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# The Blockage of Survivin and Securin Expression Increases the Cytochalasin B-Induced Cell Death and Growth Inhibition in Human Cancer Cells

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## ABSTRACT

Survivin and securin proteins are overexpressed in most cancer cells that have been shown to regulate mitotic progression. In this study, we investigated the roles of survivin and securin on cytochalasin B, a cytokinesis blocker mediating the cytotoxicity and cell growth inhibition in human cancer cells. The human lung carcinoma cell lines A549 and H1299 highly expressed survivin proteins in mitosis and concentrated on the midbodies during cytokinesis. Cytochalasin B significantly decreased cell survival, inhibited cell growth, increased the levels of $G_2$/M fractions, and induced binuclei formation in lung carcinoma cells; however, the survivin proteins were concentration-dependently increased by 1 to 5 $\mu$g/ml cytochalasin B for 24 h. It is noteworthy that the expression of securin proteins was decreased in cytochalasin B-treated lung carcinoma cells. Transfection of 20 to 40 nM survivin siRNA for 48 h significantly induced the formation of multiple nuclei and apoptosis but decreased the levels of survivin and securin proteins in A549 cells. Cotreatment with survivin small interfering RNA (siRNA) and cytochalasin B increased the cytotoxicity and cell growth inhibition. In addition, the securin-null colorectal carcinoma cells were more susceptible to the cytotoxicity after cytochalasin B and survivin siRNA treatments than the securin–wild-type cells. As a whole, our results indicate that the inhibition of survivin and securin protein expression may increase the cell death and growth inhibition after cytochalasin B treatment in human cancer cells.

Survivin proteins are expressed in most human cancer and embryonic cells but are undetectable in normal adult cells (Ambrosini et al., 1997; Li et al., 1998; Chao et al., 2004). It has been shown that survivin may inhibit apoptosis (Kawamura et al., 2003) and promote mitotic progression (Li et al., 1998; Deveraux and Reed, 1999; O'Connor et al., 2000; Wall et al., 2003; Chao et al., 2004; Kuo et al., 2004) in cancer cells. Moreover, survivin may serve as a radio- and chemoresistance factor during cancer therapy (Rodel et al., 2003; Wall et al., 2003; Chakravarti et al., 2004; Lu et al., 2004). The expression of survivin may reduce cancer cell death resulting from treatments with anticancer agents such as radiation, doxorubicin (Adriamycin), paclitaxel (taxol), or quercetin (Wall et al., 2003; Chakravarti et al., 2004; Kuo et al., 2004; Lu et al., 2004). Therefore, the depletion of survivin expression would block the antiapoptosis and cell cycle progression in cancer cells, providing an important strategy in cancer therapy.

It has been shown that survivin may regulate cytokinesis in cancer cells (Chen et al., 2000; Bolton et al., 2002; Honda et al., 2003). The survivin proteins are located on the midbodies during cytokinesis (Li et al., 1998; Kuo et al., 2004). Furthermore, survivin can interact with Aurora-B and INCENP proteins that form a chromosomal passenger protein complex that plays a crucial role in cytokinesis progression (Uren et al., 2000; Wheatley et al., 2001; Honda et al., 2003). Loss of survivin may display abnormal mitotic spindles and failure of cytokinesis (Uren et al., 2000). In addition, separase is required for the fidelity of chromosome separation in human cells (Chestukhin et al., 2003). It has been reported that separase can activate phosphatase cdc14 to regulate INCENP-Aurora B-survivin complex and promotes chromosome segregation (Pereira and Schiebel, 2003; Higuchi and Uhlmann, 2005). However, the precise mechanism of regulation of cytokinesis progression by survivin is still not clear.

In vertebrate, securin is also called the pituitary-tumor

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**ABBREVIATIONS:** MTT, 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; siRNA, small interfering RNA; F-actin, actin filament.

transforming gene or vSecurin (Pei and Melmed, 1997; Dominguez et al., 1998; Heaney et al., 1999; Saez et al., 1999; Zou et al., 1999). Securin has been found to be overexpressed in a variety of human cancer cells (Dominguez et al., 1998; Heaney et al., 1999; Saez et al., 1999; Zou et al., 1999). It has been shown that securin can promote the cell proliferation and tumorigenesis (Zhang et al., 1999; Zou et al., 1999; Hamid et al., 2005). Furthermore, securin acts as an anaphase inhibitory protein that plays an important role in preventing abnormal chromosome segregation (Nasmyth, 2001; Stemmann et al., 2001). The activity of separase is inhibited by securin in metaphase (Nasmyth, 2001; Stemmann et al., 2001). At the metaphase-anaphase transition, securin is degraded with subsequent release of separase to mediate the separation of sister chromatids by cleavage of the chromosomal cohesin (Nasmyth, 2001; Stemmann et al., 2001). Moreover, securin can prevent the anaphase progression when cellular DNA and spindle are damaged (Funabiki et al., 1996; Yamamoto et al., 1996a,b; Yanagida, 2000). In a recent study, securin and separase proteins have been found to participate in DNA repair after UV or X-ray exposures (Nagao et al., 2004). However, the interaction and regulation between securin and survivin proteins are still not clear.

Cytochalasin B, a cytokinesis inhibitor, has been shown to inhibit actin polymerization, to prevent separation of daughter cells after mitosis, and to inhibit cell cycle progression (Cooper, 1987; Fenech, 1993). However, the regulation of survivin and securin proteins on the cytochalasin B-induced cell death and growth inhibition remains unknown. Thus, we investigated the expression and possible roles of survivin and securin in the cytochalasin B-treated human cancer cells. The present data indicate that the depletion of survivin and securin proteins may increase cancer cell death and growth inhibition after cytochalasin B exposure.

## Materials and Methods

**Chemicals and Antibodies.** Cytochalasin B, Hoechst 33258, propidium iodide, 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), and the Cy3-labeled mouse anti-$\beta$-tubulin were purchased from Sigma Chemical (St. Louis, MO). BODIPY FL phallacidin (B-607) was purchased from Invitrogen (Carlsbad, CA). Anti-phospho-cdc2 (threonine-161), SignalSilence survivin siRNA, SignalSilence control siRNA, and goat anti-rabbit IgG-horseradish peroxidase were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-ERK-2, anti-survivin, goat anti-mouse IgG-horseradish peroxidase, and the fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-securin and anti-separase were purchased from Abcam (Cambridgeshire, UK). Anti-cdc2, and anti-cyclin B1 were purchased from Oncogene Sciences

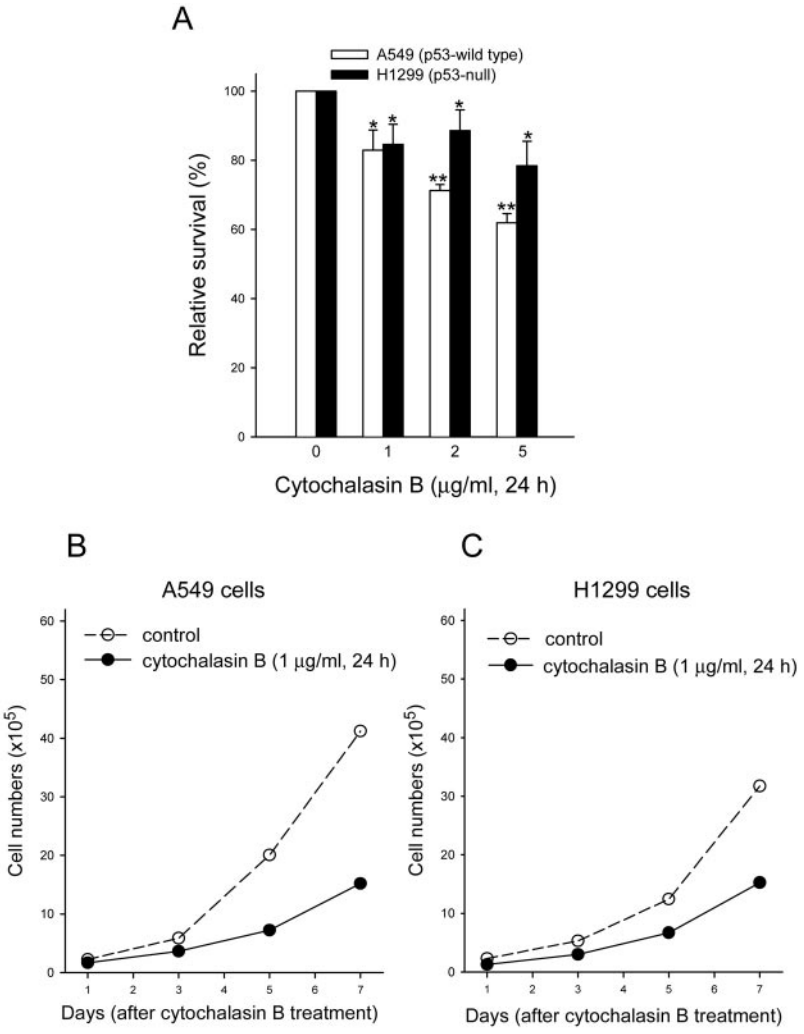


**Fig. 1.** Effects of cytochalasin B on cytotoxicity and cell growth in human lung carcinoma cells. A, the p53-functional A549 or p53-mutational SW480 cells were treated with 0 to 5 $\mu$g/ml cytochalasin B for 24 h. Cell survival was measured by MTT assay. Results were obtained from four to six experiments, and the bar represents mean ± S.E. B and C, the cells were plated at a density of $10^5$ cells/p60 Petri dish for 18 h. Then the cells were treated with 1 $\mu$g/ml cytochalasin B for 24 h. After drug treatment, the cells were washed twice with PBS and incubated for various times before they were counted by hemocytometer. Results were obtained from the average of three independent experiments. $p < 0.05$ (*) and $p < 0.01$ (**) indicate comparison between untreated samples and cytochalasin B-treated samples.

Products (Boston, MA). The Cy5-labeled goat anti-rabbit IgG was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

**Cell Culture.** The H1299 cell line, which contained a homozygous 5′ intragenic deletion of the *p53* gene and could not produce p53 mRNA and protein, was derived from a non–small-cell lung adenocarcinoma tumor of 43-year-old white man (Chen et al., 1993; Tsai et al., 1996). The A549 cell line was p53–wild-type and was derived from the lung adenocarcinoma of a 58-year-old white man (Giard et al., 1973). RKO was a colon carcinoma cell line that expressed the wild-type p53 proteins (Bhat et al., 1997). SW480, established from the colon adenocarcinoma of a 50-year-old white man, contained a G-to-A mutation in codon 273 and a C-to-T mutation in codon 309 of the *p53* gene (Rodrigues et al., 1990; Weiss et al., 1993). The securin-contained and securin-null HCT116 colorectal cancer cell lines (Jallepalli et al., 2001) were kindly provided by Dr. B. Vogelstein of The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (Baltimore, MD). The A549 and H1299 lung cancer cells, RKO and SW480 colorectal cancer cells, and HCT116 cells were maintained in RPMI-1640 medium (Invitrogen), Dulbecco's modified Eagle's medium (Invitrogen), and Macoy's 5A medium (Sigma Chemical), respectively. The complete media were supplemented with 10% fetal bovine serum. These cells were cultured at 37°C and 5% $CO_2$ in a humidified incubator (310/Thermo; Forma Scientific, Inc., Marietta, OH).

**Cytotoxicity Assay.** The cell survival was determined by the MTT colorimetric assay (Plumb et al., 1989). The cells were plated in 96-well plates at a density of $1 \times 10^4$ cells/well for 16 to 20 h. After drug treatment, the cells were washed with isotonic phosphate-buffered saline (PBS) and recultured in complete medium for 2 or 3 days. Thereafter, the medium was replaced and the cells were incubated with 0.5 mg/ml MTT in complete medium for 4 h. The surviving cells converted MTT to formazan, which produced a blue-purple color when dissolved in dimethyl sulfoxide. The intensity was measured at 565 nm using a 96-well plate reader (OPTImax; GE Healthcare). The relative percentage of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

**Apoptosis Assay.** For analysis of the percentage of apoptosis, the adherent cells were cultured on coverslips in a p60 Petri dish for 16

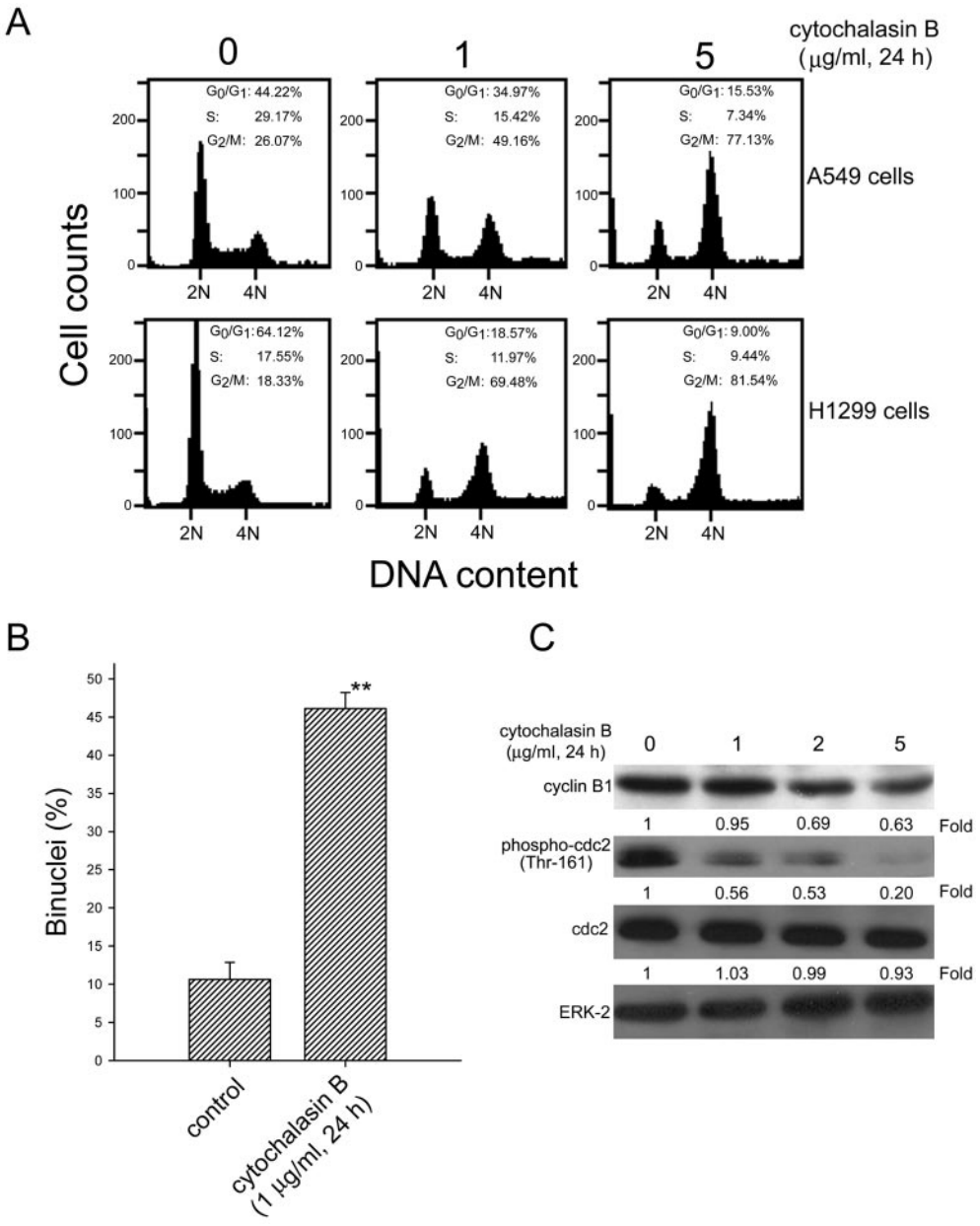


**Fig. 2.** Effects of cytochalasin B on the cell cycle progression and the levels of cyclin B1 and phospho-cdc2 (Thr-161) proteins in human lung carcinoma cells. A, the cells were treated with 0 to 5 μg/ml cytochalasin B for 24 h. At the end of treatment, the cells were trypsinized and then subjected to flow cytometry analyses. The data represented the average values from three experiments. B, the nuclei were stained with Hoechst 33258, and the percentage of binuclei was scored under a fluorescence microscope. Results were obtained from four experiments, and the bar represents mean ± S.E. $p < 0.01$ (**) indicates comparison between untreated samples and cytochalasin B-treated samples. C, the total protein extracts were prepared for immunoblot analysis using anti-cyclin B1, phospho-cdc2 (Thr-161), cdc2, and anti-ERK-2 antibodies. The relative protein levels under each treatment were the average of three independent experiments.

to 20 h before treatment. After drug treatment, the cells were washed twice with isotonic PBS, pH 7.4, and incubated with 4% paraformaldehyde solution in PBS for 1 h at 37°C. Then, the nuclei were stained with 2.5 $\mu$g/ml Hoechst 33258 for 30 min. The number of apoptotic nuclei was counted by hemocytometer under a fluorescence microscope. At least 200 cells were examined from the random fields for the calculation of apoptotic percentage in each treatment.

**Cell Cycle Analysis.** For analysis of cell cycle, the cells were plated at a density of $1 \times 10^6$ cells per p60 Petri dish in complete medium for 16 to 20 h. After drug treatment, the cells were collected and fixed with ice-cold 70% ethanol overnight at −20°C. After centrifugation, the cell pellets were treated with 4 $\mu$g/ml propidium iodide solution containing 1% Triton X-100 and 100 $\mu$g/ml RNase for 30 min. To avoid cell aggregation, the cell solutions were filtrated by nylon membrane (BD Biosciences, San Jose, CA). Thereafter, the samples were analyzed in a flow cytometer (LSR; BD Biosciences) using CellQuest software. A minimum of $10^4$ cells was analyzed for DNA content, and the percentage of cell cycle phases was quantified by a ModFit LT software (ver. 3.0; BD Biosciences).

**Cell Growth Assay.** The cells were plated at a density of $10^5$ cells per p60 Petri dish (or $10^6$ cells per p100 Petri dish) in complete medium for 16 to 18 h. After drug treatment, the cells were washed with PBS and recultured in complete medium. Thereafter, the cells were incubated for various times before they were counted by hemocytometer.

**Immunofluorescence Staining and Confocal Microscopy.** The cells were cultured on coverslips that were kept in a p60 Petri dish for 16 to 20 h before treatment. After drug treatment, the cells were washed with isotonic PBS, pH 7.4, and fixed in 4% paraformaldehyde solution in PBS for 1 h at 37°C. Then, the coverslips were washed three times with PBS, and nonspecific binding sites were blocked in PBS containing 10% FBS and 0.25% Triton X-100 for 1 h. The cells were incubated with rabbit anti-survivin (1:250), rabbit anti-separase (1:250), or mouse anti-securin (1:50) antibodies in PBS

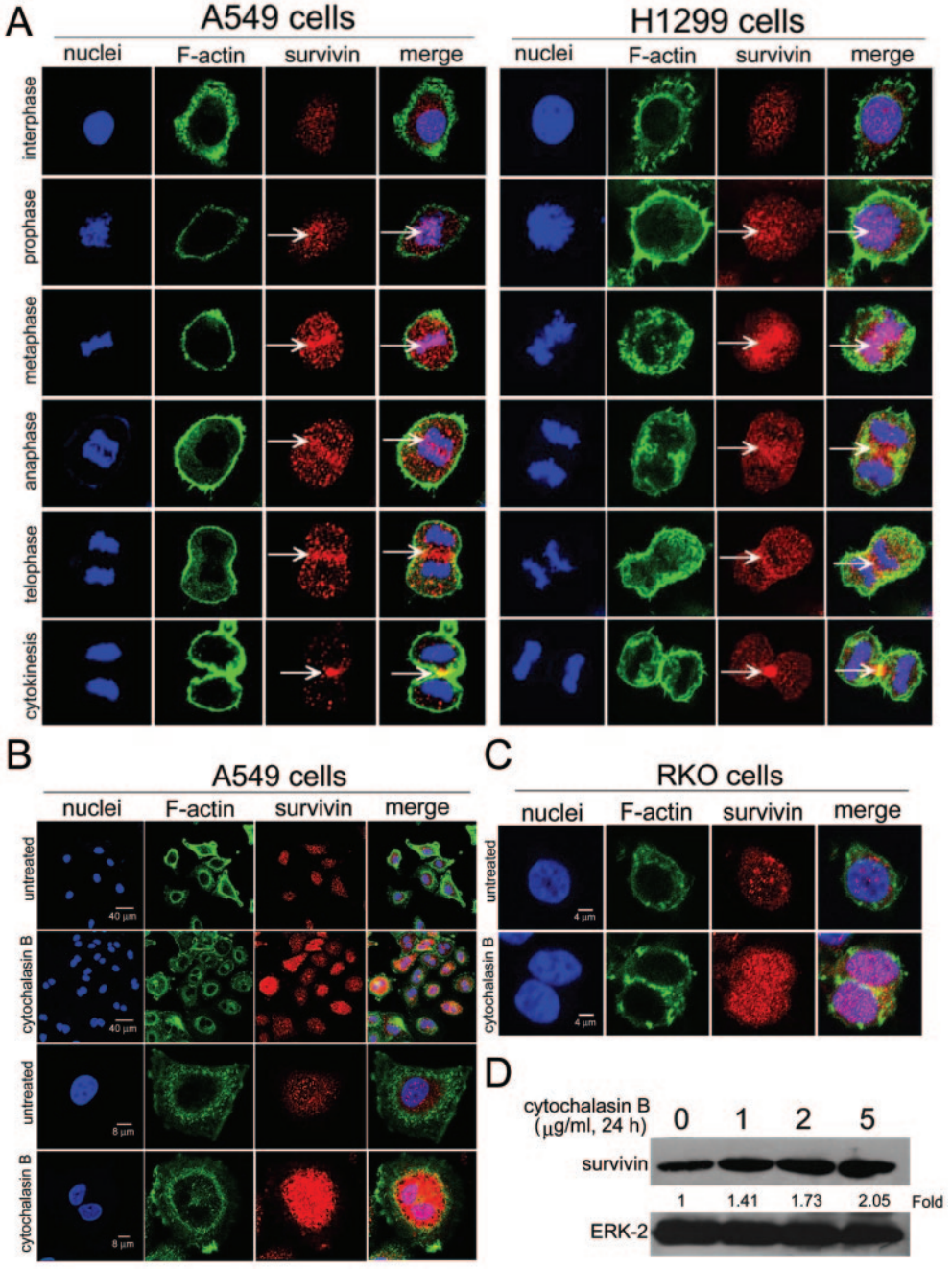


**Fig. 3.** Effects of cytochalasin B on the expression and location of survivin proteins in human cancer cells. A, the cells were incubated with rabbit anti-survivin antibody and then incubated with goat anti-rabbit Cy5. The survivin protein displayed red fluorescence with goat anti-rabbit Cy5. The F-actin was stained with BODIPY FL phallacidin, which displayed green fluorescence. Arrows indicate the location of survivin proteins. The nuclei were stained with Hoechst 33258, which displayed blue fluorescence. A549 (B) and RKO (C) cells were treated with or without 1 $\mu$g/ml cytochalasin B for 24 h. D, A549 cells were treated with 0 to 5 $\mu$g/ml cytochalasin B for 24 h. The total protein extracts were subjected to Western blot analysis using anti-survivin and anti-ERK-2 antibodies. The relative protein levels under each treatment were the average of three independent experiments.

containing 10% FBS for overnight at 4°C and washed three times with 0.25% Triton X-100 in PBS. Then, the cells were incubated with goat anti-rabbit Cy5 (1:250) or goat anti-mouse FITC (1:50) in PBS for 2.5 h at 37°C and washed three times with 0.25% Triton X-100 in PBS. The actin filament (F-actin) and β-tubulin were stained with 20 U/ml BODIPY FL phallacidin and anti-β-tubulin Cy3 (1:50) for 30 min at 37°C, respectively. The nuclei were stained with 2.5 μg/ml Hoechst 33258 for 30 min. At the end of staining, the samples were immediately examined under a confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with a UV laser (351/364 nm), an argon laser (457/488/514 nm), and a helium/neon laser (543 nm/633 nm). The fluorescence images were displayed through the frame store in the computer, and the images were merged by Leica confocal software (Lite version). The pictures were processed for presentation by Adobe Photoshop software (ver. 6.0; Adobe Systems, Mountain View, CA).

**Western Blot Analysis.** Western analyses of cyclin B1, cdc2, phospho-cdc2 (threonine-161), survivin, securin, and ERK-2 were performed using specific antibodies. After drug treatment, the cells were lysed in the ice-cold cell extract buffer, pH 7.6, containing 0.5 mM dithiothreitol, 0.2 mM EDTA, 20 mM HEPES, 2.5 mM $MgCl_2$, 75 mM NaCl, 0.1 mM $Na_3VO_4$, 50 mM NaF, and 0.1% Triton X-100. Protease inhibitors, including 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 100 μg/ml AEBSF were added to the cell suspension. The protein concentrations were determined by the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of proteins (50–100 μg/well) were subjected to electrophoresis using 10 to 12% SDS-polyacrylamide gels. After electrophoretic transfer of proteins onto polyvinylidene difluoride membranes, they were sequentially hybridized with primary antibody and followed with a horseradish peroxidase-conjugated secondary antibody. Finally, the protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA). A gel-digitizing software, Un-Scan-It gel (ver. 5.1; Silk Scientific, Inc., Orem, UT), was used to quantify the intensity of each band on the X-ray film.

**Transfection.** The cells ($5 \times 10^5$ cells/p35 dish) were transfected with control or survivin siRNAs by using Lipofectamine 2000 (Invitrogen) in serum-free DMEM for 48 h at 37°C in a $CO_2$ incubator according to the manufacturer's recommendations. The cells were then recultured in complete medium and subjected to cell growth analysis, MTT assay, immunofluorescence staining, or immunoblot as described above.

**Statistical Analysis.** Data were analyzed using Student's $t$ test or analysis of variance (a comparison of multiple groups), and a $p$ value of $<0.05$ was considered statistically significant in each experiment.

## Results

**Cytochalasin B Decreases the Cell Survival, Inhibits Cell Growth, and Increases the Levels of $G_2$/M Fractions in Human Lung Carcinoma Cells.** As shown in Fig. 1A, treatment with 1 to 5 μg/ml cytochalasin B for 24 h decreased the cell survival in a concentration-dependent manner, and approximately 60% and 80% of A549 and H1299 cells, respectively, survived after exposure to 5 μg/ml cytochalasin B for 24 h. Moreover, the cell growth in both A549 and H1299 cells was significantly inhibited after cytochalasin B treatment (Fig. 1, B and C). To further investigate cytochalasin B on the cell cycle progression, the human lung carcinoma cells were treated with cytochalasin B and analyzed by flow cytometry. As shown in Fig. 2A, cytochalasin B decreased the $G_1$ fractions but increased the $G_2$/M fractions in both A549 and H1299 cells. In addition, the formation of binuclei was increased to 45% in the cytochalasin B-treated A549 cells (Fig. 2B). Furthermore, immunoblot analysis showed that 1 to 5 μg/ml cytochalasin B for 24 h decreased the levels of cyclin B1 and phospho-cdc2 (Thr-161) proteins in A549 cells ($p < 0.05$, compared between untreated and drug-treated samples) (Fig. 2C). However, the level of total cdc2 proteins was slightly decreased by treatment with 5 μg/ml cytochalasin B for 24 h (Fig. 2C). ERK-2 protein has been used as an internal control protein in several studies (Chao et al., 2004; Kuo et al., 2004). ERK is activated through phosphorylation at Thr202/Tyr204 sites; however, ERK total protein is not altered in various treatments. Therefore, ERK-2 was used as an internal control in this study.

**Cytochalasin B Increases the Level of Survivin Proteins in Human Cancer Cells.** To examine the expression and location of survivin after cytochalasin B treatment, the lung carcinoma cells were subjected to immunofluorescence staining and confocal microscopy. The red fluorescence (Cy5) exhibited by survivin was found in both A549 and H1299 cells (Fig. 3A). The survivin proteins were highly expressed in mitotic phase, colocalized with chromosome in metaphase, and concentrated on the midbodies during cytokinesis in lung carcinoma cells (Fig. 3A, arrows). The red fluorescence intensity of survivin was significantly increased when exposed to 1 μg/ml cytochalasin B for 24 h in A549 and RKO cells (Fig. 3, B and C). Moreover, cytochalasin B (1–5 μg/ml, 24 h) concentration-dependently increased the level of survivin proteins in A549 cells ($p < 0.05$) (Fig. 3D).

**Survivin siRNA Decreases Survivin Protein Expression and Cell Survival, but Increases the Formation of Apoptosis and Multiple Nuclei in Human Lung Carcinoma Cells.** A control siRNA and a survivin siRNA were used for transfection to examine the role of survivin on the

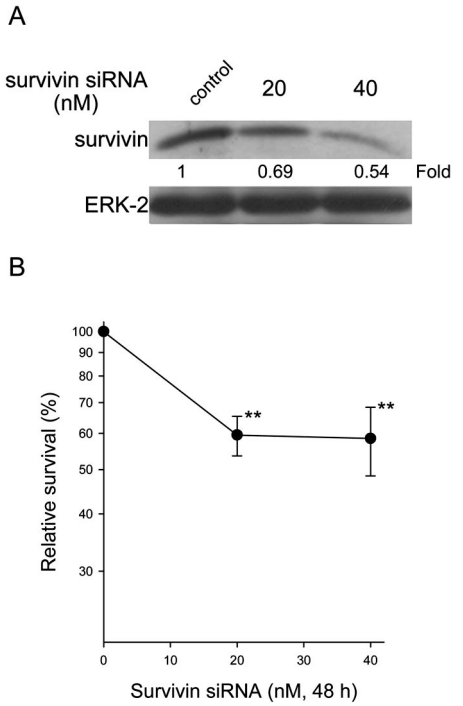


**Fig. 4.** Transfection of a survivin siRNA on the level of survivin proteins and cell survival in human lung carcinoma cells. A, A549 cells were transfected with control or survivin siRNA for 48 h. The total protein extracts were subjected to Western blot analysis using anti-survivin and anti-ERK-2 antibodies. The relative protein levels under each treatment were the average of three independent experiments. B, the cell survival was measured by MTT assay. Results were obtained from four experiments, and the bar represents the mean ± S.E. $p < 0.01$ (**) indicates comparison between control and survivin siRNA-treated samples.

cytokinesis progression and apoptosis in human lung carcinoma cells. As shown in Fig. 4A, the levels of survivin proteins were significantly decreased when transfected with 20 to 40 nM survivin siRNA for 48 h in A549 cells ($p < 0.01$). Moreover, transfection of survivin siRNA significantly inhibited the cell survival (Fig. 4B). By observation of nuclei staining, survivin siRNA increased the levels of apoptotic nuclei in A549 cells (Fig. 5A, stars). The percentage of apoptotic nuclei was counted under a fluorescence microscope. Transfection with 40 nM survivin siRNA for 48 h increased approximately 10% of apoptosis in A549 cells (Fig. 5D). At the same time, survivin siRNA induced a variety of multinuclei (> 4N) including three nuclei (Fig. 5A, arrows), four nuclei (Fig. 5B, bottom pictures), and five nuclei (Fig. 5C, bottom pictures) that indicated the blockage of cytokinesis progression in A549 cells. Counting the percentage of multiple nuclei, the transfection of 40 nM survivin siRNA for 48 h increased approximately 15% of multinuclei in A549 cells (Fig. 5E). However, the cells of multinuclei were not found after transfection with control siRNA (Fig. 5E).

**The Survivin Gene Knockdown Increases the Cytochalasin B-Induced Cell Death and Cell Growth Inhibition in Human Lung Carcinoma Cells.** We have investigated the combination of survivin siRNA and cytochalasin B on the cell survival and cell growth in human lung carcinoma cells. As shown in Fig. 6A, transfection of survivin siRNA (20 nM, 48 h) or treatment with cytochalasin B (1 $\mu$g/ml, 48 h) significantly decreased the cell survival in A549 cells. Moreover, cotreatment with survivin siRNA and cytochalasin B increased the decrease of cell survival in A549 cells (Fig. 6A). To examine the combined effect of survivin siRNA and cytochalasin B on the cell growth, the cells were

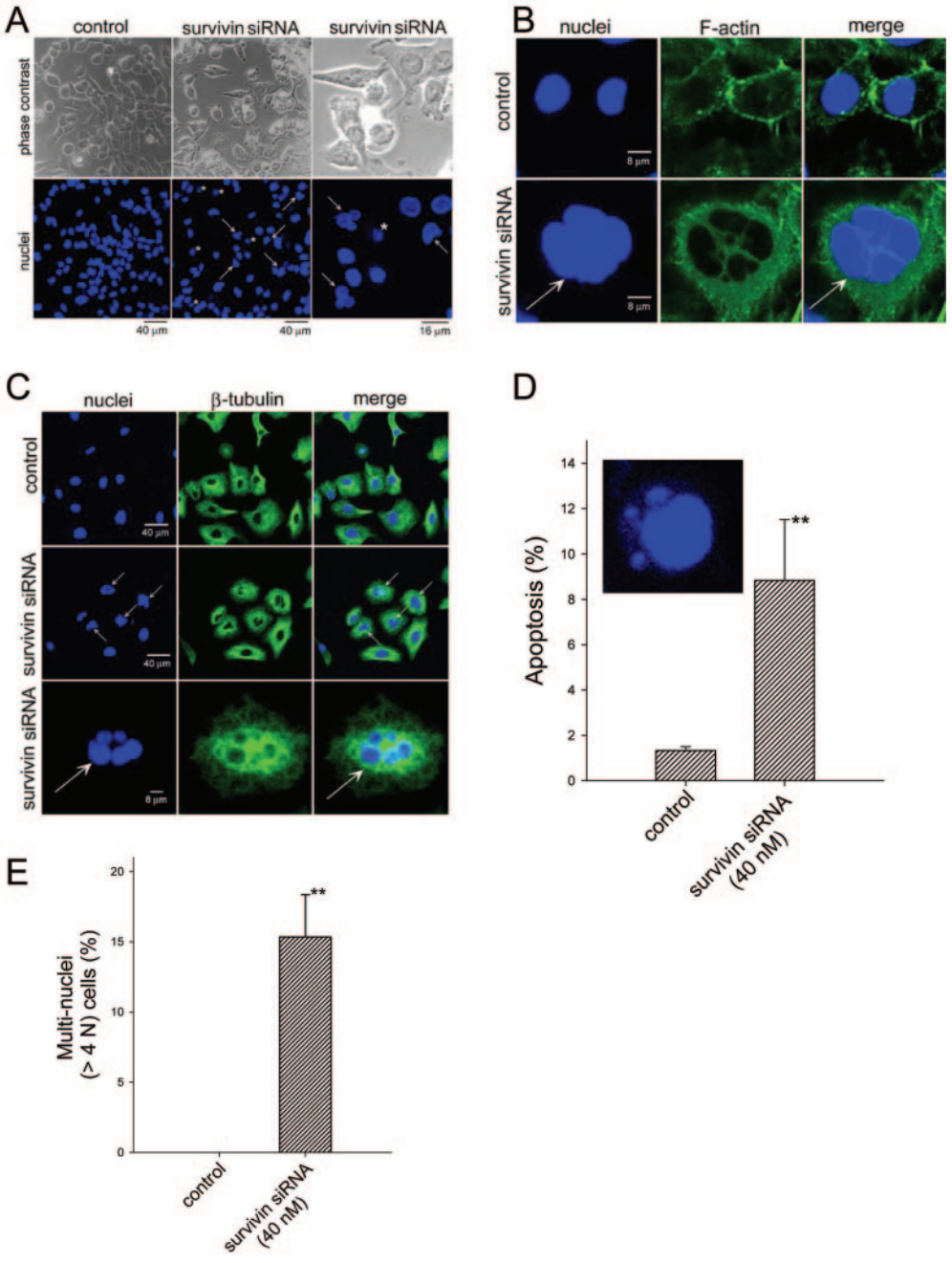


**Fig. 5.** Transfection of a survivin siRNA on the levels of apoptosis and multinuclei in human lung carcinoma cells. A-C, A549 cells were transfected with 40 nM survivin siRNA for 48 h. The $\beta$-tubulin, F-actin, and nuclei were stained with the Cy3-labeled mouse anti-$\beta$-tubulin, BODIPY FL phallacidin, and Hoechst 33258, respectively. Stars indicate the apoptotic nuclei and arrows indicate the multinuclei. D, the percentage of apoptosis was scored the apoptotic nuclei under a fluorescence microscope. Results were obtained from three experiments and the bar represents mean ± S.E. E, the percentage of multinuclei (> 4N) was scored under a fluorescence microscope. Results were obtained from three experiments, and the bar represents mean ± S.E. $p < 0.01$ (**) indicates comparison between control and survivin siRNA-treated samples.

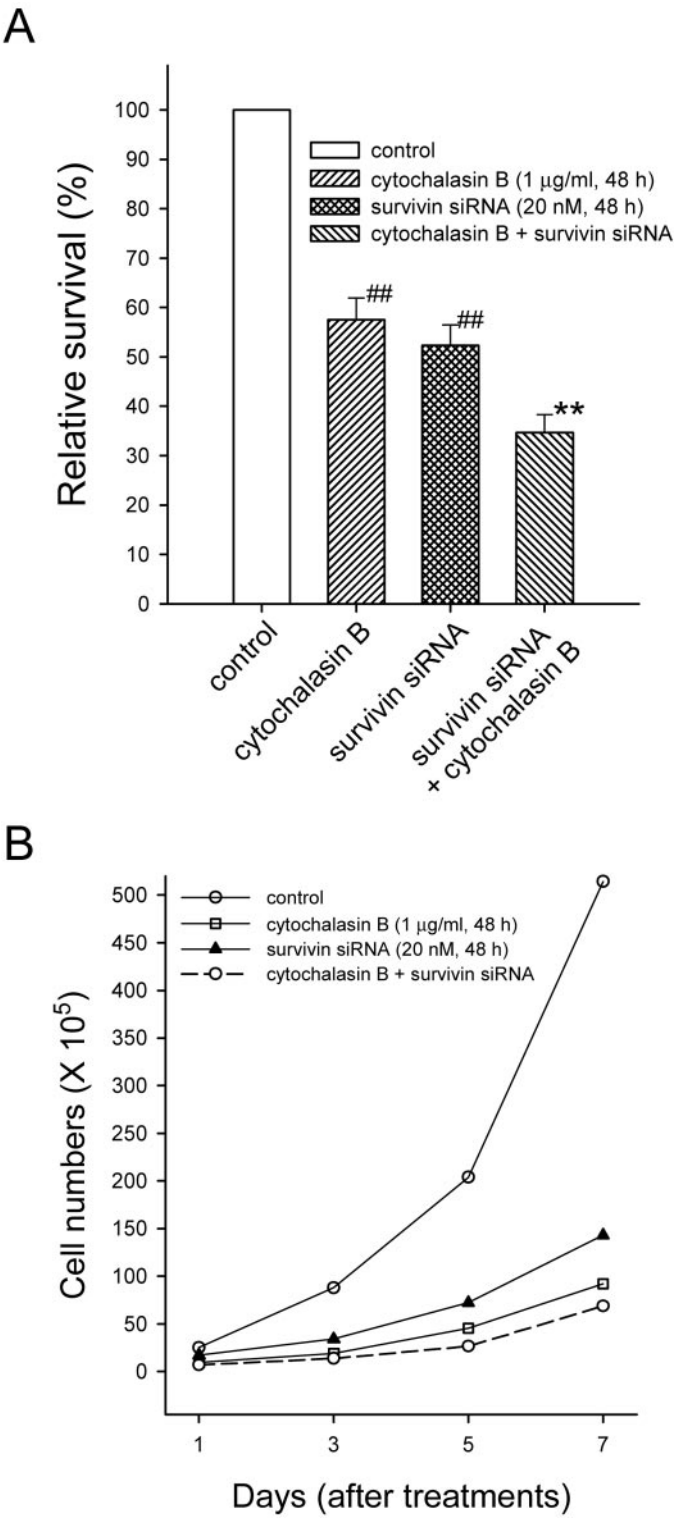


**Fig. 6.** Cotreatment of survivin siRNA and cytochalasin B on the cytotoxicity and cell growth in A549 cells. A, the cell survival was measured by MTT assay. Results were obtained from four experiments, and the bar represents ± S.E. $p < 0.01$ (##) indicates comparison between control and transfection of survivin siRNA or cytochalasin B treatment. $p < 0.01$ (**) indicates comparison between control and survivin siRNA transfected-cells by cotreatment with cytochalasin B. B, the cells were plated at a density of $8 \times 10^5$ cells/p100 Petri dish for 18 h. Then the cells were cotreated with 20 nM survivin siRNA and 1 μg/ml cytochalasin B for 48 h. After drug treatment, the cells were washed with PBS and incubated for various times before they were counted by a hemocytometer.

plated at a density of $8 \times 10^5$ cells per p100 dish and were cotreated with survivin siRNA and cytochalasin B. As shown in Fig. 6A, both survivin siRNA and cytochalasin B significantly inhibited the cell growth in A549 cells ($p < 0.01$). Furthermore, the combination of survivin siRNA and cytochalasin B than the survivin siRNA or cytochalasin B alone increased the higher cell growth inhibition ($p < 0.05$) (Fig. 6B).

**The Expression and Location of Survivin and Securin Proteins in a Variety of Human Cancer Cells.** To examine the expression and location of securin and separase proteins relative to survivin protein in human cancer cells, the cells were subjected to immunoblot and immunofluorescence staining. As shown in Fig. 7A, a variety of human cancer cell lines including lung cancer (A549 and H1299) and colon cancer (RKO and SW480) expressed high levels of survivin and securin proteins. Moreover, we found that the securin-contained HCT116 cells but not the securin-null cells expressed the securin proteins (Fig. 7A). In these cell lines, the p53 proteins were expressed in the A549, RKO, SW480, and HCT116 cells but not H1299 cells (Fig. 7A). However, it has been shown that SW480 cells contained a G-to-A mutation in codon 273 and a C-to-T mutation in codon 309 of the p53 gene (Rodrigues et al., 1990; Weiss et al., 1993). The survivin proteins were expressed in all of the above cell lines, but the p53-mutated cells were expressed at relatively higher levels than the p53-normal cells (Fig. 7A). Furthermore, the survivin proteins were highly expressed in mitotic phase and concentrated on the midbodies during cytokinesis (Fig. 7B, arrows). It is noteworthy that the green fluorescence exhibited by securin and the red fluorescence (Cy5) exhibited by separase proteins were found on the nuclei of interphase in A549 cells (Fig. 7, B and C, arrows). In addition, the securin and separase proteins were located on the cytoplasm of mitosis phases (Fig. 7, B and C). The securin and separase were apparently not colocalized with survivin at the telophase and cytokinesis phases in A549 cells (Fig. 7, B and C, arrows).

**Cytochalasin B and Survivin siRNA Decrease the Securin Protein Expression in Human Lung Carcinoma Cells.** To investigate the effects of cytochalasin B or the survivin gene knockdown on the levels of securin protein expression, the cells were treated with cytochalasin B or transfected with survivin siRNA and subjected to immunoblot analysis and immunofluorescence staining. As shown in Fig. 8A, the levels of securin proteins were concentration-dependently decreased when exposed to 1 to 5 μg/ml cytochalasin B for 24 h in A549 cells ($p < 0.01$). Moreover, the green fluorescence (FITC) exhibited by securin was decreased by 5 μg/ml cytochalasin B for 24 h in A549 cells (data not shown). It is interesting that the red fluorescence (Cy5) exhibited by separase proteins was concentrated between the binuclei in the cytochalasin B-treated cells (Fig. 8B, arrows). In addition, the levels of securin proteins were decreased when transfected with 20 to 40 nM survivin siRNA for 48 h in A549 cells ($p < 0.05$) (Fig. 8C). The level of apoptosis was induced by transfection with survivin siRNA (40 nM, 48 h) in A549 cells (Fig. 8D, arrow). Meanwhile, survivin siRNA decreased the green fluorescence intensity of securin and the red fluorescence of separase in A549 cells (Fig. 8D). Furthermore, transfection with 40 nM survivin siRNA for 48 h additively

decreased the level of securin proteins in the cytochalasin B-treated A549 cells (data not shown).

**The Blockage of Securin Protein Increases Cell Death in Both Cytochalasin B- and Survivin siRNA-Treated Human Colorectal Carcinoma Cells.** To investigate the role of securin on cell survival, securin–wild-type and securin-null HCT116 cell lines were examined after transfection with survivin siRNA or treatment with cytochalasin B. As shown in Fig. 9A, both the securin–wild-type and securin-null cells expressed survivin proteins that were located on the midbodies during cytokinesis. However, the securin-null cells grew more slowly than the securin–wild-type cells after being cultured for 6 to 8 days (Fig. 9B). In addition, the securin-null and securin–wild-type cells were treated with survivin siRNA or cytochalasin B, and the percentage of cell survival was estimated by MTT assay. Survivin siRNA (20–40 nM, 48 h) and cytochalasin B (1–5 $\mu$g/ml, 24 h) concentration-dependently induced cytotoxicity in both the securin-null and securin–wild-type cells (Fig. 10, A and B). Moreover, the securin-null cells were more susceptible to cell death than the securin–wild-type cells after cytochalasin B and survivin siRNA treatments (Fig. 10, A and B).

## Discussion

In this study, survivin proteins were highly expressed during mitosis and on the midbodies during cytokinesis in a variety of cancer cell lines, including A549 (p53-containing) and H1299 (p53-null) lung carcinoma cells and HCT116 (securin–wild-type and securin-null) colorectal carcinoma cells. Cytochalasin B decreased the cell survival, inhibited the cell growth, increased the levels of $G_2$/M fractions, and induced the formation of binuclei in human lung carcinoma cells; however, survivin protein levels were increased by cytochalasin B treatment. It has been shown that survivin expression can reduce the cancer cell death by treated with a variety of anticancer agents (Wall et al., 2003; Chakravarti et al., 2004; Kuo et al., 2004; Lu et al., 2004). Moreover, the technique of specific survivin gene knockdown by transfection with a survivin siRNA increased the cytochalasin B-induced cell growth inhibition and cell death in human lung carcinoma cells. Thereafter, the blockage of securin proteins in human colorectal cancer cells increased the cytotoxicity after cytochalasin B and survivin siRNA treatments. It has been shown that the gene knockdown of securin inhibited the

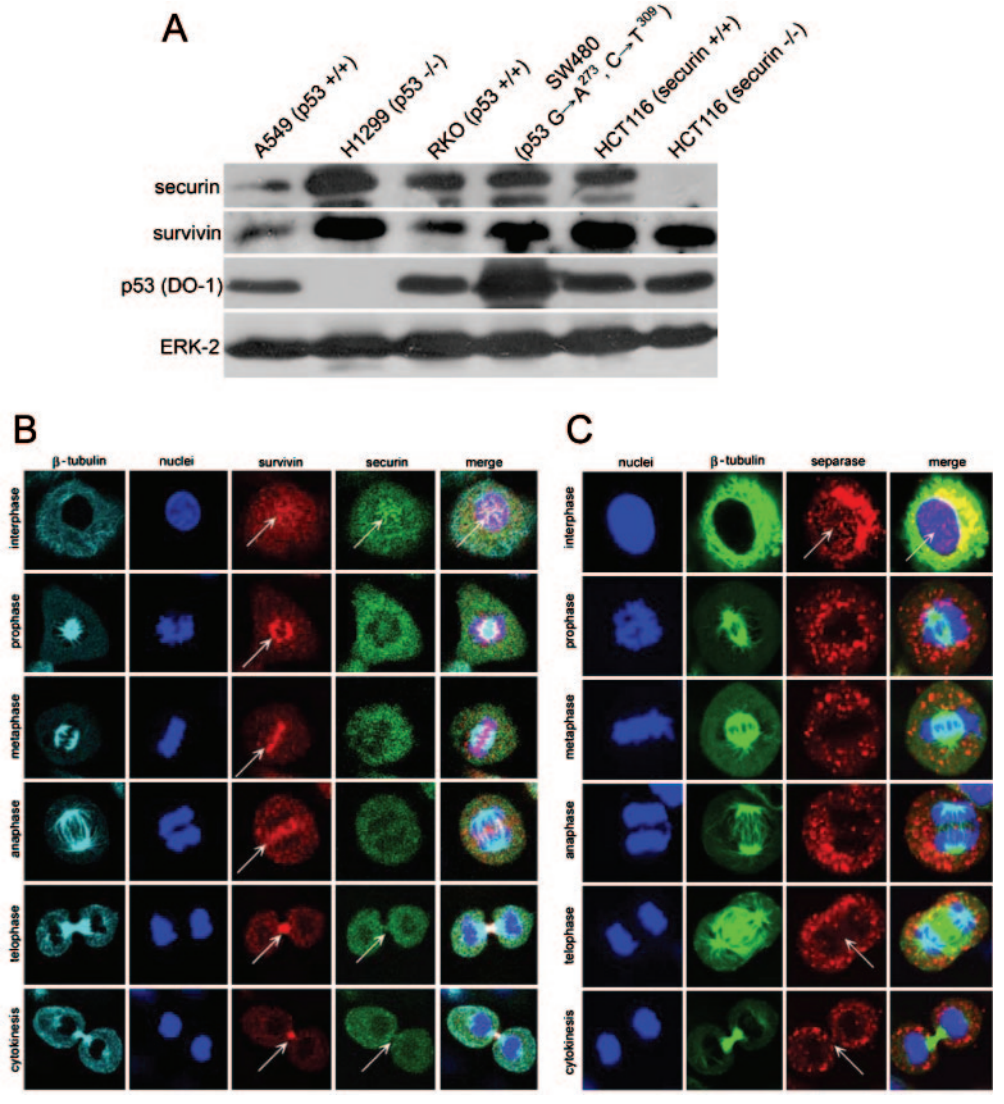


**Fig. 7.** The expression and location of survivin, securin, and separase proteins in human cancer cells. A, the total protein extracts were subjected to Western blot analysis using anti-survivin, anti-securin, anti-p53 (DO-1), and anti-ERK-2 antibodies. B, the cells were incubated with rabbit anti-survivin and mouse anti-securin antibodies and then incubated with goat anti-rabbit Cy5 and goat anti-mouse FITC. The survivin protein displayed red fluorescence with goat anti-rabbit Cy5. The securin protein displayed green fluorescence with goat anti-mouse FITC. C, the separase protein displayed red fluorescence with goat anti-rabbit Cy5. The $\beta$-tubulin and nuclei were stained with the Cy3-labeled mouse anti-$\beta$-tubulin and Hoechst 33258, respectively. Representative Western blot and immunofluorescence data were shown from one of three separate experiments with similar findings.

proliferation of glioma cells (Tfelt-Hansen et al., 2004). Therefore, we suggest that survivin and securin expression may resist the cell growth inhibition and apoptosis in the cytochalasin B-treated human cancer cells.

It is interesting that both cytochalasin B and transfection of a survivin siRNA blocked cytokinesis and decreased securin protein expression in human lung carcinoma cells; however, cytochalasin B caused binuclei induction, but survivin siRNA induced the formation of multiple nuclei. Cytochalasin B is known to inhibit actin polymerization, to prevent separation of daughter cells after mitosis, and to lead to binucleated cells (Cooper, 1987; Fenech, 1993). Nevertheless, the levels of survivin proteins were increased in the cytochalasin B-treated cells. Moreover, we found that the separase proteins were apparently concentrated between the binuclei of the cytochalasin B-treated cells. In contrast, survivin siRNA decreased the level of separase proteins and induced the formation of multiple nuclei in human lung carcinoma cells. Indeed, loss of survivin may display abnormal mitotic spindles and failure of cytokinesis (Uren et al., 2000). Furthermore, it has been reported that separase activated phosphatase cdc14 to regulate INCENP-Aurora B-survivin complex and promoted chromosome segregation (Pereira and Schiebel, 2003; Higuchi and Uhlmann, 2005). Separase is required for the fidelity of chromosome separation in human cells (Chestukhin et al., 2003). We suggest that survivin siRNA and cytochalasin B mediate the blockage of cytokinesis via two different pathways, and the survivin and separase proteins may cooperatively regulate the cytokinesis and cell growth in human cancer cells. However, the precise role of separase on the regulation of survivin siRNA- and cytochalasin B-induced cell growth inhibition and cell death needs further investigation.

The activity of separase is inhibited by binding to securin protein in metaphase (Nasmyth, 2001; Stemmann et al., 2001). Securin acts as an anaphase inhibitory protein that plays an important role in preventing abnormal chromosome segregation (Nasmyth, 2001; Stemmann et al., 2001). At the metaphase-anaphase transition, the securin is degraded and separase is released to mediate the separation of sister chromatids in anaphase by cleavage of the chromosomal cohesin (Nasmyth, 2001; Stemmann et al., 2001). However, the levels of securin were decreased in both the survivin siRNA and cytochalasin B-treated cells. The securin-null cells were more susceptible to the cell death than the securin–wild-type cells after survivin siRNA or cytochalasin B treatments. The combination of cytochalasin B and survivin siRNA significantly increased the cell death and growth arrest. Moreover, transfection with survivin siRNA additively increased the loss of securin in the cytochalasin B-treated cells. In several studies, securin has been found to be overexpressed in a variety human cancer cells (Dominguez et al., 1998; Heaney et al., 1999; Saez et al., 1999; Zou et al., 1999) and can mediate the proliferation and tumorigenesis of cancer (Kakar and Jennes,

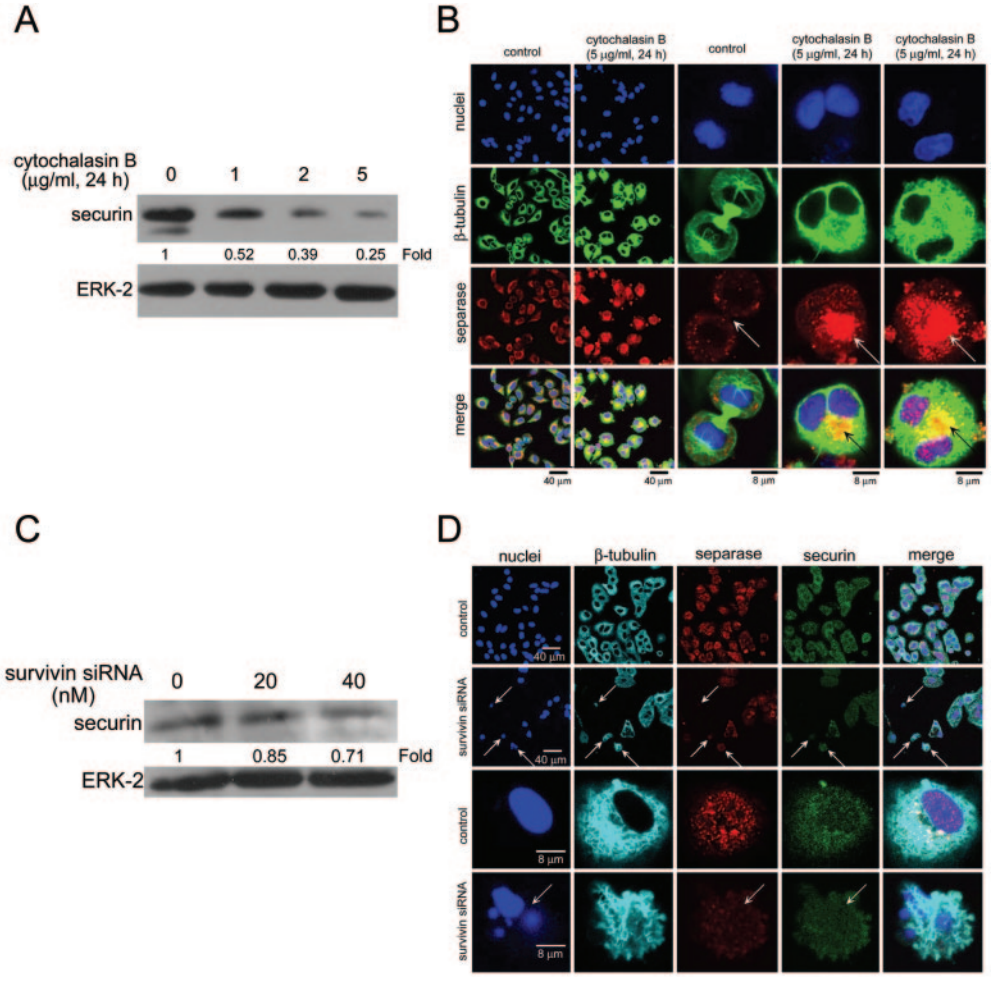


**Fig. 8.** Effects of cytochalasin B and survivin siRNA on the expression of securin and the location of separase in human lung carcinoma cells. A and B, A549 cells were treated with or without cytochalasin B for 24 h. C and D, A549 cells were transfected with control or survivin siRNA for 48 h. The total protein extracts were subjected to Western blot analysis using anti-securin and anti-ERK-2 antibodies. The separase proteins displayed red fluorescence with goat anti-rabbit Cy5. The securin protein displayed green fluorescence with goat anti-mouse FITC. The β-tubulin and nuclei were stained with the Cy3-labeled mouse anti-β-tubulin and Hoechst 33258, respectively. The relative protein levels under each treatment were the average of three independent experiments. Representative immunofluorescence data were shown from one of three separate experiments with similar findings.

1999; Zhang et al., 1999). Together, we suggest that combination of cytochalasin B and survivin siRNA enhances the decrease of securin protein expression and induces the cytotoxicity and cell growth inhibition in human cancer cells.

p53, a tumor suppressor protein, can regulate cell cycle arrest, apoptosis, and DNA repair in a variety of cells (Levine, 1997; Hofseth et al., 2004). We found that survivin was expressed and concentrated on the midbodies during cytokinesis in both A549 (p53-containing) and H1299 (p53-null) lung carcinoma cells. Cytochalasin B increased the levels of $G_2$/M fractions and caused the binuclei formation in A549 and H1299 cells. Therefore, our data indicate that cytochalasin B-mediated inhibition of cytokinesis results from a p53-independent pathway. However, the H1299 cells were more resistant to cytochalasin B-induced cytotoxicity than the A549 cells. In addition, the level of survivin proteins of H1299 cells was relatively higher than that of A549 cells. It has been shown that p53 can down-regulate the survivin protein levels in human cancer cells (Hoffman et al., 2002). Moreover, the inhibition of survivin protein expression may increase the level of p53 proteins and induce cell death in cancer cells (Beltrami et al., 2004). Therefore, the loss of functional p53 protein expression may reduce the cell death in the cytochalasin B- and survivin siRNA-treated cells.

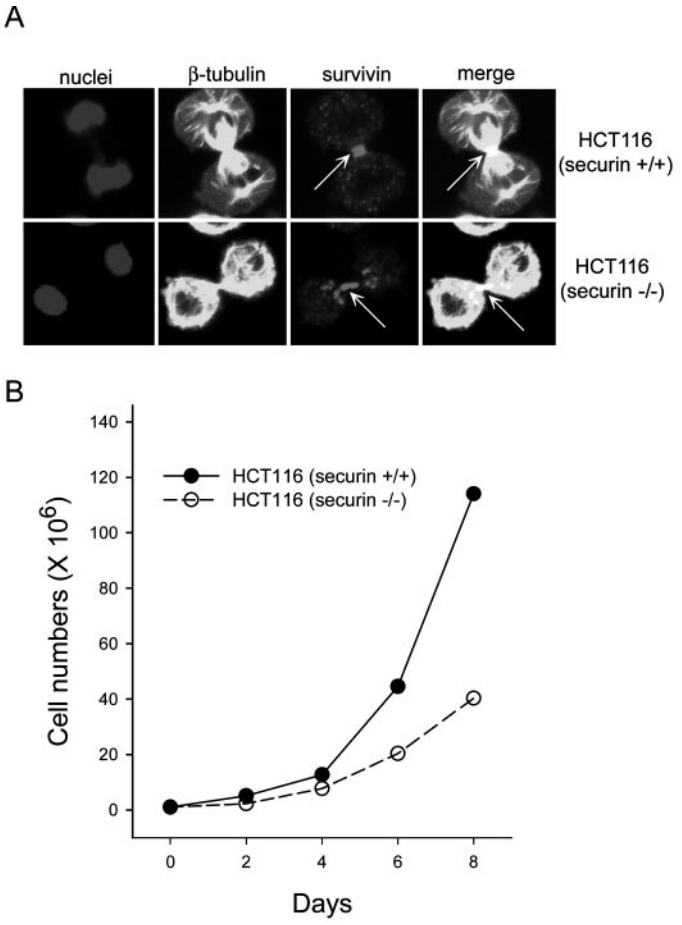


**Fig. 9.** The expression of survivin protein and the cell growth in the securin–wild-type and securin-null HCT116 colorectal carcinoma cells. A, the cells were fixed in 4% paraformaldehyde solution in PBS for 1 h at 37°C. The cells were incubated with rabbit anti-survivin antibody and then incubated with goat anti-rabbit Cy5. The β-tubulin and nuclei were stained with the Cy3-labeled mouse anti-β-tubulin and Hoechst 33258, respectively. B, the cells were plated at a density of $10^6$ cells/p100 Petri dish, and incubated for various times before they were counted by a hemocytometer. Results were obtained from the average of three experiments.

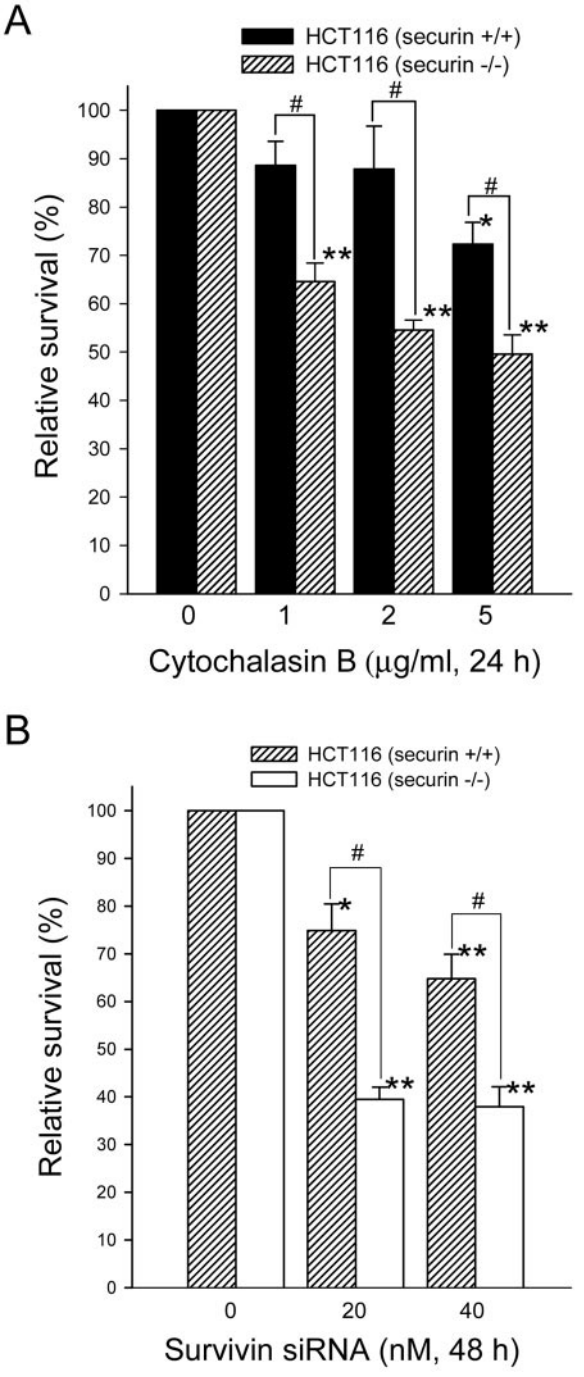


**Fig. 10.** Effects of survivin siRNA and cytochalasin B on the cytotoxicity in the securin–wild-type and securin-null HCT116 colorectal carcinoma cells. A, the cells were treated with 0–5 μg/ml cytochalasin B for 24 h. The cell survival was measured by MTT assay. Results were obtained from four to six experiments and the bar represents the mean ± S.E. B, the cells were transfected with survivin siRNA for 48 h. Results were obtained from four experiments, and the bar represents the mean ± S.E. $p < 0.05$ (*) and $p < 0.01$ (**) indicate comparisons between the control and transfection of survivin siRNA or cytochalasin B treatment in the securin–wild-type or securin-null HCT116 cells. $p < 0.05$ (#) indicates comparison between the securin–wild-type and securin-null HCT116 cells by the control and transfection of survivin siRNA or cytochalasin B treatment.

In conclusion, we propose that the survivin and securin proteins play an important role in cell cycle progression and resist the cytotoxicity of the cytochalasin B-exposed cells. The blockage of these proteins may increase the cell growth inhibition and apoptosis in human cancer cells. Understanding the mechanisms by which survivin and securin regulate the cell cycle progression and apoptosis, and using an effective method to block their gene and protein expression in human cancer cells, may provide a novel strategy in cancer therapy.

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