate a simple strategy for rapidly screening effective siRNAs against Plk1 but also suggest that Plk1 might be an attractive therapeutic target in the treatment of human laryngeal carcinoma.

## RESULTS

### Rapid screening of effective siRNAs against Plk1

To rapidly screen the effective siRNAs against Plk1, the negative control and Plk1 siRNAs were transiently transfected into both HeLa and Hep-2 cells. According to our and others observations, we deduce that cells transfected with effective Plk1 siRNAs would be arrested in mitotic phase with typical round shape. As expected, twenty-four to seventy-two hours after transfection, the Plk1-depleted HeLa and Hep-2 cells displayed significant abnormal morphological changes whereas the control cells showed a typical polygonal and intact appearance, and a normal growth phenotype (Fig. 1). Twenty-four hours after Plk1 siRNA transfection, cells partially detached from the culture plates, and floated on the media with round shape, strongly suggesting the cell cycle arrest at mitosis phase. Forty-eight and seventy-two hours after transfection, cells became less confluent, and completely detached from the culture plates, forming floating aggregates, suggesting the significant growth inhibition and cell death (Fig. 1). All the four Plk1 siRNAs showed remarkable growth inhibitory effects in both HeLa and Hep-2 cells, strongly suggesting their ability to inhibit Plk1 expression.

### Validation of effective siRNAs against Plk1

To further validate and evaluate the phenotype-based screening results, the conventional method, real time RT-PCR, was used to detect the knockdown effects of endogenous Plk1 expression after Plk1 siRNA transfection. As shown in Fig. 2A, Real time RT-PCR analysis indicated that the Plk1 expression was significantly inhibited at mRNA level after transfection, whereas the expression of GAPDH was unchanged.

Moreover, flow cytometry was also used to determine the cell cyle distribution after Plk1 depletion. As shown in Fig. 2B, cell cycle distribution analysis demonstrated that all the Plk1 siRNA-transfected cells showed an obvious increase in the percentage of cells with 4N DNA content and/or sub-G1 DNA content, strongly suggesting the phenotype of mitotic arrest and/or apoptosis induced by Plk1 depletion.

Taken together, these results consistently validated the feasibility and reliability of the simple cellular morphology-based strategy for rapid functional screening of effective siRNAs against Plk1 in mammal cells.

### Plk1 depletion inhibits proliferation and decreases viability in Hep-2 cells

Notably, the Plk1-targeted therapeutic effects have been investigated in many types of human cancers but not laryngeal carcinoma. Therefore, to both investigate the therapeutic implication of Plk1 siRNA in laryngeal carcinoma and further validate the above mentioned Plk1 siRNA screening results, we selected Plk1 siRNA-607 to check the growth inhibitory effects of Plk1 depletion in human laryngeal carcinoma Hep-2 cells in details.

At first, we determined the effect of Plk1 depletion on the growth and viability of Hep-2 cells. Real time RT-PCR and Western blot analysis demonstrated that the Plk1 expression was significantly inhibited at both mRNA and protein levels after transfection, whereas the expression of GAPDH or actin was unchanged. The expression level of Plk1 was efficiently
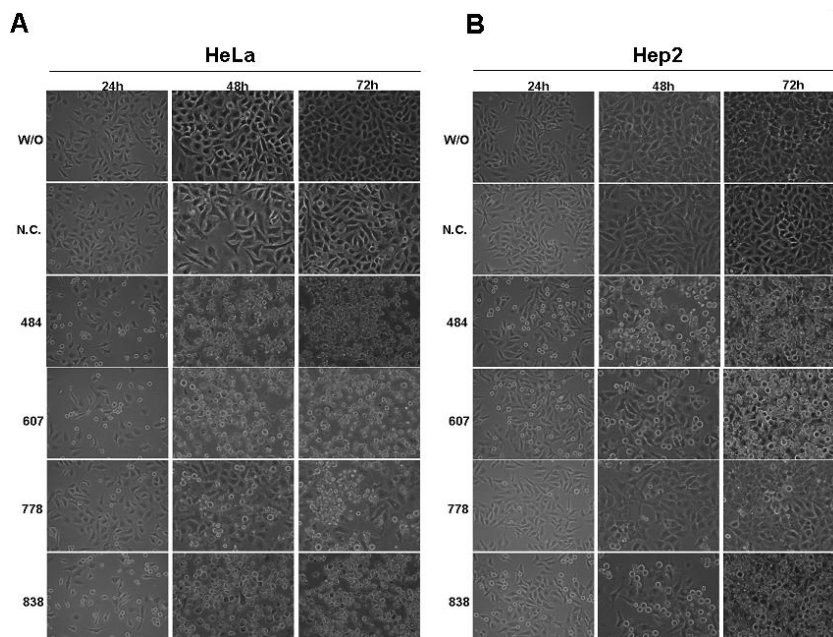


**Fig. 1.** Morphology-based screening of effective siRNAs against Plk1. HeLa (A) and Hep-2 (B) cells were transiently transfected with the control siRNA and different Plk1 siRNA candidates, respectively. Cellular morphology was monitored under phase-contrasted microscope at the indicated times after siRNA transfection (×200).

reduced after transfection (Fig. 3A). As indicated in Fig. 3B, Plk1 siRNA-607 strongly inhibited the growth rate of Hep-2 cells whereas control siRNA did not affect the cellular proliferation. As indicated in Fig. 3C, Trypan blue exclusion assay
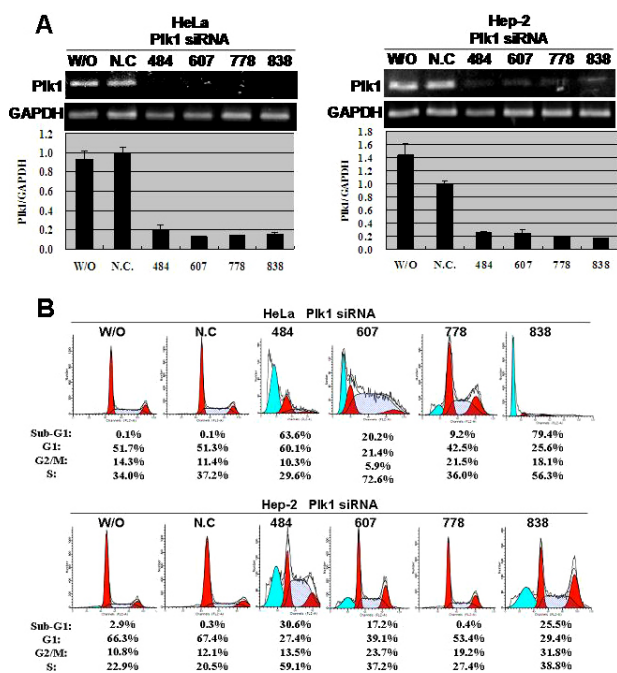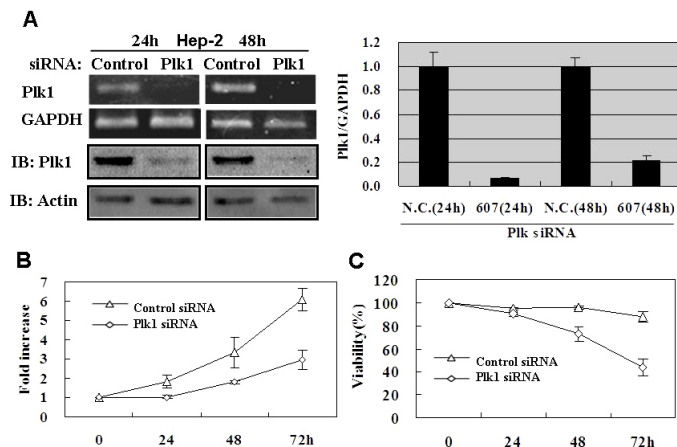


**Fig. 2.** Validation of effective Plk1 siRNAs-mediated Plk1 depletion. (A) Plk1 siRNA-mediated Plk1 depletion. HeLa and Hep-2 cells were transiently transfected with the control siRNA and different Plk1 siRNA candidates, respectively. Four-eight hours after transfection, total RNA were prepared and subjected to real time RT-PCR as described in Material and Methods. GAPDH was used as an internal control. (B) Cell cycle analysis. Four-eight hours after siRNA transfection, HeLa and Hep-2 cells were collected, and subjected to FACS analysis. The positions and percentages of G1, G2/M, and sub-G1 populations are shown.

demonstrated that, compared with control siRNA which showed very little effect on cell viability, Plk1 siRNA-607 significantly reduced the cell viability in Hep-2 cells. Notably, the Plk1 siRNA-607-transfected Hep-2 cells also showed a typical Plk1 depletion induced morphological changes as aforementioned.

## Plk1 depletion induces mitotic cell cycle arrest in Hep-2 cells

Next, we analyzed the effect of Plk1 depletion on cell cycle progression using flow cytometry. As shown in Fig. 4A, Plk1 depletion induced an obvious increase in the percentage of cells with 4N DNA content. Twenty-four hours after transfection, approximately 84% of Plk1 siRNA-607-transfected Hep-2 cells had a 4N DNA content compared with only 16% in control siRNA-transfected cells. The phosphorylation of histone H3, a mitosis marker, was then checked to confirm the phenotype of mitotic arrest in Plk1 siRNA-607-transfected cells (18). As shown in Fig. 4B, a significant accumulation of phosphorylated histone H3 was observed in Plk1 siRNA-607-transfected Hep-2 cells, suggesting that Plk1 siRNA-607 causes mitotic arrest in Hep-2 cells. Consistently, the expression of mitotic cyclin, cyclin B1, also remarkably accumulated in Plk1 siRNA-607-transfected Hep-2 cells.

## Plk1 depletion induces apoptosis in Hep-2 cells

Furthermore, we carefully determined whether or not Plk1 depletion resulted in apoptosis in human laryngeal carcinoma Hep-2 cells because Plk1 depletion has been shown to induce apoptosis in many other cancer cells (7-9, 15). Consistently, forty-eight hours after transfection, about 78% of Plk1 siRNA-607-transfected cells displayed sub-G1 DNA content, strongly suggesting the induction of apoptosis in Hep-2 cells (Fig. 4A). To further confirm this phenotype, Annexin V-FITC and PI double staining with flow cytometry was used to check apoptosis in Plk1-depleted cells. As expected, approximately 19.7% and 14.5% of Plk1 siRNA-607-transfected cells displayed early apoptotic (FITC$^+$/PI$^-$) and late apoptotic/secondary necrotic



**Fig. 3.** Plk1 depletion results in a significant decrease in the viability and growth of hep-2 cells. (A) siRNA- mediated Plk1 depletion. Hep-2 cells were transiently transfected with the control siRNA and Plk1 siRNA- 607, respectively. Twenty-four and four-eight hours after transfection, total RNA and whole cell lysates were prepared and subjected to RT-PCR and immunoblotting, respectively. For RT-PCR, GAPDH was used as an internal control. For immune blot analysis, equality of protein loading was confirmed by the expression of actin. (B) Plk1 depletion reduced cell proliferation. Following siRNA transfection, cells were collected and counted at the indicated time points. The Y axis represents average folds of increase in cell numbers. The error bar represents the standard error of three independent experiments. (C) The effect of Plk1 depletion on cell viability. Cell viability was measured using Trypan Blue Exclusion analysis as described in Material and Methods. Cell viability data are expressed as the percent viable cells out of the total number of cells. The error bar represents the standard error of three independent experiments.
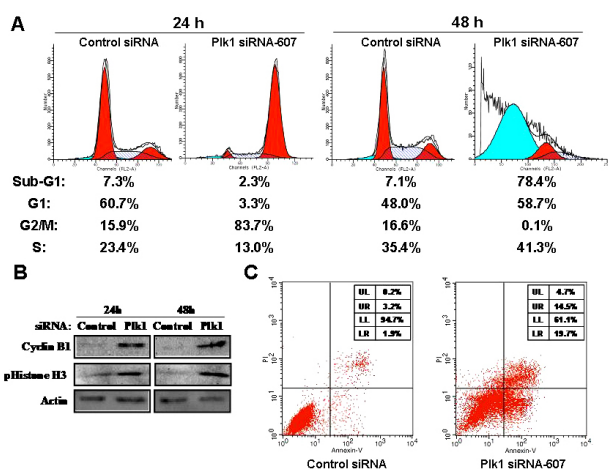
A



| | 24 h | | 48 h | |
|---|---|---|---|---|
| | Control siRNA | Plk1 siRNA-607 | Control siRNA | Plk1 siRNA-607 |
| Sub-G1: | 7.3% | 2.3% | 7.1% | 78.4% |
| G1: | 60.7% | 3.3% | 48.0% | 58.7% |
| G2/M: | 15.9% | 83.7% | 16.6% | 0.1% |
| S: | 23.4% | 13.0% | 35.4% | 41.3% |

B



C



**Fig. 4.** Plk1 depletion induces mitotic arrest and apoptosis in Hep-2 cells. (A) Cell cycle analysis. Hep-2 cells were transiently transfected with the control siRNA and Plk1 siRNA-607, respectively. Twenty-four and four-eight hours after transfection, cells were collected and subjected to FACS analysis. (B) Western blot analysis. Following siRNA transfection, whole cell lysates were prepared and subjected to immunoblotting. Equality of protein loading was confirmed by the expression of actin. (C) Hep-2 cells were transiently transfected with the control siRNA and Plk1 siRNA-607, respectively. Forty-eight hours after transfection, cells were collected, stained with Annexin V-FITC and propidium iodide (PI) and subjected to apoptosis analysis. Quadrants: lower left, viable (intact) cells (FITC$^-$/PI$^-$); lower right, early apoptotic cells (FITC$^+$/PI$^-$); upper right, late apoptotic and secondary necrotic cells (FITC$^+$/PI$^+$).

phenotype forty-eight hours after transfection, whereas only 1.9% and 3.2% of control cells had this phenotype (Fig. 4C).

## DISCUSSION

For the first part of the present study, we proposed and demonstrated a simple cellular morphology-based strategy for rapid screening effective siRNAs against Plk1. The successful screening and validation of four newly designed and synthesized Plk1 siRNA candidates further confirmed the feasibility and reliability of this strategy. Conventionally, the tedious RT-PCR and/or western blotting analysis was the most commonly used method for screening and validating effective siRNAs against specific target genes including Plk1 (15, 19). Recently, several other validation methods are developed to experimentally determine the efficiencies of in silico-selected siRNAs (20-22). Notably, all these methods ingeniously designed to use fluorescent reporter gene for monitoring the efficiency of siRNAs. Indeed, these methods do avoid using the tedious RT-PCR and/or Western blotting analysis, but they all need to do additional time-consuming lab works such as cloning the target cDNA and constructing a new vector. In contrast to these methods, obviously, our morphology-based screening strategy is easy, simple, and cost-effective. Conceivably, this strategy

could be also used to screen any other molecules targeting Plk1 including small molecule chemical inhibitors and other oligonucleotides including ribozymes, antisense oligonucleotides, and microRNAs.

For the second part of the present study, we further used the newly identified effective siRNA against Plk1, Plk1 siRNA-607, to investigate the effects of Plk1 depletion on human laryngeal carcinoma Hep-2 cells. As demonstrated by numerous studies, Plk1 is overexpressed in human tumors and has prognostic value in many different types of cancers, indicating its involvement in carcinogenesis and its potential as a therapeutic target (3, 4). In laryngeal carcinoma, Knecht et al reported that Plk1 mRNA is overexpressed in human head and neck tumors including laryngeal carcinoma and the overexpression of Plk1 mRNA correlates to prognostic parameters and the survival of patients with head and neck cancer (7). However, its therapeutic potential has not been investigated in laryngeal cancer until now. Our present in vitro study showed that the Plk1 siRNA-607 significantly inhibited the Plk1 expression at both mRNA and protein levels, and subsequently caused significant growth retardation in Hep-2 cells. According to our results, mitotic cell cycle arrest was the early events and the first major phenotype in Plk1-depleted Hep-2 cells. Additionally, as shown by the appearance of a sub-G1 population in cell cycle profiles, and the increase of Annexin V-FITC positive cells, apoptosis was the second major phenotype observed in Plk1-depleted Hep-2 cells. Besides, we also found that Plk1 depletion caused the failure of cytokinesis in Hep-2 cells and sensitized the cells to chemotherapeutic agents such as cisplatin and adriamycin (data not shown). These results are consistent with our and others previous reports observed in other cancer cells and strongly suggest that inhibition of Plk1 would be also a promising option for the treatment of human laryngeal carcinoma (5, 11, 15, 23, 24).

Intriguingly, based on the cell cycle distribution analysis, different siRNAs exert differential effects on cell cycle profiles. In both HeLa and Hep-2 cells, Plk1 siRNA-484 ,607 and 838 caused the most significant effects of mitotic arrest and apoptotic cell death whereas Plk1 siRNA-778 has the least effects on cell cycle and apoptosis (Fig. 2B). Obviously, this differential effect is not associated with the extent of Plk1 depletion as the four different siRNAs caused the similar knock-down effects of Plk1 expression in both cells (Fig. 2A). Given that Yamada et al suggested the existence of alternative splicing for Plk1, we deduced that the above-mentioned phenomena probably result from differential targeting of the four siRNAs on different splicing variants of Plk1 (25). However, we do not rule out the other possibility that this differential effect is associated with the possible differential off-target effects of siRNAs.

In summary, our present study not only provides a simple porphology-based strategy for rapidly screening effective siRNAs against Plk1 but also implicates that Plk1 may also serve as a potential target in the treatment of laryngeal carcinoma. The four novel validated siRNAs against Plk1 in this study could be

further developed for the treatment of human malignancies including laryngeal carcinoma.

## MATERIALS AND METHODS

### Design and synthesis of Plk1 siRNA
*Plk1* mRNA reference sequence, NM_005030, was obtained from GenBank, and used as template to design several novel candidate siRNAs against Plk1. siRNAs were chemically synthesized by Shanghai GenePharma.

The siRNA sequences were listed as following.
Negative control siRNA: 5'-UUCUCCGAACGUGUCACGUTT-3';
Plk1-siRNA-607: 5'-AUGAAGAUCUGGAGGUGAATT-3';
Plk1-siRNA-778: 5'-AUACCUUGUUAGUGGGCAATT-3';
Plk1-siRNA-838: 5'-GGAUCAAGAAGAAUGAAUATT-3';
Plk1-siRNA-484: 5'-GGAGGAAAGCCCUGACUGATT-3'.

All the siRNAs were chemically synthesized and purified by high-performance liquid chromatography (HPLC), and checked for purity and integrity by PAGE (20%) under denaturing conditions.

### Cell culture and siRNA transfection
Human laryngeal carcinoma cell line, Hep-2, and human cervical carcinoma cell line, HeLa, were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and penicillin (100 IU/ml)/streptomycin (100 mg/ml). Cells were maintained at 37°C in a water-saturated atmosphere of 5% $CO_2$ in air.

The siRNAs were transfected into the indicated cells using Lipofectamine RNAiMAX reagent (invitrogen) according to the manufacturer's instructions. Cells were collected and subjected to subsequent analysis 24 to 72 h after transfection.

### Cell growth and viability assay
Trypan blue exclusion assay was used to determine the effects of Plk1 depletion on the cellular growth and viability. Briefly, twenty-four to seventy-two hours after siRNA transfection, both floating and attached cells were collected and re-suspended in phosphate-buffered saline (PBS) solution. An aliquot of cell suspension was mixed with Trypan blue solution (0.4% in PBS, Sigma, St. Louis, MO), and the number of cells (viable-unstained and nonviable-blue) were counted under microscope.

### RNA isolation and RT-PCR
Total RNA was prepared from the indicated cells using the TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. cDNA was generated from 1 µg of total RNA using SuperScript II reverse transcriptase and random primers following the manufacturer's conditions (Invitrogen). Real time PCR was carried out by using the SYBR® Premix Ex Taq™ (Perfect Real Time, TAKARA) following the manufacturer's instructions. The relative expression level of the Plk1 gene compared with that of the housekeeping gene, GAPDH, was calculated by the $2^{-\Delta\Delta Ct}$ method (26). The primer sequences used were as follows: Plk1, 5'-GGCAACCTTTTCCTGAATGA-3' and 5'-AATGGACCACACATCCACCT-3'; GAPDH, 5'-ACCTGACCTGCCGTCTAGAA-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. The PCR was run for 30 to 40 cycles with a 95°C denaturing step (5 s), a 60°C annealing step (15 s), and a 72°C extension step (15 s), plus final incubation at 72°C for 10 min. For semi-quantitative RT-PCR, the amplified products were separated by a 2.0% agarose gel electrophoresis and checked by ethidium bromide staining.

### Immunoblotting analysis
Following the transfection of cells with control or Plk1 siRNA, the cells were collected and lysed in lysis buffer containing 25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100 and protease inhibitor mixture (Sigma) at 4°C for 30 min followed by brief sonication. After centrifugation at 14,000 rpm at 4°C for 10 min to remove insoluble materials, supernatants were collected and the protein concentrations were measured by Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA). Equal amounts of whole cell lysates were boiled in an SDS-sample buffer for 5 min, separated by a 8-10% SDS-PAGE, and electro-transferred onto Immobilon-P membranes (Millipore, Bedford, MA). After saturation, the membranes were blocked with TBS-T containing 5% non-fat dry milk at room temperature for 1 h, and subsequently incubated with the monoclonal anti-Plk1 (A-1, Zymed, San Francisco, CA), polyclonal anti-Phospho-Histone H3 (Upstate, Lake Placid, NY), or polyclonal anti-actin (20-33, Sigma) primary antibody, followed by incubation with a secondary horseradish peroxidase (HRP)-conjugated antibody (Cell Signaling, Beverly, MA). After washing with TBS-T, the membranes were developed using an enhanced chemifluorescence detection system (Amersham Biosciences, Uppsala, Sweden).

### Cell cycle analysis
Both floating and attached cells were collected by trypsin digestion and low speed centrifugation, washed with cold PBS, and fixed in ethanol at 4°C for at least 4 h. The fixed cells were collected by brief centrifugation and resuspended in PBS containing 0.1% BSA and 0.01% $NaN_3$. The cells were then treated with RNase A and stained with propidium iodide (PI) for 15 min at room temperature, respectively. The samples were finally subjected to cell cycle analysis on a FACScan flow cytometer (BD Bioscience, San Jose, CA).

### Apoptosis analysis
An Annexin V-FITC apoptosis detection kit (BD Pharmingen) was used to detect early apoptotic activity according to the manufacturer's instructions, with slight modifications. The cells were harvested, washed twice with ice-cold PBS, and resuspended in binding buffer. Annexin V-FITC and propidium iodide (PI) were then added and incubated for 15 min at room temperature in the dark. Cells were finally analyzed for apop-

tosis by flow cytometry.

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