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Survivin mediates self-protection through ROS/cdc25c/CDK1 signaling pathway during tumor cell apoptosis induced by high fluence low-power laser irradiation

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ABSTRACT

Survivin, an important member of inhibitor-of-apoptosis (IAP) family, can be up-regulated by various pro-apoptotic stimuli, such as UV, photodynamic therapy (PDT) and cisplatin. High fluence low-power laser irradiation (HF-LPLI) is a newly discovered pro-apoptotic stimulator. The anti-apoptotic mechanism of survivin during HF-LPLI-induced apoptosis is still not investigated. Here, we report that HF-LPLI up-regulates survivin activity through reactive oxygen species (ROS)/cdc25c protein phosphatase (cdc25c)/cyclin-dependent kinase (CDK1) signaling pathway in human lung adenocarcinoma cells (ASTC-a-1). The up-regulation of survivin activity can reduce HF-LPLI-induced apoptosis, while down-regulation of the activity can promote the apoptosis. In addition, activated survivin delays mitochondrial depolarization, cytochrome *c* release, caspase-9 and Bax activation, all of which are typical pro-apoptotic events of cell apoptosis induced by HF-LPLI. On the basis of the present studies, we conclude that survivin can mediate self-protection during tumor cell apoptosis caused by HF-LPLI.

1. Introduction

Low-power laser irradiation (LPLI) can regulate several biological processes [1], including cell viability [2], cell proliferation [3], and cell apoptosis [4,5]. The biological effects of LPLI depend on some proteins, like protein kinase Cs (PKC) [6], protein kinase B (Akt/PKB) [7], Src tyrosine kinases [8], interleukin-8/1α (IL-8/1α), and Ras and coworkers [9]. It has been reported that laser irradiation has a stimulatory effect on cell proliferation at relatively low fluence, but an inhibitory effect at higher fluence [10]. LPLI at high fluence interfered with cell cycling and inhibited cell proliferation, thus could be used to control certain types of hyperplasia [11]. This phenomenon is consistent with our early results of ASTC-a-1 cell apoptosis induced by HF-LPLI at the fluence of 60 and 120 J/cm^2 [12].

Survivin is a member of the inhibitors of apoptosis protein (IAPs) family [13]. Survivin is dramatically overexpressed in most human cancers and correlates with unfavorable prognosis, resistance to therapy, and accelerated rates of recurrences [14]. Survivin expresses in several subcellular compartments, like cytosol, mitochondria and nucleus [15]. One of the critical requirements for survivin stability and function was recently identified in the phosphorylation on Thr-34 by cyclin-dependent kinase 1 (CDK1) [16], and a phosphorylation-mimetic survivin mutant strongly inhibited p53-induced apoptosis [17]. This step has also been exploited for anticancer therapy, and inducible expression or adenoviral delivery of dominant-negative mutant survivin (T34A-survivin) prevented

Abbreviations: HF-LPLI, high fluence low-power laser irradiation; ASTC-a-1, human lung adenocarcinoma cells; ROS, reactive oxygen species; IAPs, inhibitors of apoptosis protein; XIAP, X-linked inhibitor-of-apoptosis protein; $\Delta\Psi_m$, mitochondrial transmembrane potential; DHA, dehydroascorbic acid; Rh123, rhodamine 123; CCK-8, Cell Counting Kit-8; CDK1, cyclin-dependent kinase 1; Bax, Bcl-2-associated X protein; PDT, photodynamic therapy; MPT, mitochondrial permeability transition; cdc25c, cdc25c protein phosphatase.

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phosphorylation of endogenous survivin, which resulted in caspase-9-dependent apoptosis [18,19]. It has been known that the activity of CDK1 is regulated by cdc25c phosphatase. The abrogation of Nox4-generated reactive oxygen species (ROS) resulted in the inhibition of cdc25c activity [20]. Therefore, it is reasonable to speculate that the level of cdc25c activity is regulated by ROS.

HF-LPLI induced apoptosis through mitochondrial permeability transition (MPT) which is mediated by a high-level of intracellular ROS generation. The decrease of mitochondrial transmembrane potential ($\Delta\Psi_m$) caused by MPT triggers outer mitochondrial permeabilization and subsequently release of cytochrome *c*, followed by caspase cascade reaction [5]. Bax is also activated during HF-LPLI-induced apoptosis. The ability of cells to evade apoptosis is a molecular trait probably common to all human cancers [21]. This leads to aberrantly extended cell viability, which translates in accelerated disease progression, and resistance to therapy [22]. Among the regulators of apoptosis involved in cancer, interests have been recently focused on survivin [14]. The possibility of investigating the anti-apoptotic mechanism of survivin has been intensely investigated. In these studies, molecular antagonists of survivin including antisense molecules or dominant-negative mutants cause tumor cell apoptosis, enhance chemotherapy-induced cell death, and result in anticancer activity *in vivo* [23].

In the present study, we show that HF-LPLI activated survivin by inducing increase in its phosphorylation levels in Thr-34 during tumor cell apoptosis. Activated survivin obviously delayed a series of pro-apoptotic events caused by HF-LPLI, including the decease of $\Delta\Psi_m$, the release of cytochrome *c*, the activation of Bax and caspase-9. In contrast, the decline in the survivin phosphorylation levels through T34A-survivin overexpression facilitated HF-LPLI-induced cell apoptosis. Our findings demonstrate that survivin mediates self-protection through ROS/cdc25c/CDK1 signaling pathway during tumor cell apoptosis induced by HF-LPLI.

2. Materials and methods

2.1. Materials

Dehydroascorbic acid (DHA) was purchased from Sigma (St. Louis, MO, USA). Anti-phospho-Survivin (Thr-34) antibody and anti-Survivin antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-GFP antibody was obtained from Proteintech Group, Inc. (Campell Park, Chicago, USA). DNA Extraction kit was purchased from Qiagen (Valencia, CA, USA). Rhodamine 123 (Rh123) and tetramethylrhodamine methyl esters (TMRM) were purchased from Molecular Probes Inc. (Eugene, OR, USA). Flavopiridol was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Thymidine was purchased from Amresco (Solon, OH, USA).

2.2. Cell culture and transfection

ASTC-a-1 cells were grown in DMEM (GIBCO, Grand Island, NY, USA) medium supplemented with 15% fetal bovine serum (FBS) (Sijiqing, Hangzhou, China), 50 units/ml penicillin, and 50 g/ml streptomycin, in 5% CO_2, and 95% air at 37 °C in a humidified incubator. In all experiments, 70–85% confluent cultures were used.

Transient transfections were performed with 1 μg of expression vectors using the Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions in serum-free medium. The serum-free medium was replaced with fresh culture medium after 5 h and incubated for an additional 24 h for expression. The efficiency of transfection was assessed by co-transfection with a green fluorescent protein (GFP) reporter vector and analyzing for GFP expression by flow cytometry 24 h after transfection.

2.3. HF-LPLI treatment and cell viability assays

ASTC-a-1 cells were cultured at a density of 4×10^3 cells/well in 96-well microplates. Twenty-four hours after transfection with plasmid DNA of GFP-WT-survivin (a kind gift from Dr. O. Inanami, Hokkaido University, Japan), the cells were irradiated using a fiberoptic light delivery system (635 nm, semiconductor laser, NL-FBA-2.0-635, nLight Photonics Corporation, Vancouver, WA, USA) at a dose of 120 J/cm^2. The interval wells were filled with black ink. After irradiation, the cells were maintained in DMEM with 15% FBS and were returned to the incubator for a further culture. The irradiation was performed on monolayer cells. In all cases, non-irradiated cells were kept in the same conditions as the treated cells. Cell viability was assessed with Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) at 5 and 10 h after the laser irradiation. At the indicated time, CCK-8 was added to cells and incubated for 1.5 h. OD_{450} was read with a 96-well plate reader (DG5032, Hua dong, Nanjing, China). The value was directly proportional to the number of viable cells in a culture medium and the cell proliferation.

2.4. HF-LPLI treatment for single cell analysis

For irradiation of cells, a 633-nm He–Ne laser inside a confocal laser scanning microscope (LSM510-ConfoCor2) (Zeiss, Jena, Germany) was used in HF-LPLI treatment. Laser irradiation was performed through the objective lens of the microscope. In this setup, only the cells under observation were irradiated by the laser. A minitype culture chamber with CO_2 supply (Tempcontrol 37–2 digital, Zeiss, Germany) was used in order to keep cells under normal culture conditions (37 °C, 5% CO_2) during irradiation. Under the HF-LPLI treatment, the cells in selected area were irradiated at a fluence rate 0.2 W with a total fluence of 120 J/cm^2.

2.5. Laser scanning confocal microscopy (LSCM)

To image single cells, the confocal laser scanning microscope system (LSM510-ConfoCor2) (Carl Zeiss, German) was used. All images were acquired after laser irradiation with a Plan–Neofluar 40×/NA1.3, oil-immersed objective lens. The specific imaging process is as follows. The excitation wavelengths were 514 nm for pYFP-survivin and

pYFP-Bax, 488 nm for pGFP-cyt *c*, 458 nm for pCFP-Bax, 543 nm for TMRM and pDsRed-mit. The emission detection filters were band pass 520–555 nm for pYFP-survivin and pYFP-Bax, band pass 500–550 nm for pGFP-cyt *c*, band pass 470–500 nm for pCFP-Bax, band pass 565–615 IR for TMRM, long pass 560 nm for pDsRed-mit. To quantify the results, the fluorescence emission intensities (including the background fluorescence) were obtained with Zeiss Rel 3.2 image processing software (Carl Zeiss, German).

2.6. Fluorescence resonance energy transfer (FRET) analysis

Plasmid DNA of SCAT9, a FRET probe (a kind gift from Dr. M. Miura, Osaka University, Japan) consists of a donor (enhanced cyan fluorescent protein, ECFP) and an acceptor (Venus, a mutant of yellow fluorescent protein). The linking sequence contains a caspase-9 cleavage, LEHD. The activation of caspase-9 leads to the cleavage of the linker, thus, effectively reduces the FRET. Using FRET technique based on SCAT9, the spatiotemporal dynamics of caspase-9 activity in individual living cells can be monitored in real-time.

FRET was performed on a commercial laser scanning microscopes (LSM510/ConfoCor2) combination system (Carl Zeiss). The excitation wavelengths were 458 nm for SCAT9, 514 nm for Venus. The emission fluorescence channels were 470–500 nm for CFP, 535–545 for Venus. The quantitative analysis of the fluorescence images was performed using Zeiss Rel 3.2 image processing software (Carl Zeiss, German).

2.7. Flow cytometry

Cell apoptosis was assessed with AnnexinV-FITC apoptosis detection kit (BD Biosciences, San Diego, CA, USA) 5 h after the laser irradiation. Cells were collected and washed with PBS, then stained with 5 μl AnnexinV-FITC and 10 μl PI (10 μg/ml) for 30 min at room temperature in the dark. Cells were subjected to fluorescence activated cell sorting (FACS) analysis using a flow cytometer (FACS. Canto^{TM} IIBD, USA).

The $\Delta\Psi_m$ was also measured by flow cytometry (FCM). Cells were grown at a density of 1×10^5 cells on 30-mm diameter cellular culture utensils. After treatment with laser irradiation for 0, 1, 2 and 4 h, cells were collected and washed with PBS for three times. Cells were stained with 1 μM Rh123 for 30 min. After that, cells were collected and were washed twice with PBS. Fluorescence emitted from the Rh123 was detected with the flow cytometer.

2.8. Spectrofluorometric analysis

ASTC-a-1 cells transfected with pSCAT9 were cultured for 24 h and then treated with HF-LPLI (120 J/cm^2) or UV-C irradiation. Five hours after irradiation, the cells were transferred into a 96-well flat-bottomed microplate. The microplate was then placed inside the sample chamber of a luminescence spectrometer (LS55, PerkinElmer, Wellesley, MA, USA). The fluorescence emission spectra were then acquired. The step length of the scanning spectra was 2 nm. The excitation wavelength of SCAT9 was 434 ± 5 nm and the emission fluorescence channel was band pass 454–600 nm.

2.9. Western blotting analysis

After treatments, cells were collected, washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4), and lysed with ice-cold lysis buffer for 30 min on ice. The lysates were collected at 4 °C, and the protein concentration was determined. Equivalent samples were subjected to SDS–PAGE on 15% gel. The proteins were then transferred onto polyvinylidene fluoride membranes, and probed with primary antibody: anti-phospho-survivin (Thr-34), anti-Survivin, anti-GFP and anti-β-actin, followed by IRDye 800 secondary antibodies and Alexa Fluor 680 secondary antibodies. Detection was performed using the Li-COR Odyssey Infrared Imaging System (Li-COR, Inc., USA).

2.10. Colonogenic assay

Colonogenic assays were carried out on growing cells. Briefly, ASTC-a-1 cells was plated onto a 6-well plate at a density of 5000 cells/well in DMEM containing 15% FBS.

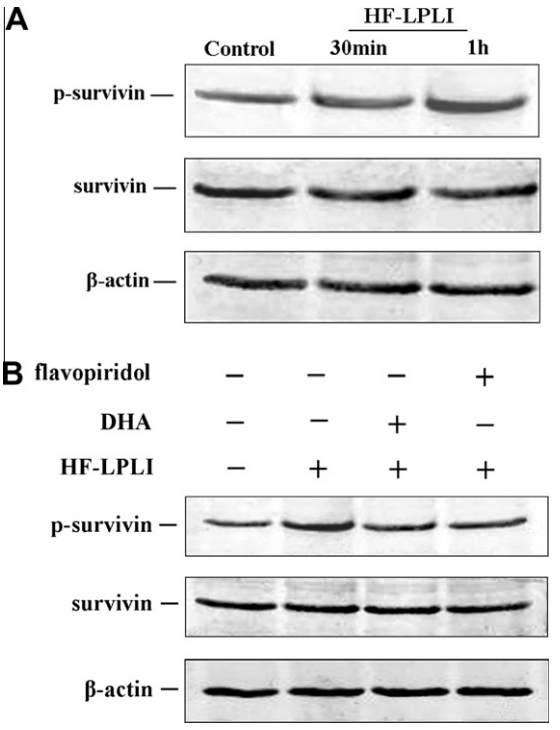


Fig. 1. HF-LPLI increases survivin phosphorylation, which is mediated by ROS and CDK1 in ASTC-a-1 cells. (A) ASTC-a-1 cells were pre-synchronized by thymidine (2 mM), and were exposed to 120 J/cm^2 of HF-LPLI. Cell lysates were collected at 30 min or 1 h after light exposure and assayed for phosphorylated survivin (p-survivin), total survivin and β-actin levels. (B) After incubation with DHA for 30 min or flavopiridol for 24 h, pre-synchronized ASTC-a-1 cells were irradiated and analyzed for survivin phosphorylation by Western blotting analysis. Survivin and β-actin were used as loading controls. Results represent one of three replicates.

Before the induction of apoptosis, cells were transfected with WT-survivin or T34A-survivin for survivin overexpression. Twenty-four hours after transfection, cells were treated with HF-LPLI. Colonies formed within 1–2 weeks. The cells were then fixed in 50% methanol in PBS followed by 100% methanol for 10 min each and stained with Giemsa (Sigma, St. Louis, MO, USA) and then individual colonies were counted.

2.11. Statistics analysis

All assays were repeated independently for a minimum of three times. Data were represented as mean ± SD. Statistical analysis was performed with Student's paired *t*-test. Differences were considered statistically significant at $^{*,\#}P < 0.05$. The symbol $*$ denoted the difference between experimental group and control group, while # indicated the discrepancy between two experimental groups connected by a line.

3. Results

3.1. HF-LPLI activates survivin through phosphorylation on Thr-34 by ROS and/or CDK1 in ASTC-a-1 cells

As is known to all, survivin is activated through phosphorylation on Thr-34, which is necessary for the anti-apoptotic function of survivin [16]. A variety of anticancer agents and exogenous stress conditions, including Adriamycin, taxol, PDT and UV, can increase survivin expression and phosphorylation [24,25]. So we initially examined whether HF-LPLI could affect the phosphorylation of survivin on Thr-34. ASTC-a-1 cells were pre-synchronized by thymidine (2 mM), and irradiated with HF-LPLI at a fluence of 120 J/cm^2. The fluence can induce ASTC-a-1 cell apoptosis as demonstrated before [4]. Western blotting analysis showed that the phosphorylation levels of survivin increased remarkably 1 h after the treatment, while the total amount of survivin remained unchanged (Fig. 1A). It was also shown a moderate increase in the level of protein phosphorylation 30 min after the treatment as compared with control. Non-irradiated cells under the same experimental procedures were used as control.

Previous studies have shown that survivin physically associates with the cyclin-dependent kinase 1 (p34^{cdc2}), and is phosphorylated on Thr-34 by CDK1, *in vitro* and *in vivo* [16]. It is also known that a high-level intracellular ROS generation has been observed after HF-LPLI treatment [5]. Therefore, we sought to determine whether survivin phosphorylation

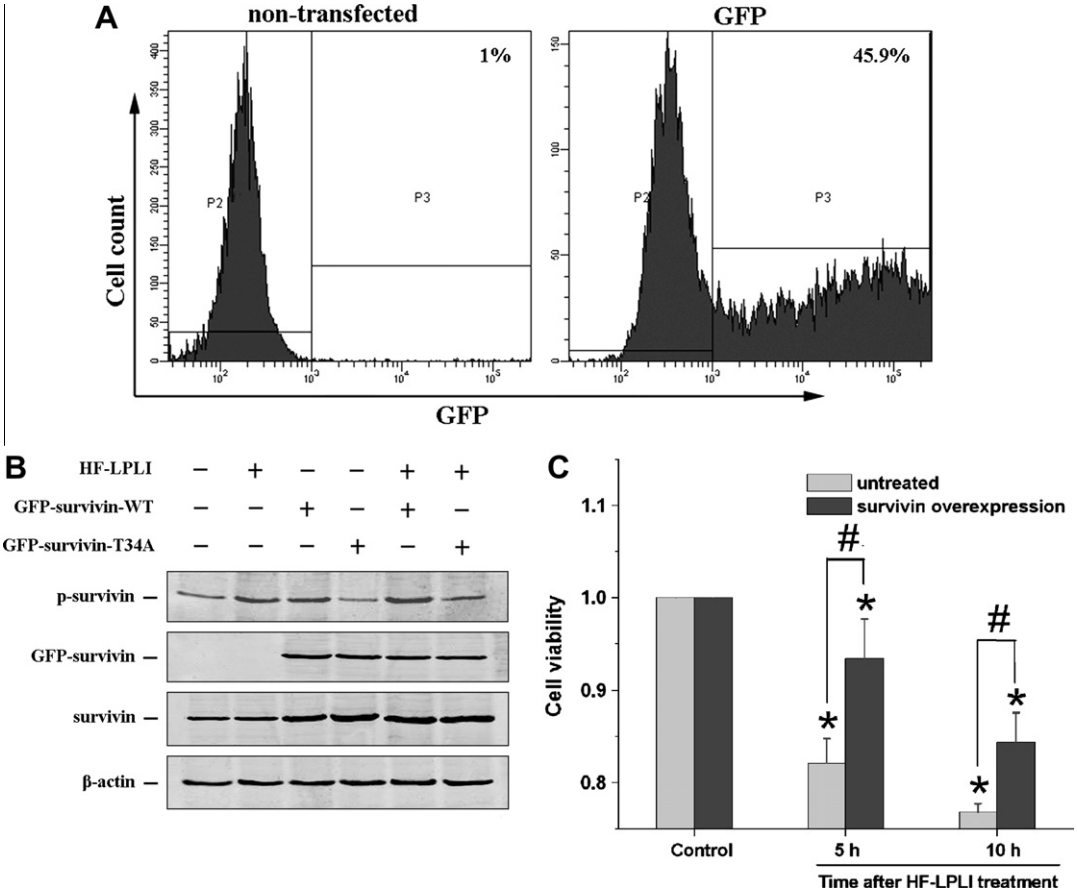


Fig. 2. The relationship between the level of survivin phosphorylation and cell viability under HF-LPLI treatment. (A) GFP fluorescence in ASTC-a-1 cells was assayed by flow cytometry 24 h post-transfected with GFP expression plasmid pGFP. (B) The levels of phosphorylated survivin with overexpression of WT-survivin or T34A-survivin. Pre-synchronized ASTC-a-1 cells with different treatments were collected at 1 h post-irradiation, and were analyzed by Western blotting analysis. The expression of exogenous survivin was assessed by anti-GFP antibody. Survivin and β-actin were used as loading controls. Results represent one of three replicates. (C) CCK-8 assayed the viability of ASTC-a-1 cells with survivin overexpression or not at 5 h and 10 h after HF-LPLI treatment. Cells without HF-LPLI treatment were control. (D and E) Five hours after HF-LPLI treatment, cells transfected with WT/T34A-survivin were stained with Annexin V-FITC and PI as described before. (D) The fluorescences of FITC and PI were quantified by flow cytometry. (E) The comparison among the percentages of apoptotic cells under different conditions ($^{*,\#}P < 0.05$).

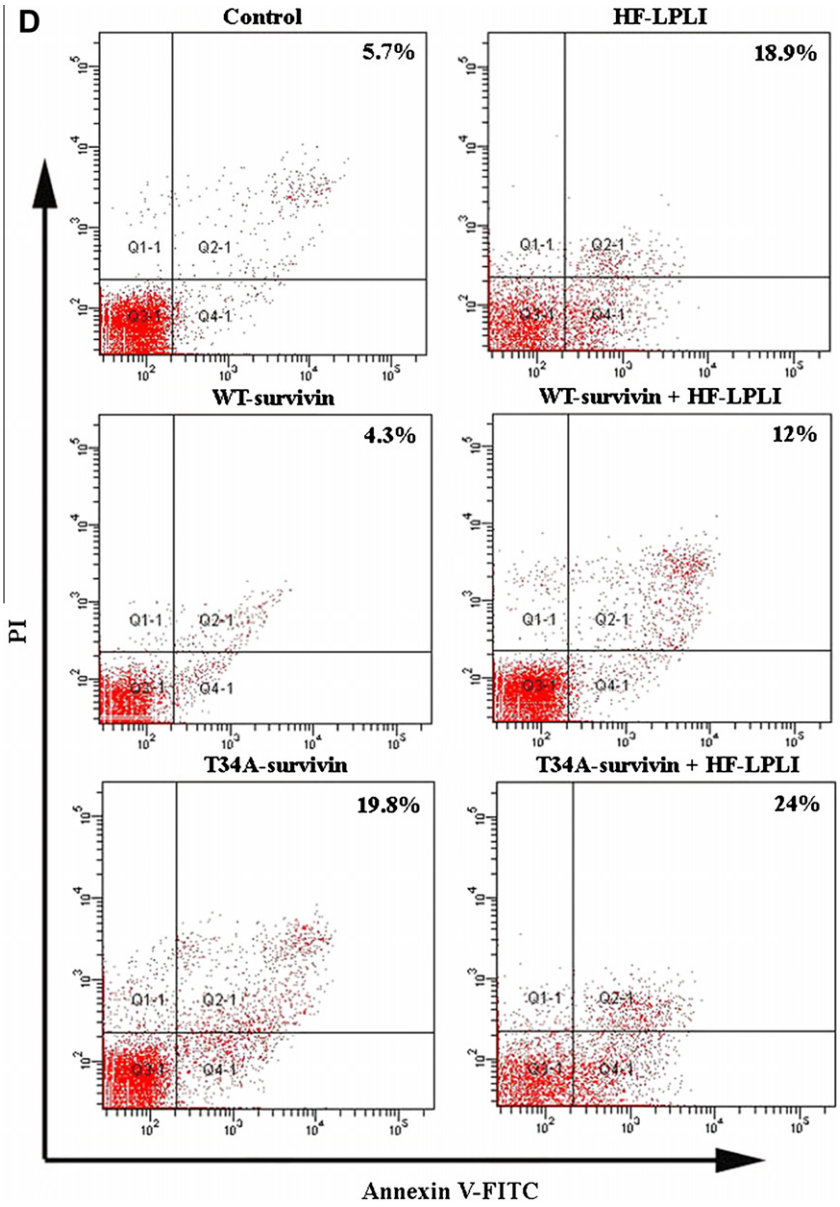


Fig. 2 (*continued*)

was dependent on ROS generation and CDK1 activity. After pre-incubated with CDK1 inhibitor flavopiridol (100 nM) or ROS scavenger DHA (100 μM), pre-synchronized ASTC-a-1 cells were treated with HF-LPLI and analyzed 1 h later by Western blotting. As shown in Fig. 1B, the level of survivin phosphorylation was significantly decreased in the presence of either flavopiridol or DHA as compared with the group which was treated with HF-LPLI only. We therefore speculated that flavopiridol and DHA inhibited survivin phosphorylation in HF-LPLI-treated cells.

Based on our results, it is reasonable to speculate that HF-LPLI activates survivin through phosphorylation on Thr-34. Both the ROS and CDK1 activity are involved in survivin activation.

3.2. Up-regulation of survivin activity suppresses HF-LPLI-induced apoptosis, while down-regulation of survivin activity enhances the apoptosis

The efficiency of transient transfection was determined by assaying GFP expression by flow cytometry and comparing with non-transfected cells. Flow cytometric analysis of GFP expression indicated that the transfection efficiency was greater than 40% (Fig. 2A). To determine the effect of survivin overexpression on survivin phosphorylation, we transfected two GFP-fused plasmids, GFP-WT-survivin and GFP-T34A-survivin, into ASTC-a-1 cells. Then the cells were synchronized by thymidine (2 mM), followed by HF-LPLI treatment. One hour later, the levels of survivin phosphorylation were confirmed by Western blotting analysis. As shown in Fig. 2B, 1 h after HF-LPLI treatment, the cells with overexpression of GFP-WT-survivin showed an obviously high level of survivin phosphorylation in comparison with non-overexpressed cells. In contrast, overexpression of GFP-T34A-survivin evidently reduced the survivin phosphorylation levels (Fig. 2B). This is probably because that the survivin dominant-negative mutant T34A competes with endogenous survivin for access to kinases and thereby prevents the phosphorylation of endogenous survivin [26].

To define the protective role of survivin on apoptosis, we used CCK-8 method to determine viabilities of cells with survivin overexpression. Non-irradiated cells were used as control. Cells were assessed 5 and 10 h post-irradiation. Cell viability was gradually decreased 10 h post HF-LPLI treatment (Fig. 2C). It was also shown that the decrease of cell

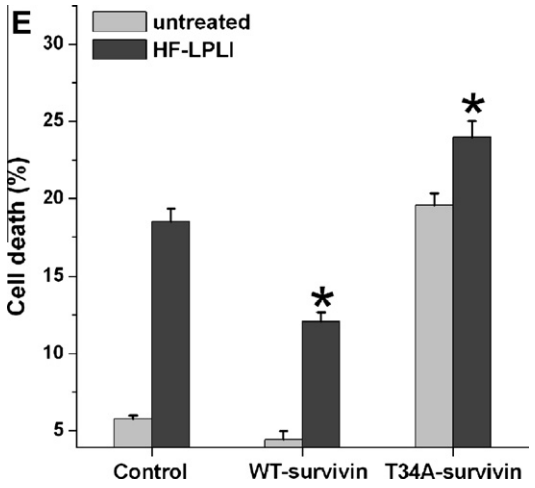


Fig. 2 (*continued*)

viability was significantly prevented when survivin overexpression was applied to the cells, indicating that survivin overexpression suppressed apoptosis.

To further investigate the anti-apoptotic function of survivin, ASTC-a-1 cells were transfected with WT/T34A-survivin, followed by HF-LPLI treatment. Cells were stained with AnnexinV-FITC and PI at 5 h post-irradiation, and their fluorescences were quantified by flow cytometry for cell apoptosis assessment. As shown in Fig. 2D, under HF-LPLI treatment, the percentage of apoptosis in T34A-overexpressing cells (24.0%) was obviously higher than that in non-transfected cells (18.9%), while the apoptotic percentage in WT-overexpressing cells (12.0%) was much lower. The data revealed that the overexpression of T34A-survivin significantly enhanced HF-LPLI-induced apoptosis, whereas the overexpression of WT-survivin remarkably suppressed the apoptosis (Fig. 2E). These results suggest that under HF-LPLI treatment, survivin inhibits apoptosis, which is dependent on the activation of survivin through phosphorylation on Thr-34.

3.3. Survivin postpones mitochondrial depolarization induced by HF-LPLI

The decrease of the $\Delta\Psi_m$ may disrupt the mitochondria structure, and cause the release of pro-apoptotic factors [4]. For monitoring the real-time changes in $\Delta\Psi_m$, cells stained with TMRM were treated with HF-LPLI and imaged by confocal microscopy. As shown in Fig. 3A, immediately after laser irradiation, the mitochondrial TMRM fluorescence emission intensity decreased rapidly and reached the lowest plateau 45 min post-treatment, while in control cells, the intensity of TMRM remained unchanged.

It was recently shown that survivin-ΔEx3, a kind of survivin splice variant, maintained $\Delta\Psi_m$ and inhibited the generation of ROS during lymphotoxin-β receptor–mediated cell death [27]. To determine whether survivin has a similar inhibitory effect on $\Delta\Psi_m$, ASTC-a-1 cells were transfected with YFP-survivin, followed by incubated with TMRM for 30 min and then treated with HF-LPLI. It could be observed that after laser irradiation, the starting time of the $\Delta\Psi_m$ decrease in cells transfected with WT-survivin (YFP-survivin) was about 20 min later than that of cells without survivin overexpression (Fig. 3A).

To further validate our speculation about the inhibitory effect of survivin on $\Delta\Psi_m$ reduction, we used flow cytometry to confirm the collapse of $\Delta\Psi_m$ at 0, 1, 2 and 4 h after HF-LPLI treatment. As shown in Fig. 3B, the percentage of cells exhibiting high mitochondrial transmembrane potential were 86.5%, 89.1%, 83.1% at 2 h post-irradiation in "non-transfected" group, "WT-survivin" group, and "T34A-survivin" group, respectively. The data revealed that the decrease speed of $\Delta\Psi_m$ in cells of "WT-survivin" group was much slower than that in cells of "non-transfected" group while T34A-survivin overexpression sped up the decrease of $\Delta\Psi_m$ induced by HF-LPLI (Fig. 3C).

All these results strongly demonstrate that under HF-LPLI treatment, survivin delays the decrease of $\Delta\Psi_m$ and that phosphorylation deficiency of survivin on Thr-34, that is to say, inactivation of survivin, removes this inhibition effect on $\Delta\Psi_m$ reduction.

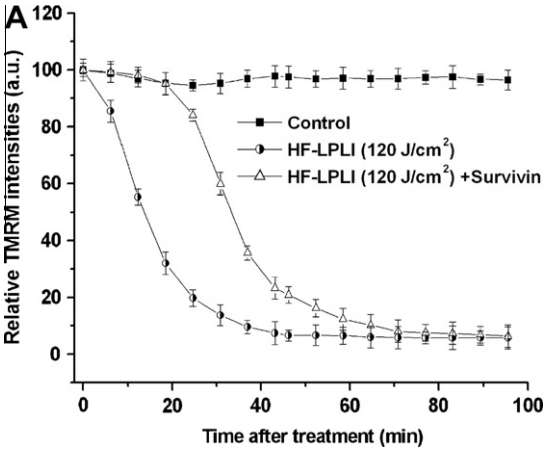


Fig. 3. Survivin delays the decrease of $\Delta\Psi_m$ induced by HF-LPLI treatment, whereas inhibition of survivin phosphorylation speeds up $\Delta\Psi_m$ decrease. (A) ASTC-a-1 cells were loaded with TMRM probe and then were irradiated and imaged (ex. 543 nm, em. BP 565-615 nm). The relative fluorescence intensities of TMRM ($n = 5$) were taken from three groups of time-course images of the cells with different treatments (Control, HF-LPLI, HF-LPLI + Survivin). Each curve represents an average of about 10 cells obtained from five independent experiments. Relative fluorescence intensities in each curve at 0 min after irradiation are normalized to 100. (B and C) Cells were stained with Rh123, which indicated $\Delta\Psi_m$, and then were irradiated and assayed 0, 1, 2 or 4 h later, respectively. (B) Fluorescence emitted from the Rh123 was detected by flow cytometry. (C) The trend of changes over time in the percentage of cells exhibiting high mitochondrial membrane potential under different treatments.

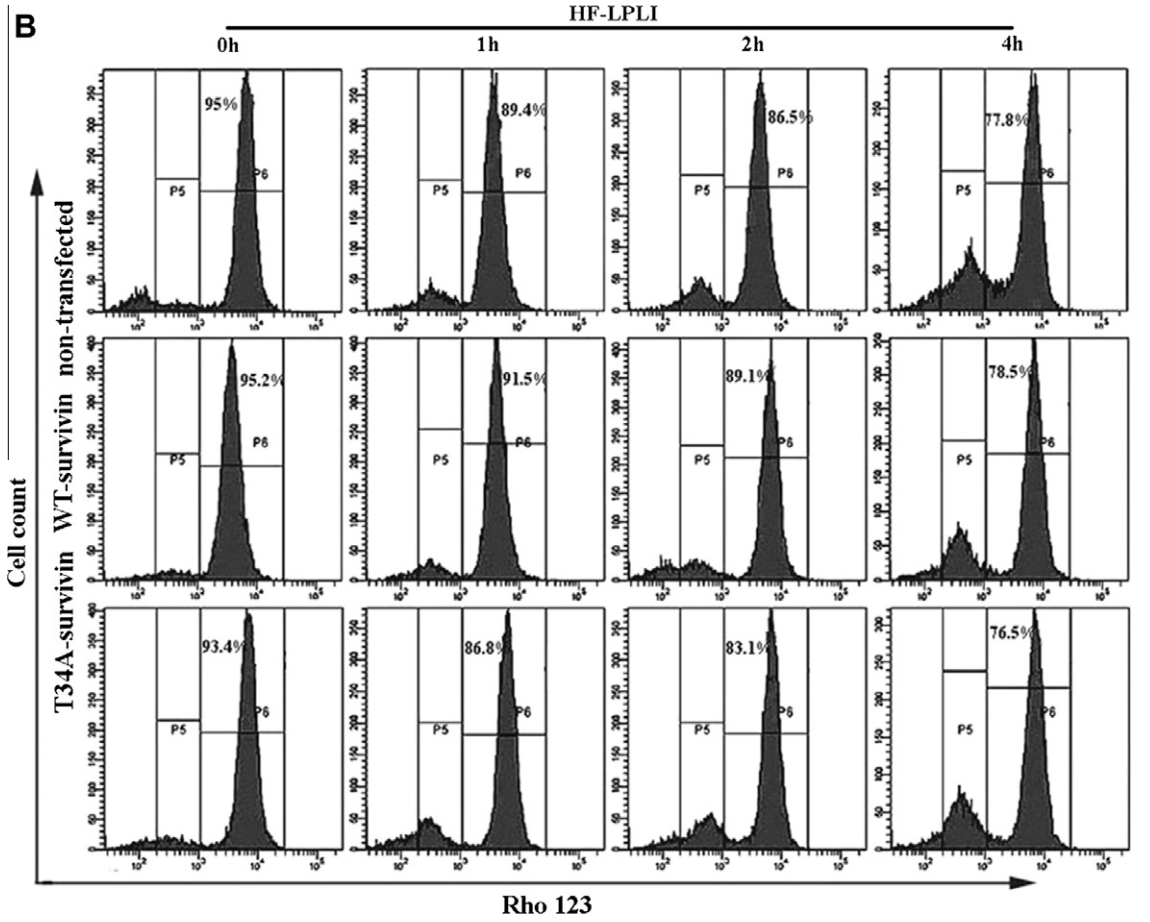
3.4. Survivin delays cytochrome c release induced by HF-LPLI

It was reported that survivin-ΔEx3 prevented the release of cytochrome *c* from Hep3BT2 cells after LIGHT/IFN-γ treatment [27]; therefore, we asked whether survivin could also inhibit the release of cytochrome *c* after HF-LPLI treatment. To address this question, GFP-cyt *c* and YFP-survivin were doubly transfected into ASTC-a-1 cells and then the cells were irradiated. In non-irradiated cells, GFP-cyt *c* was co-localized with DsRed-mit, a marker for mitochondria. As shown in Fig. 4, after HF-LPLI treatment, the starting time of the cytochrome *c* release from mitochondria in cells transfected with WT-survivin (YFP-survivin) (~74 min) was much later than that in non-overexpressing cells. The data demonstrate that survivin delays cytochrome *c* release induced by HF-LPLI for about 10 min.

3.5. Survivin delays Bax activation induced by HF-LPLI

Because survivin prevented the accumulation of pro-apoptotic Bim and Bax at mitochondria during DNA-damage induced apoptosis, such as doxorubicin and etoposide [28], we studied whether survivin overexpression could influence Bax activation after HF-LPLI treatment. 120-mJ/cm^2 UV-C irradiation, a DNA-damage stress which could induce Bax translocation [29], was used as a positive control. To determine the inhibitory effect of survivin on Bax activation, ASTC-a-1 cells were co-transfected with CFP-Bax and YFP-survivin, followed by UV-C irradiation. As shown in the lower part of Fig. 5A, CFP-Bax maintained a diffuse distribution in the whole cell, a non-actived state, even at 5 h after UV-C irradiation. These results suggest that survivin overexpression inhibits the Bax activation upon UV stimulation.

Previous reports demonstrated that Bax signaling pathway was involved in HF-LPLI-induced apoptosis [5]. To explore the inhibitory effect of survivin on Bax activation, ASTC-a-1 cells doubly transfected with CFP-Bax and YFP-survivin were treated with HF-LPLI and imaged by confocal microscopy (Fig. 5C). As shown in Fig. 5C and 5D, the activation of Bax occurred at about 115 min after HF-LPLI treatment, whereas the Bax activation happened at ~4 h post-irradiation in survivin-overexpressing cells. The data suggest that survivin delays Bax activation under HF-LPLI treatment.



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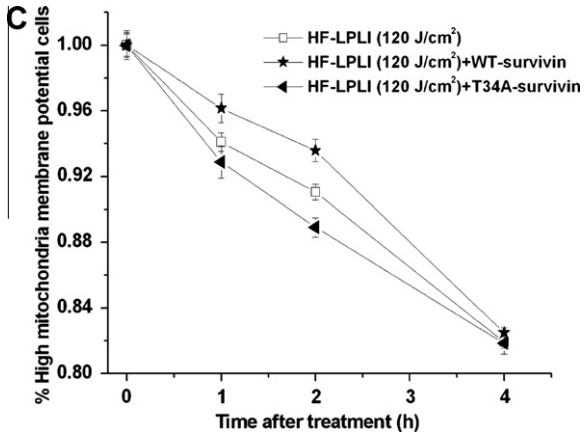

Fig. 3 (*continued*)

3.6. Survivin inhibits caspase-9 activation induced by HF-LPLI

Previous works showed that HF-LPLI could induce cell apoptosis via the mitochondrial signaling pathway (mitochondria/caspase-3) [4]. To further investigate the mechanism involved in the apoptotic process upon HF-LPLI treatment, FRET imaging was used to determine the activation of caspase-9 in ASTC-a-1 cells transfected with SCAT9 reporters. The typical time-course FRET/CFP ratio images of cells showed an intensity decrease

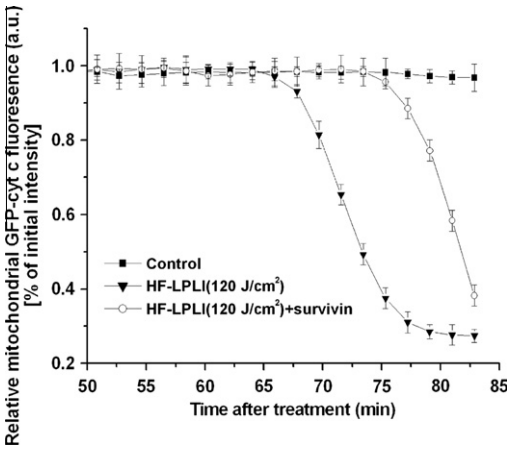

Fig. 4. Survivin delays the HF-LPLI-induced cytochrome *c* release from mitochondria. ASTC-a-1 cells were co-transfected with pDsRed-mit (red emission) for mitochondrial localization and pGFP-cyt *c* (green emission) and then irradiated. Fluorescence images were acquired by confocal microscopy. Relative mitochondrial GFP-cyt *c* fluorescences (% of initial intensity) were taken from three groups of time-course images of the cells with different treatments (Control, HF-LPLI, HF-LPLI + survivin). Each curve represents an average of about 10 cells obtained from five independent experiments. Mitochondrial GFP-cyt *c* fluorescence intensity in each curve at the first time point is normalized to 1.

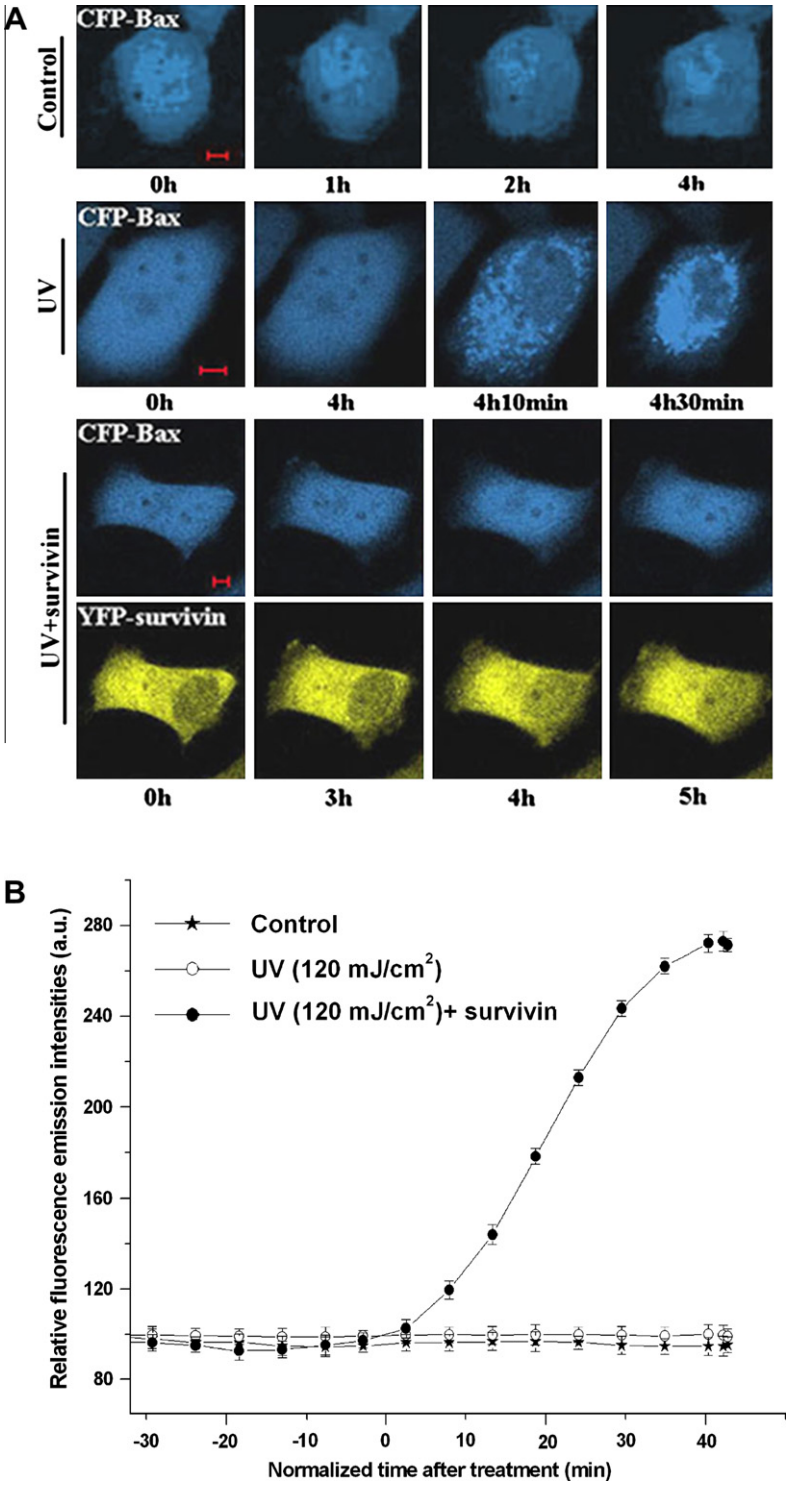

Fig. 5. Spatial and temporal changes in Bax subcellular localization after UV or HF-LPLI treatment. (A) Typical time-lapse confocal imaging of living cells transiently transfected with CFP-Bax and/or YFP-survivin under 120-mJ/cm^2 UV-C irradiation. Similar results were obtained from five independent experiments. (B) Time courses of Bax translocation after different treatments. Each curve represents an average of 10 cells from five independent experiments. (C) Typical time-lapse confocal imaging of living cells under various treatments. Similar results were obtained from five independent experiments. (D) Time courses of Bax translocation after different treatments. Each curve represents an average of ~10 cells from five independent experiments. CFP/YFP-Bax fluorescence intensity in each curve at the first time point is normalized to 100. Bar = 5 μm.

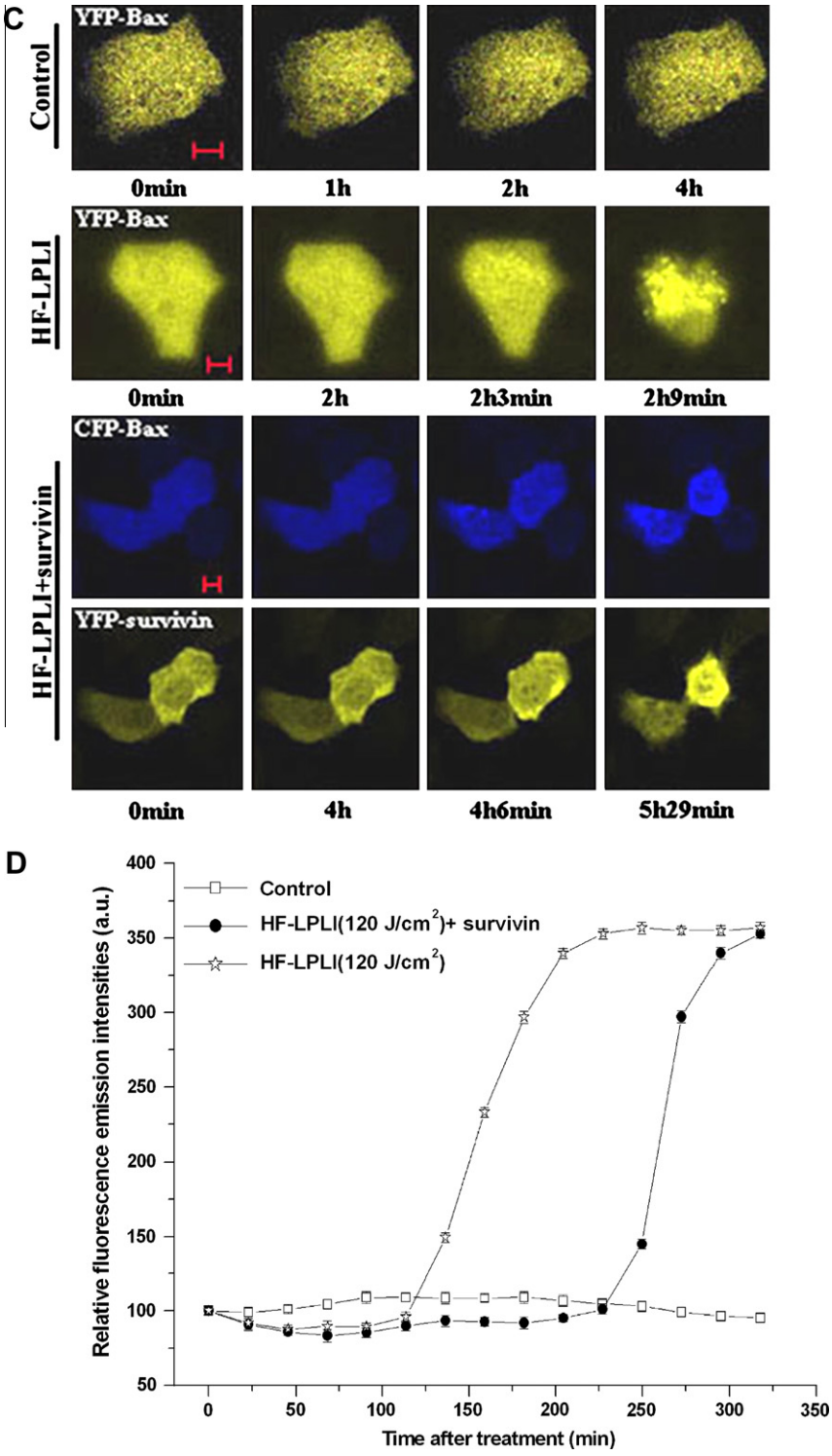

Fig. 5 (*continued*)

in Fig. 7A (bottom), indicating the caspase-9 activation. As shown in Fig. 7B, the average time of the onset of caspase-9 activation was about 75 min after HF-LPLI treatment. The caspase-9 activation first increased gradually for about 175 min and then increased sharply. These results show that HF-LPLI can activate caspase-9.

It is known that survivin suffers phosphorylation on Thr-34 by CDK1. This phosphorylation site is ideally positioned in the crystal structure to stabilize potential anti-apoptotic protein–protein interactions mediated by the survivin BIR [14]. Loss of Thr-34 phosphorylation resulted in the dissociation of a survivin–caspase-9 complex, and caspase-9-dependent

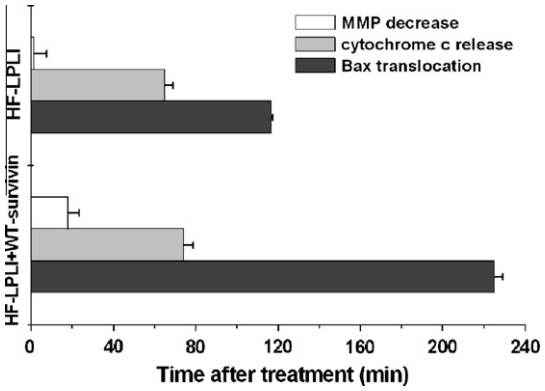

Fig. 6. The comparison of the starting times for mitochondria membrane potential ($\Delta\Psi_m$) decrease, cytochrome *c* release and Bax translocation induced by HF-LPLI with survivin overexpression or not. The data showed that survivin delays the onset of MMP decrease for about 17 min, cytochrome *c* release for about 10 min, Bax translocation for about 110 min.

apoptosis in various cancer-cell types [16]. To examine the inhibitory effect of survivin on caspase-9 activation, spectrofluorometric analysis, a technique for monitoring the overall profile of FRET fluorescence emission from a group of cells, was applied to measure the activation of caspase-9. Five groups of ASTC-a-1 cell samples were all transfected with SCAT9 reporters, and two of them were co-transfected with T34A-survivin and WT-survivin, respectively. Next, the samples were treated with HF-LPLI or UV-C irradiation. Five hours later, all groups were detected with a luminescence spectrometer for fluorescence emission spectra. The ratio of FRET/CFP is inversely proportional to the caspase-9 activity. Under HF-LPLI treatment, the caspase-9 activity in the cells overexpressing WT-survivin was much lower than that in non-overexpressing cells, while the activity of caspase-9 in T34A-survivin expressing cells was the highest of all (Fig. 7C).

These results suggest that survivin inhibits caspase-9 activation induced by HF-LPLI, and the inhibition of survivin activity by T34A-survivin promotes caspase-9 activation.

3.7. Up-regulation of survivin activity reduces the efficacy of HF-LPLI treatment, while down-regulation of survivin activity improve the efficacy

The perhaps most important parameter for the efficacy of HF-LPLI treatment is its long-term effect on cancer cell viability [30]. Therefore, we used colonogenic assays to test whether the regulation of survivin activity during a pulse of HF-LPLI treatment would have a bearing on the outcome in a long-term assay that challenges the capacity of a cell to survive and thrive after an insult instead of querying a specific molecular mechanism (Fig. 8). Cells were treated with HF-LPLI and/or WT/T34A-survivin overexpression as detailed in Section 2. After that they

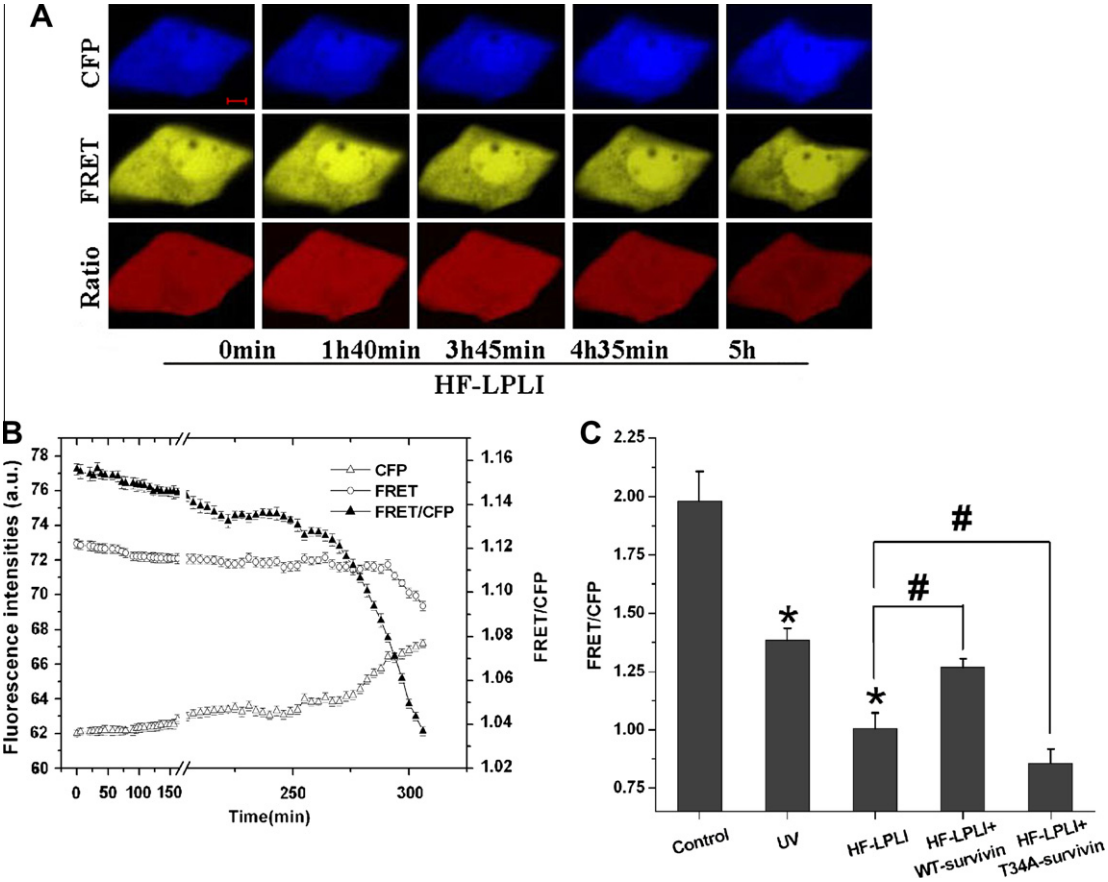

Fig. 7. Real-time monitoring of caspase-9 activation in ASTC-a-1 cells treated with HF-LPLI of 120 J/cm^2. (A) Typical time-lapse imaging of the cells transfected with SCAT9. The fluorescence images of CFP, FRET and ratio channels were recorded with LSCM microscope. Bar = 5 μm. (B) Quantitative analysis of CFP, FRET intensities and FRET/CFP ratio ($n = 5$). (C) Spectrofluorometric analysis of caspase-9 activation at 5 h after HF-LPLI treatment in living cells. The cells were excited at the wavelength of CFP (434 ± 5 nm), resulting in a CFP emission peak (476 nm) and FRET emission peak (528 nm) caused by FRET from CFP. The fluorescence emission spectra were obtained by luminescence spectrometer. This bar graph shows the comparison of the FRET/CFP ratio. Data are from three independent experiments ($^{*,\#}P < 0.05$).

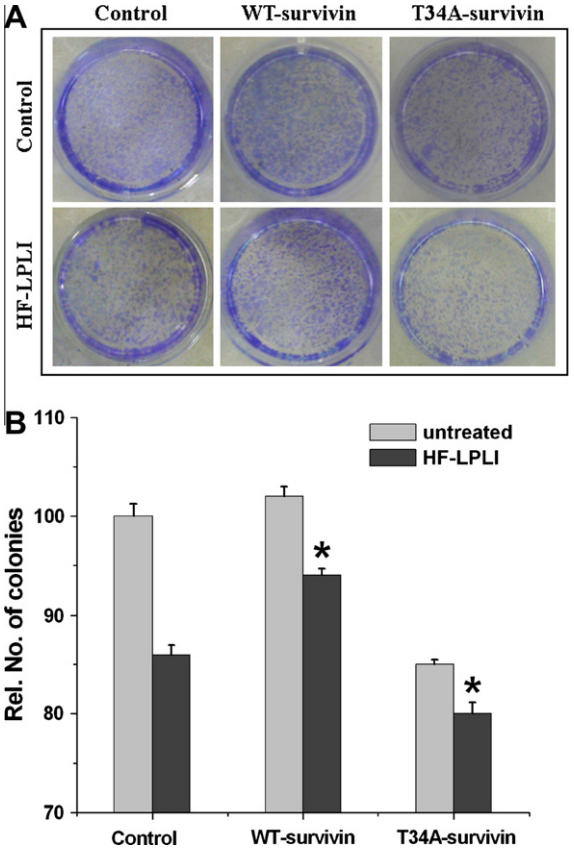

Fig. 8. The effect of WT/T34A-surivin overexpression on HF-LPLI-induced reduction of colonogenic survival. (A) ASTC-a-1 cells were transfected with WT-survivin or T34A-survivin for survivin overexpression and then were treated with HF-LPLI. Colonies formed within 1–2 weeks. After stained with Giemsa dyes, colonies in the plates were observed on an inverted light microscope. (B) Quantitative analysis of the number of colonies under different treatments. Data shown are representative of three independent experiments ($^{*}P < 0.05$).

were seeded into normal growth medium and the outgrowth of cell colonies was scored 10–14 days later. HF-LPLI treatment significantly reduced colony formation. Obviously, up-regulation of survivin activity by WT-survivin overexpression increased the number of colonies in HF-LPLI treated ASTC-a-1 cells suggesting that overexpression of WT-survivin reduces the efficacy of HF-LPLI treatment. In contrast, down-regulation of survivin activity by T34A-survivin overexpression decreased the colony yield in response to HF-LPLI demonstrating that overexpression of T34A-survivin improves the efficacy of HF-LPLI treatment.

4. Discussion

We show for the first time that HF-LPLI can obviously activate survivin through phosphorylation on Thr-34 residues in ASTC-a-1 cells (Fig. 1A). Protein phosphorylation has been involved in the regulation of cell death pathways, influencing subcellular localization and cytoprotection [24]. Survivin is a relatively short-lived protein ($t^{1/2}$ = 30 min) [31]. A crucial requirement for the stabilization and anti-apoptotic function of survivin is phosphorylation on Thr-34 by $p34^{cdc2}$, also known as cyclin-dependent kinase 1 (CDK1) [16,24]. CDK1 is activated by cdc25c protein phosphatase that dephosphorylates CDK1 on Tyr-15 residues [20]. As abrogation of ROS leads to inactivation of cdc25c [20], a possible speculation is the existence of a signal transmission from ROS to cdc25c-CDK1. Based on the fact that a high-level of intracellular ROS generation can be triggered by HF-LPLI, the working hypothesis of this study is that HF-LPLI-induced up-regulation of survivin activity through phosphorylation on Thr-34 is mediated by ROS/cdc25c/CDK1 signaling pathway. As shown in Fig. 1B, both inhibition of CDK1 by flavopiridol and abrogation of ROS by DHA decreased survivin activity. Therefore, we conclude that the ROS/cdc25c/CDK1 signaling pathway contributes to survivin activation by HF-LPLI treatment. Data shown in Fig. 1B also revealed that the suppression effect on survivin activity by ROS scavenger was much stronger than that by CDK1 inhibitor. So it will be of interest in future studies to identify other signaling pathway(s) mediating a signal transmission from ROS to survivin phosphorylation connection.

It has been reported that survivin can promote cell proliferation and play a major role in antagonizing mitochondrial-dependent apoptosis [31]. Here, we investigate the relationship between survivin activity and apoptotic rate induced by HF-LPLI. It was shown that WT-survivin overexpression up-regulates survivin activity and inhibits cell apoptosis caused by HF-LPLI, whereas T34A-survivin overexpression remarkably down-regulates the activity and promotes the apoptosis (Fig. 2). Survivin is undetectable in most terminally differentiated normal tissues but is highly expressed in many malignant tumors [15]. Patients with tumors expressing high levels of survivin generally have a poor prognosis concomitant with resistance to radiotherapy and chemotherapy, and a reduced apoptotic index. Therapeutic procedures targeting survivin could therefore reduce the survival phenotype observed in tumor tissue without influencing normal tissue [32]. In this regard, various strategies are being tested to target survivin, including (a) use of antisense molecules and dominant-negative mutants of survivin; (b) pharmacologic inhibition of survivin phosphorylation. Our discovery that HF-LPLI-activated survivin can suppress apoptosis caused by HF-LPLI, while inactivated survivin can promote the apoptosis leads to the hypothesis that survivin functioned as a self-protection mediator of HF-LPLI-induced apoptosis.

To address the underlying mechanism of the anti-apoptotic function of survivin under HF-LPLI treatment, we investigate the effects of survivin on mitochondrial depolarization, cytochrome *c* release, Bax and caspase-9 activation. Our results show that under HF-LPLI treatment, survivin delays $\Delta\Psi_m$ decrease for about 17 min, cytochrome *c* release for about 10 min, Bax activation for about 110 min (Fig. 6) and also obviously inhibits caspase-9 activation (Fig. 7C). Because HF-LPLI-induced cell apoptosis is tightly controlled at the level of mitochondria [4], the ability of phosphorylated mitochondrial survivin to inhibit some pro-apoptotic protein, such as Smac/DIABLO [27], and to stabilize some anti-apoptotic protein, like XIAP [33,34], might explain, at least in part, why the increase of survivin activity delays mitochondrial apoptotic events, such as $\Delta\Psi_m$ collapse. Our earlier studies have demonstrated that under HF-LPLI treatment, Bax activation occurs at the end-stage of apoptotic process and is independent of

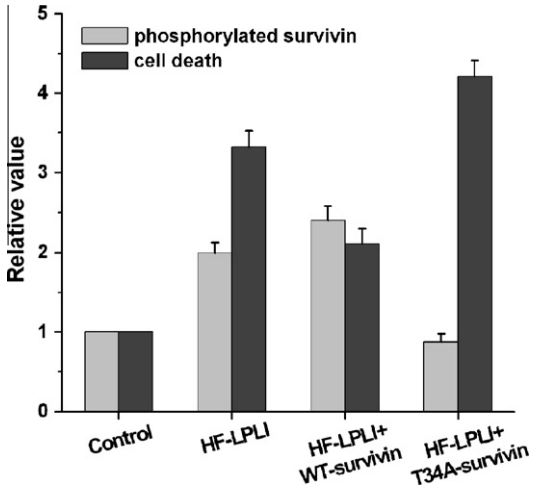

Fig. 9. Analysis on correlation of the survivin phosphorylation level and the percentage of apoptotic cell death induced by HF-LPLI. Values were normalized according to the control sample lanes that were arbitrarily set as 1.

mitochondrial injury. This was consistent with our results that the delay duration in Bax activation (~110 min) was much longer than that in mitochondrial signaling pathway (~17 min), suggesting that the mechanism of the delay in Bax activation was rather different. The molecular basis for these observations is of interest for further investigation.

Some biological systems exhibit self-protection when stimulated. For example, PDT, an effective clinical option for treating solid tumors, induces considerable stress within the tumor microenvironment. A consequence of the stress is the induction of a survival phenotype associated with increased expression of angiogenic growth factors and anti-apoptotic molecules [35]. Our previous studies suggest that HF-LPLI is promising for high treatment efficacy for future cancer therapy without photosensitizer administration. The anti-apoptotic protein, survivin, deserves attention as a target for cancer therapy due to its high expression levels in tumors. It has been reported that the expression of survivin in tumors is correlated with drug resistance and/or shorter survival in patients with non-small cell lung cancer and neuroblastoma [36]. HF-LPLI (120 J/cm^2) can increase survivin activity, and thus elevate its anti-apoptotic function. However, up-regulation of survivin activity remarkably reduced the pro-apoptotic effect (Fig. 2) and efficacy (Fig. 8) of HF-LPLI. We also found that there was a negative correlation between survivin activity and apoptotic rate under HF-LPLI treatment (Fig. 9). The data obtained in current study identify a target to accelerate tumor cell apoptosis through down-regulating survivin activity for the possible clinical applications of HF-LPLI.

Conflict of interest

All authors declare no conflicts of interest and acknowledge no financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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