ARTICLE
Journal of Cellular Biochemistry 105:381–390 (2008)

Journal of Cellular Biochemistry

Silencing Survivin Gene Expression Promotes Apoptosis of Human Breast Cancer Cells Through a Caspase-Independent Pathway

Diego O. Croci,[1] Ingrid S. Cogno,[2] Natalia B. Rumie Vittar,[2] Edgardo Salvatierra,[3] Felipe Trajtenberg,[4] Osvaldo L. Podhajcer,[3] Eduardo Osinaga,[4] Gabriel A. Rabinovich,[1*] and Viviana A. Rivarola[2**]

[1]*Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina*

[2]*Departamento Biología Molecular, Universidad Nacional de Río Cuarto, 5800 Río Cuarto, Argentina*

[3]*Laboratorio de Terapia Molecular y Celular, Fundación Instituto Leloir, CONICET, Universidad de Buenos Aires, Buenos Aires, Argentina*

[4]*Facultad de Medicina, Departamento de Bioquímica Laboratorio de Oncología, Universidad de la República, Montevideo, Uruguay*

ABSTRACT

Survivin is recognized as an attractive target in cancer therapy because of its selective overexpression in the majority of tumors. Upregulated expression of this protein correlates with increased tumor grade, recurrence risk and decreased cancer patients survival. In this study, we assessed the efficacy of two survivin-specific small interfering RNA (siRNA) constructs to inhibit T47D human breast cancer cell growth. After siRNA transfection, T47D cells showed a significant reduction in proliferation and survival exhibiting clear signs of apoptosis. pSil_1 that targeted exon 1 exhibited a stronger inhibitory effect on cell growth, and increased cell apoptosis compared to pSil_30 that targeted exon 4. Cell apoptosis was found to be mediated by translocation of the mitochondrial apoptosis inducing factor (AIF), while no changes were observed in caspase-3 activation and Bid cleavage. Thus, silencing survivin expression using siRNA strategies represents a suitable therapeutic approach to selectively modulate the survival and growth of human breast cancer cells. J. Cell. Biochem. 105: 381–390, 2008.

KEY WORDS: APOPTOSIS; APOPTOSIS INDUCING FACTOR (AIF); BREAST CANCER; SMALL INTERFERING RNA (siRNA); SURVIVIN

Apoptosis plays an important physiological role in different processes, from embryonic development to maintenance of adult tissue homeostasis [Hengartner, 2000]. This evolutionary conserved genetic program of cell death is characterized by unique biochemical and morphological features that distinguish this process from other types of cell death such as necrosis or autophagia [Hengartner, 2000]. Defects in physiological pathways of apoptosis contribute to many human disorders including autoimmunity,

Diego O. Croci and Ingrid S. Cogno contributed equally to this work.

Gabriel A. Rabinovich and Viviana A. Rivarola jointly supervised this work.

Grant sponsor: Agencia Nacional de Promoción Científica y Tecnológica (PICTO); Grant sponsor: Cancer Research Institute; Grant sponsor: Fundación Sales; Grant sponsor: Agencia Nacional de Promoción Científica y Tecnológica; Grant number: PICT 2003-05-13787; Grant sponsor: Universidad de Buenos Aires; Grant number: M091; Grant sponsor: Fundación Rene Baron; Grant sponsor: Amigos del Instituto Leloir para la Lucha contra el Cáncer (AFULIC); Grant sponsor: Comisión Honoraria de Lucha Contra el Cáncer (Uruguay); Grant sponsor: Programa de Cooperación Bilateral Argentina-Uruguay.

*Correspondence to: Gabriel A. Rabinovich, Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina. E-mail: gabyrabi@ciudad.com.ar

**Correspondence to: Viviana A. Rivarola, Departamento Biología Molecular, Universidad Nacional de Río Cuarto, 5800 Río Cuarto, Argentina. E-mail: vrivarola@exa.unrc.edu.ar

Received 4 February 2008; Accepted 13 May 2008 • DOI 10.1002/jcb.21836 • 2008 Wiley-Liss, Inc.

Published online 13 June 2008 in Wiley InterScience (www.interscience.wiley.com).

neurodegeneration and cancer [Reed, 1999]. The molecular executors of the apoptotic machinery are the caspases (cysteine proteases that cleave after aspartic acid), which are constitutively inactive as zymogens (pro-caspases), but can trans-process each other, to produce fully active proteases, which are responsible of generating a proteolytic cascade that ultimately leads to apoptotic cell death [Salvesen and Dixit, 1997].

Two major pathways may lead to apoptosis in most cell types. The "extrinsic" pathway is triggered by the binding of ligands to extracellular membrane receptors (death receptors) which leads to activation of caspase-8. [Cryns and Yuan, 1998]. The "intrinsic" apoptotic pathway involves the mitochondria, which responds to pro-apoptotic signals by releasing cytochrome *c*, which binds and activates the apoptotic protease activating factor-1 (Apaf-1). These sequential events promote the assembly of a multiprotein caspase-activating complex (apoptosome), thus leading to activation of caspase-9 and the initiation of the proteolytic cascade [Cryns and Yuan, 1998].

The apoptotic machinery is regulated by several proteins belonging to the Bcl-2 family, which include anti-apoptotic (Bcl-2, Bcl-xl, Mcl-1) and pro-apoptotic (Bax, Bak, Bcl-xs, Bad and Bid) mediators which differentially affect mitochondrial homeostasis and cytochrome *c* release [Adams and Cory, 1998]. Other proteins responsible of regulating apoptotic mechanisms are the inhibitors of apoptosis protein (IAPs) family, including ML-IAP, XIAP, cIAP1, cIAP2, NIAP, apollon and survivin. They block a common step downstream of mitochondrial cytochrome *c* release by inhibiting the terminal effectors caspase-3 and -7, and interfering with caspase-9 activity and processing [Deveraux and Reed, 1999].

Although caspase activation is considered a hallmark of apoptotic cell death, other less defined cell death pathways have been described which do not require caspase activation [Sperandio et al., 2000]. In particular, some apoptotic stimuli activate apoptosis inducing factor (AIF), a mitochondrial flavoprotein that induces cytochrome c release and subsequent caspase activation; AIF also translocates to the nucleus and causes nuclear fragmentation which is not blocked by caspase inhibitors [Susin et al., 1999].

Recently, a great interest has emerged in survivin as a diagnostic marker and potential drug target by virtue of its overexpression in most tumor cell types. This protein has been shown to play an important role in tumorigenesis and to a lesser extent in terminally differentiated normal tissue [Hengartner, 2000]. Survivin has a dual function inside the cell by suppressing apoptosis and regulating cell cycle progression [Reed, 1999; Altieri, 2004; Canovas and Guadagno, 2007]. Overexpression of survivin in tumor cells correlates with poor prognosis in patients with different tumor types [Altieri, 2003; Li et al., 2005; Hinnis et al., 2007]. Taken together, these observations suggest that targeting survivin might provide therapeutic benefits in clinical settings.

In this context, studies which evaluate the efficacy of different strategies for silencing survivin would help for the development of novel anti-tumor strategies. In recent years, the available strategies for modulating apoptosis by counteracting survivin expression include mostly the use of antisense oligonucleotides, pharmacological inhibitors and ribozymes [Plescia et al., 2005; Yan et al., 2006; Pennati et al., 2007; Zaffaroni et al., 2007]. However, recent evidence highlights the efficacy of strategies using the interference RNA (RNAi) technology that induce strong inhibition of specific gene expression [Elbashir et al., 2001]. Several studies in experimental human tumor models have demonstrated the feasibility of this approach for the inhibition of survivin [Uchida et al., 2004; Huynh et al., 2006]. Carvalho and colleagues were the first to use siRNA to specifically repress survivin in HeLa cells. The authors showed that survivin-depleted cells experienced a significant delay in their proliferative response. Finally, an enhanced apoptotic response was also observed in several carcinoma cell lines transfected with survivin-specific siRNAs [Uchida et al., 2004; Huynh et al., 2006; Kappler et al., 2007].

The aim of the present study was to evaluate the efficacy of two vectors encoding siRNA specific for survivin in their capacity to modulate the apoptotic machinery in human breast cancer cells and to analyze the cellular mechanisms involved in these effects.

MATERIALS AND METHODS

CELL CULTURES

The metastatic human breast carcinoma cell line T47D, was cultured in DMEM complete growth medium: DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 200 mM L-glutamine (Sigma), and antibiotic-antimycotic mixture (Gibco). Cells were maintained in a humidified 37°C incubator with 5% CO_2.

PLASMID VECTORS

A DNA vector-based siRNA was designed to knockdown *Survivin*. Design of specific siRNA was performed using Ambion Target Finder (http://www.ambion.com/techlib/misc/sirna_finder.html). BLAST search was used for selecting two target non-crossmatching sequences among the siRNA candidates generated. The 21-nucleotide target sequences to the exon 1 (5′-GGACCACCGCATCTC-TACATT-3′) and exon 4 (5′-GAATTTGAGGAAACTGCGAAG-3′) of the human *SURVIVIN* mRNA were selected and named pSil_1 and pSil_30, respectively. Both sequences are directed against all *Survivin* mRNA splicing variants. We used siRNA plasmid vectors against green fluorescent protein (GFP) as a control (5′-GCTGACCC-TGAAGTTCATCT-3′) named pSil_C.

TRANSFECTION

Cells (2×10^5/well) were incubated in 6-well plates. Cells were cultured in antibiotics-free medium before the start of transfection. Survivin-specific siRNA or pSil_C was mixed with transfection reagent Lipofectamine™ Plus in OptiMEM medium (Invitrogen) according to the manufacturer's instructions. After transfection for 6 h at 37°C, the cells were cultured in DMEM GlutaMAX medium (Gibco) containing 10% FBS (Gibco). At 24, 48, and 72 h after transfection, cells were harvested and stored at −20°C until the time of protein analyses.

COLONY FORMING ASSAYS

Colony forming assays were performed to evaluate the long-term survival of treated cells. Twenty-four hours after transfection, cells were seeded onto 6-well culture plates at a concentration of 10^2 or 10^3 cells per well and were cultured for 12 days. Following

removal of the medium, the wells were washed twice with PBS. Glutaraldehyde (1.25% in PBS) was added to each well and the plates were incubated for 30 min at room temperature to allow for cell fixation. After two washes with distilled water, 0.05% methylene blue solution was added to each well and plates were incubated for 30 min at room temperature to facilitate staining of the colonies. After two rinses with distilled water, plates were dried and photographed, and the number of visible colonies (more than 10 cells) was determined.

IMMUNOBLOT ANALYSIS

Total cell lysates from transfected cells were extracted with lysis buffer containing 20 mM HEPES pH 7.5; 1.5 mM KCl; 1mM EDTA; 1mM EGTA; 0.15% Triton-X100; 1 mM PMSF; 1 mM DTT; and a cocktail of protease inhibitors (Sigma). The samples were centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants were collected and protein concentration was determined by the MicroBCA assay according to the manufacturer's instruction (Pierce). Equal amounts of protein (30 μg/well) were separated on 14% sodium dodecyl sulfate polyacrylamide gels for detection of survivin, AIF, caspasa-3 and Bid. Subsequently, the resolved proteins were transferred onto Immobilon-P membrane (Sigma) to 250 mA for 1 h; immunoblots were sequentially incubated in 5% skimmed milk blocking solution at room temperature for 1 h. The primary antibodies were rabbit anti-survivin (FL-142; Santa Cruz Biotechnology), goat anti-caspase-3 (L-18; Santa Cruz Biotechnology, Inc.), goat anti-Bid (C-20; Santa Cruz Biotechnology, Inc.), rabbit anti-AIF (H-300; Santa Cruz Biotechnology, Inc.) or rabbit anti-actin (H-196; Santa Cruz Biotechnology, Inc.) polyclonal antibodies. After washing three times with 0.05% Tween-PBS solution at room temperature for 15 min, membranes were incubated with anti-rabbit (1:3,000; BioRad) and anti-goat (1:4,000; Vector Labs.) secondary antibodies. Finally, detection was carried out using the enhanced chemoluminiscence (ECL) kit (Amersham) according to the manufacturer's instructions. Nuclear and mitochondrial extracts were used to analyze the release of AIF from mitochondria to nucleus, and protein bands were compared with the expression of histone (FL-219; Santa Cruz) marker of nuclear fraction and Complex I marker of mitochondrial fraction.

APOPTOSIS ASSAYS

The frequency of apoptotic cells was assessed by flow cytometry using a FACAria (BD Biosciences) after staining the cells with FITC-annexin V (BD Biosciences). Briefly, T47D cells were collected, washed twice with PBS (centrifuged 5 min at 2,000 rpm each time) and resuspended in FITC-annexin V (BD Biosciences) according to the manufacturer's instructions. Then, cells were incubated for 15 min at RT in the dark. Cell-associated fluorescence was then analyzed using WinMDI2.8 software.

CYTOFLUORIMETRIC ANALYSIS OF CASPASE-3 ACTIVATION

pSil-treated and control cells were washed twice with PBS and the percentage of cells with active caspase-3 was assessed using the PE-conjugated monoclonal active caspase-3 antibody kit (BD Pharmigen) according to the manufacturer's instructions and analyzed on a FACSAria flow cytometer (BD Biosciences). Staurosporine (STS; 1μM) was used as positive control of apoptosis and caspase-3 activation. Cell-associated fluorescence was then analyzed using WinMDI2.8 software.

IMMUNOFLUORESCENCE

T47D cells were grown on culture slides (BD Falcon), and when reached 60-80% confluence, the transfection protocol with pSil_C, pSil_1 and pSil_30 was performed. Seventy-two hours after treatment, cells were washed three times with PBS and fixed with 2% of paraformaldehyde for 20 min at 4°C. Cells were permeabilized with ethanol:acetic acid 2:1 for 5 min at −20°C and then washed and incubated in PBS blocking solution (10% FBS, 1% BSA) for 30 min at RT. Next, cells were incubated with rabbit polyclonal anti-survivin (FL-142), anti-AIF (H-300) (both from Santa Cruz Biotechnology, Inc.) and monoclonal PE-conjugated anti-active caspase-3 (BD Pharmigen) for 45 min. at 37°C. After washing, cells were incubated with FITC-conjugated anti-rabbit-IgG (1:200; Sigma) for 45 min at 37 °C. Cells were counter-stained with PI or 4′6-diamino-2-phenylindol (DAPI Sigma) and samples were mounted on microscope slides with anti-fading agent (90% glycerol, pH 9.0). The fluorescence signal was visualized in a confocal laser microscope (NIKON C1).

STATISTICAL ANALYSIS

Statistical significance was calculated with the Student's *t*-test and Mann–Whitney *U*-test using Prism software (GraphPad). *P* values of 0.05 or less were considered significant.

RESULTS

siRNA-MEDIATED SILENCING OF SURVIVIN EXPRESSION IN BREAST CANCER CELLS

In an attempt to downregulate the expression of the antiapoptotic protein survivin, T47D human breast cancer cells were transfected with plasmids encoding siRNA against exon 1 (pSil_1) and 4 (pSil_30) within the *Survivin* transcript, as well as a control plasmid. In all cases the plasmid concentrations were 1.6 μg/ml and the efficiency of transfection was higher than 70% (Fig. 1A). The expression levels of survivin were evaluated at 48 and 72 h post-transfection by Western blot and confocal microscopy. After 72 h the expression of survivin was significantly down-regulated in T47D cells transfected with pSil_1 and pSil_30 compared with cells transfected with control vector (pSil_C). Densitometric analysis revealed that the levels of survivin in cells transfected with pSil_1 and pSil_30 were reduced to values of 48% and 70%, respectively, indicating that pSil_1 was more efficient than pSil_30 to silence survivin gene expression (Fig. 1B). Transfection of pSil_C, a control vector expressing siRNA against the green fluorescent protein, had no effect on survivin expression (Fig. 1B). There was no significant difference in the expression levels of survivin 48 h following transfection with either pSil_1, pSil_30, or control vector (data not shown). To confirm these results, confocal microscopy analysis of survivin expression was performed. Psil_C-treated cells (Fig. 1C) or non-transfected cells (data not shown) showed strong cytoplasmatic expression of survivin. On the other hand, cells

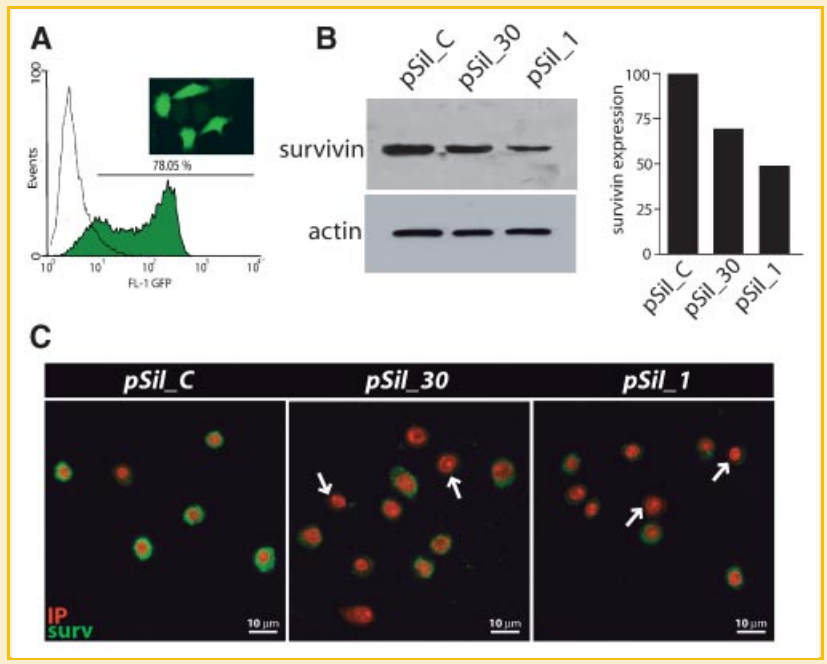

Fig. 1. si-RNA-mediated silencing of survivin in T47D cells. Cells were transfected with pSil_C, pSil_1 and pSil_30. A: Efficiency of transfection of T47D cells modified by pSil and GFP encoding-vectors by flow cytometry analysis and confocal microscopy (inset). In all cases more than 70% of cells were GFP positive. Data are representative of three independent experiments. B: Immunoblot analysis of survivin expression in T47D cells 72 h post-transfection. Data are representative of three independent experiments. C: Confocal microscopy of survivin expression in cells treated with pSil plasmids. White arrow indicates the absence of survivin expression. Data are representative of three independent experiments performed in triplicates. Scale bars 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

treated with pSil_1 and Psil_30 showed a significant reduction of survivin immunoreactivity (Fig. 1C) in agreement with the results obtained by immunoblot analysis.

siRNA-MEDIATED SURVIVIN SILENCING RESULTS IN ATTENUATED GROWTH CAPACITY

To analyze the biological consequences of silencing survivin expression, we assessed tumor cell growth in vitro using a colony forming assay. For this purpose, transfected T47D cells were incubated for 12 days. Following colonies staining with methylene blue, we observed that transfection of T47D cells with pSil_1 or pSil_30, resulted in a marked reduction of the colony growth capacity of these cells (47.2% and 56.5%, respectively) when compared to pSil_C-transfected cells (Fig. 2). Transfection with pSil_C yielded no significant effect on the growth of T47D cells. Once again, pSil_1 exhibited a stronger inhibitory effect on cell growth compared to pSil_30 (Fig. 2). Thus, siRNA-mediated survivin silencing results in attenuated growth capacity in human breast cancer cells.

siRNA-MEDIATED SURVIVIN SILENCING RESULTS IN INCREASED TUMOR CELL APOPTOSIS

Since down-regulation of survivin expression results in reduced growth capacity of T47D cells, we further analyzed whether this inhibitory effect occurred through apoptotic cell death. For this purpose, the frequency of "early" apoptotic cells was analyzed by flow cytometry using annexin-V staining. Cells treated with the siRNA constructs for 48 h showed a modest increase in the frequency of apoptosis in comparison to cells transfected with the control vector pSil_C (Fig. 3A; upper panels). By contrast, we found a marked increase in the frequency of apoptotic cells in T47D transfected with pSil_1 and pSil_30 compared to those observed following transfection with control vectors. Percentages of apoptotic cells at 72 h were 26.2% when pSil_30 was used compared to 36% when cells were transfected with pSil_1 (Fig. 3A; lower

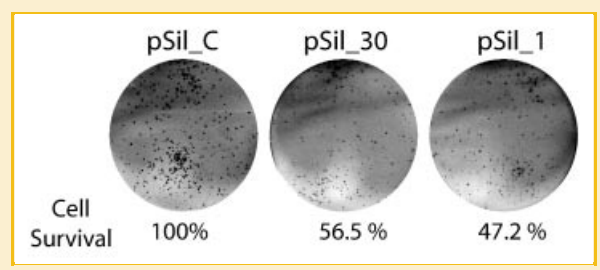

Fig. 2. Impact of siRNA-mediated silencing of survivin in the growth of T47D breast cancer cells. T47D cells were seeded onto six-well culture plates at a concentration of 10^2 or 10^3 cells per well and following transfection with different constructs (pSil_C, pSil_1 and pSil_30). Transfected cells were cultured for 12 days. Colonies were stained by methylene blue and photographs were taken. Cell survival was determined as the percentage of colonies respect to the control vector. Data are representative of three independent experiments ($^*P < 0.05$; pSil_30 vs. pSil_C and pSil_1 vs. pSil_C).

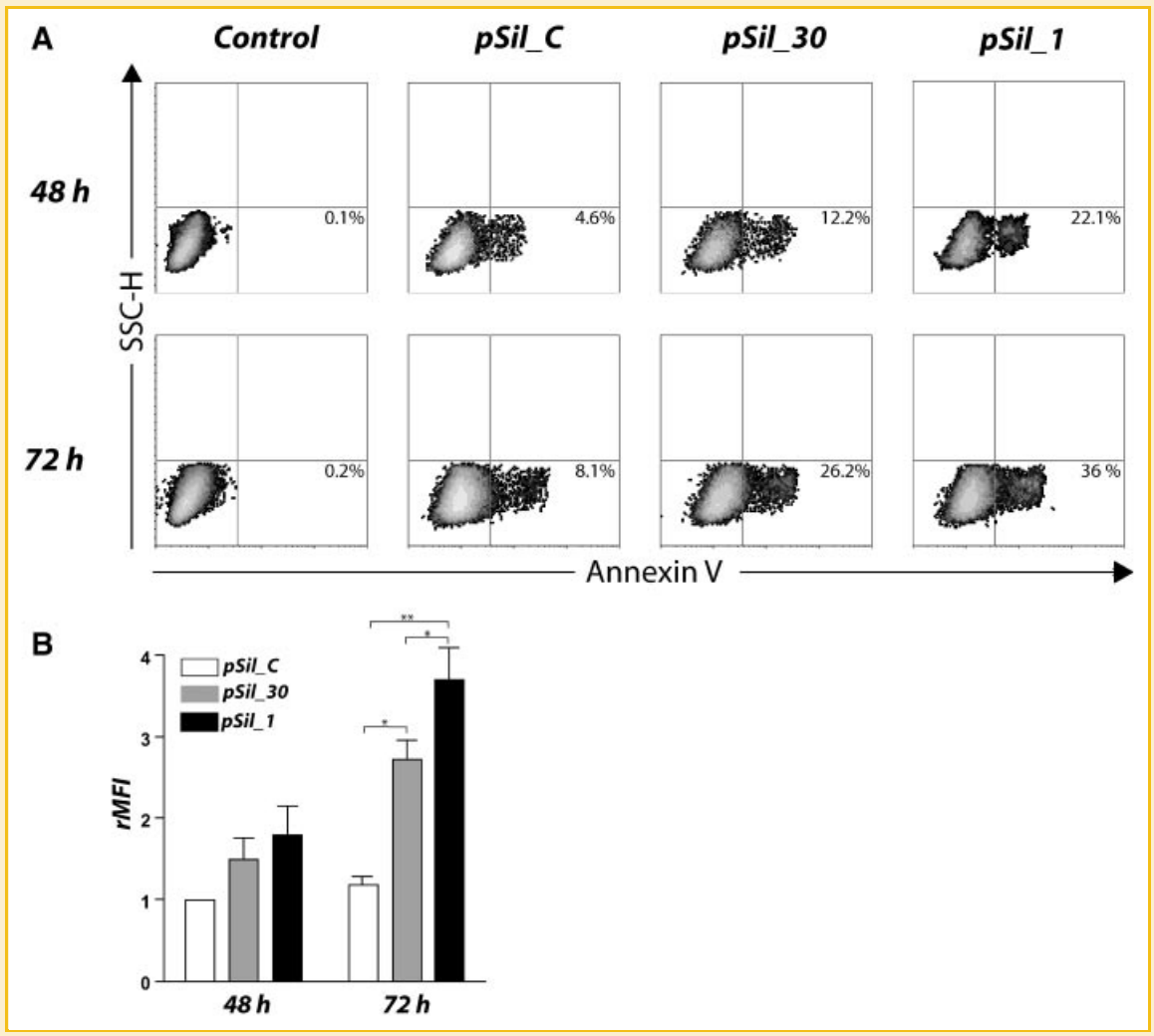

Fig. 3. Tumor cell apoptosis following siRNA-mediated silencing of survivin. T47D cells were transfected with pSil_C, pSil_1 and pSil_30 as described in Materials and Methods Section. Transfected cells were cultured for 48 and 72 h, stained with FITC-annexin V and analyzed by flow cytometry. A: Flow cytometry analysis of the percentages of apoptotic cells assessed by annexin-V staining (*x*-axis) and side-scatter (SSC-H) analysis. B: Comparative analysis of apoptosis in different transfected cells. Data represent mean fluorescence intensity relative to pSil_C ± SEM. rMFI is calculated as MFI of the population treated with iRNAs–MFI of control untreated cells/MFI of control untreated cells. $^{*}P < 0.05$, $^{**}P < 0.01$.

panels) demonstrating a direct correlation between the induction of apoptosis and survivin silencing. Apoptosis induced by pSil_30 was significantly reduced compared to pSil_1 ($P < 0.05$). Interestingly, 72 h following transfection the mean fluorescence intensity in pSil_1-transfected cells was found to be 3-fold higher compared to cells transfected with the control vector, suggesting that the induction of apoptosis was a direct consequence of the extent of survivin silencing (Fig. 3B).

TUMOR CELL APOPTOSIS INDUCED BY SURVIVIN SILENCING DOES NOT INVOLVE CASPASE-3 AND BID CLEAVAGE

Silencing survivin results in reduced survival of tumor cells, although the precise mechanisms involved in this effect are still in debate [Altieri, 2004; Liu et al., 2004; Li and Ling, 2006]. Therefore, we investigated possible pathways by which survivin would modulate tumor cell survival.

To establish whether apoptosis triggered by pSil_1 and pSil_30 was mediated by caspase-3 activation, we evaluated the status of activation of this enzyme following different treatments [Liu et al., 2004]. As shown in Figure 4, siRNA-mediated silencing of survivin expression did not affect expression (Fig. 4A) or activation (Fig. 4B,C) of executor caspase-3. To rule out the possibility that caspase-3 would be constitutively inactive in T47D cells, we incubated the cells with STS at a concentration of 1 μM for 6 h. As shown in Figure 4B,C, STS-induced apoptosis of T47D cells was accompanied by caspase-3 activation. In addition, cells treated for 72 h with pSil_1 and pSil_30 bound FITC-labeled annexin V but they were not capable of activating caspase-3 (Fig. 4B), which indicates a caspase-3-independent apoptotic pathway. To determine whether silencing

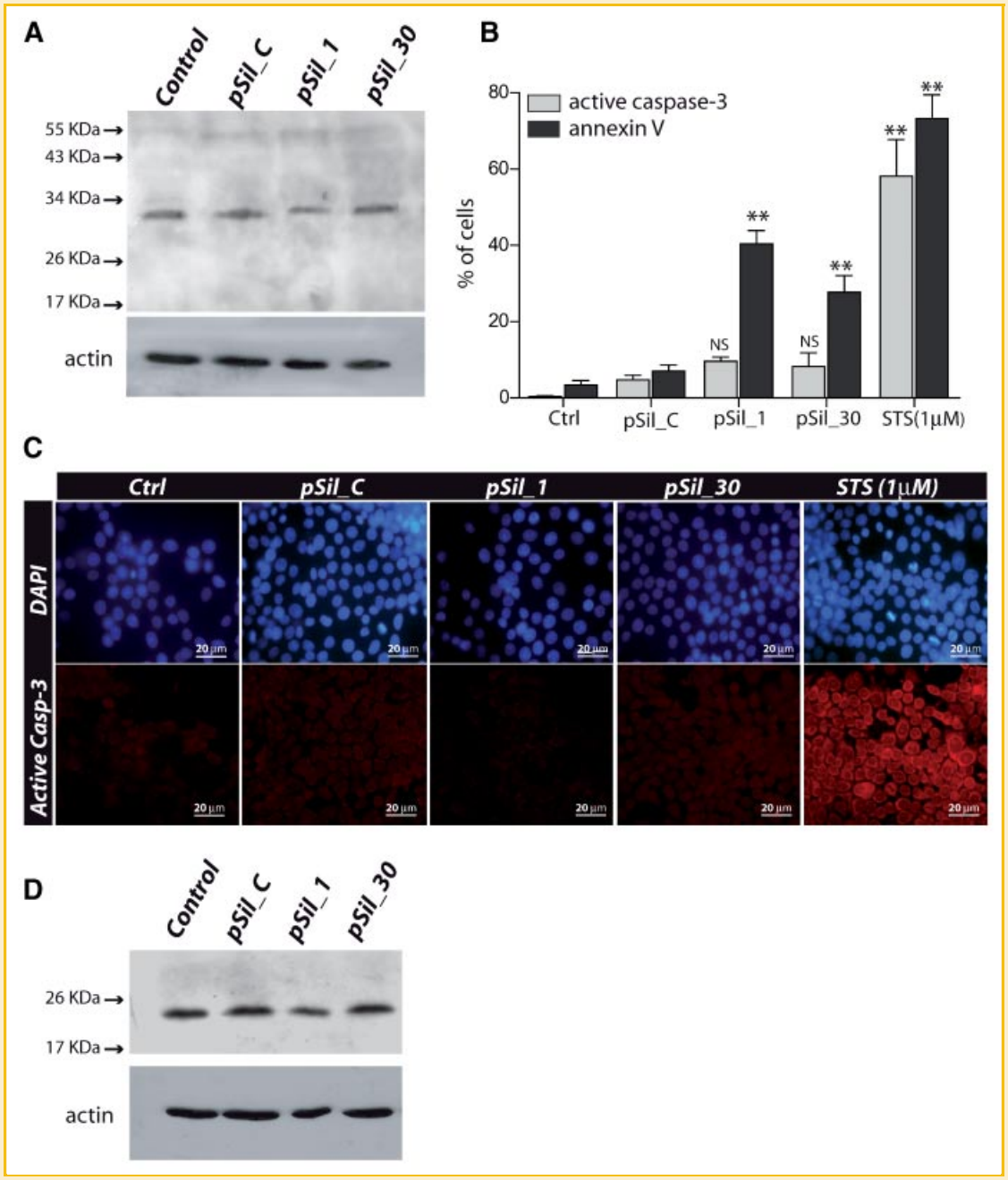

Fig. 4. Effect of pSil_1 and pSil_30 constructs on caspase-3 and Bid activation. T47D cells were transfected with pSil_C, pSil_1 and pSil_30 to silence survivin. A: After 72 h cells were collected and caspase-3 cleavage was analyzed by immunoblot analysis. B: Analysis of caspase-3 activation was performed using a PE-conjugated monoclonal anti-active caspase-3 antibody and early apoptotic cells were detected by annexin V binding in cells treated with the distinct constructs ($^{**}P < 0.01$ vs. pSil._C). Confocal microscopy of active-caspase-3 in pSil-treated cells. Staurosporine (1 μM) was used as a positive control of caspase-3 activation and nuclear DAPI was used as a counterstaining procedure. D: Immunoblot analysis of Bid (23 kDa) expression showing the absence of truncated activated Bid (17 kDa) in cells treated with pSil vectors. pSil_C was used as a control vector. Data are the mean ± SD of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

survivin affected the extrinsic apoptotic pathway, we also evaluated the expression of the proapoptotic protein Bid. Remarkably, targeting survivin did not result in any detectable cleavage of the pro-apoptotic protein Bid (Fig. 4D). Thus, tumor cell apoptosis induced by survivin silencing involves caspase-3 and Bid-independent pathways.

SILENCING SURVIVIN RESULTS IN NUCLEAR TRANSLOCATION OF MITOCHONDRIAL AIF

Given the lack of involvement of caspase-3 and Bid activation in T47D cell apoptosis induced by survivin silencing, we evaluated whether other molecular mechanisms leading to apoptosis may participate in this process. For this purpose, we evaluated the translocation of AIF

from the mitochondria to the nucleus, an event which has been shown to contribute to apoptosis through a caspase-independent pathway [Daugas et al., 2000]. Upon induction of apoptosis by survivin silencing with either pSil_1 or pSil_30, a large fraction of AIF staining was found in the nucleus, as determined by confocal microscopy and immunoblot analysis (Figure 5). Noteworthy, we were able to detect translocation of AIF to the nucleus in cells transfected with pSil_1 (77%) and pSil_30 (52%) (Fig. 5B). In addition, AIF translocation induced by survivin silencing was confirmed by immunoblot analysis of nuclear and mitochondrial fractions (Fig. 5C). This finding correlated with the increased frequency of apoptosis and the efficacy of siRNA transfection (Figs. 1A and 3A). Importantly, there was no significant translocation of AIF in pSil_C-transfected cells (Fig. 5A,C). Collectively, these data indicates that targeting survivin using siRNA-encoding vectors results in reduced growth and increased apoptosis of human breast cancer cells through a mechanism involving AIF redistribution from the mitochondria to the nucleus but independent of caspase-3 activation and Bid cleavage.

DISCUSSION

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, has received increasing attention as a candidate target for anticancer therapies due to its selective expression in tumors versus normal tissues, its distribution and subcellular localization, and the emerging evidence for its dual role in the control of both cell apoptosis and proliferation [Altieri, 2001; Jiang et al., 2001; Lens et al., 2006; Canovas and Guadagno, 2007]. Survivin is expressed during embryonic development, is completely downregulated in terminally differentiated adult tissues, and is overexpressed in transformed cell lines and a variety of human tumors [Altieri, 2008]. Several reports have demonstrated survivin expression in the majority of human tumor types including lung, breast, colon, esophagus, pancreas, bladder, uterus, ovary, liver and skin cancer [Jiang et al., 2001; Meng et al., 2004; Li and Ling, 2006]. Moreover, up-regulated expression of survivin has been consistently associated with tumor resistance to chemotherapeutic drugs [Grossman and Altieri, 2001; Chawla-Sarkar et al., 2004; Nomura et al., 2005], decreased survival of patients, and poor prognosis [Grossman et al., 1999; Li et al., 2005; Hinnis et al., 2007]. In this context, therapeutic strategies aiming to counteract survivin expression and activity in tumor cells have been proposed with the dual goal of suppressing tumor growth through an increase in spontaneous apoptosis, and enhancing tumor cell response to apoptosis-inducing agents [Jiang et al., 2001; Altieri, 2008]. Different types of survivin antagonists have been reported, including antisense oligonucleotides [Olie

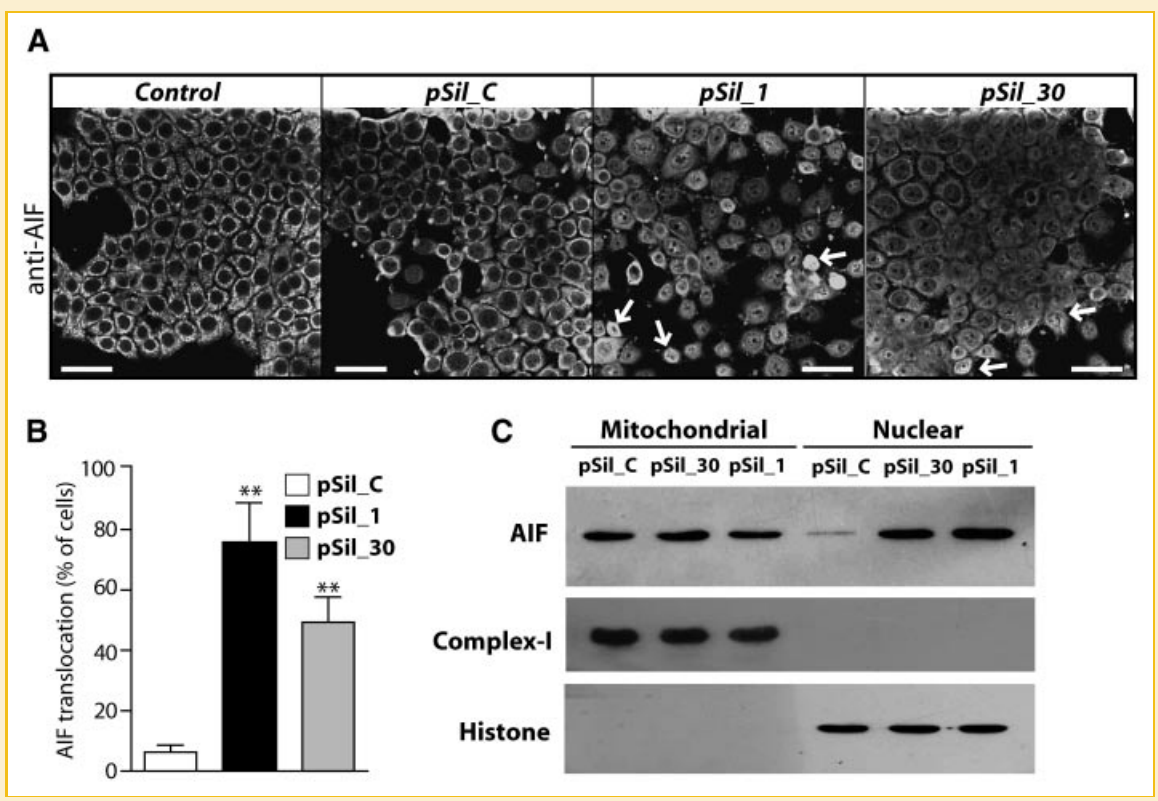

Fig. 5. Nuclear translocation of AIF in survivin-targeted cells. A: Confocal laser microscopy of T47D cells treated with pSil_C, pSil_30 or pSil_1 for 72 h. Staining of AIF in pSil_C transfected cells was predominantly detected in the cytosol. Nuclei immunoreactivity was only observed in pSil_1- and pSil_30-transfected cells (arrows). Non-transfected cells are shown as controls. Bars represent 20 μm. B: Percentage of cells showing AIF redistribution in the nuclei. Data are shown as mean ± SEM of at least 50 cells per experiment. Four independent experiments were performed (**$P < 0.01$ vs. pSil_C). C: Immunoblot analysis of AIF expression in the mitochondrial and nuclear fractions. Nuclear and mitochondrial proteins were prepared from cells 72 h after transfection and subjected to immunoblot analysis of AIF, histones (controls of nuclear fractions) and Complex I (controls of mitochondrial fractions). Data are representative of three independent experiments.

et al., 2000], ribozymes [Zaffaroni et al., 2007], small interfering RNAs (siRNAs) [Chawla-Sarkar et al., 2004; Kappler et al., 2004] and dominant-negative mutants, as well as cyclin-dependent kinase inhibitors [Pennati et al., 2007].

In the present study, we used the siRNA gene-silencing technology as an efficient approach to counteract *Survivin* gene expression in breast cancer cells [Duxbury and Whang, 2004; Hannon, 2002] and successfully transfected T47D cells with novel plasmids expressing siRNA against survivin. Breast cancer cells transiently expressing these constructs displayed a marked reduction in cell growth compared to control cells. Moreover, introduction of survivin-specific siRNAs, elicited an increased frequency of tumor cells undergoing apoptosis. We have found that pSil_1, that targeted exon 1, was the most potent approach to efficiently down-regulate survivin expression and trigger apoptosis.

The silencing effect observed could be explained by a successful siRNA uptake by target cells during prolonged times of culture (72 h). Despite the fact that the duration of gene silencing in rapidly dividing cells is short, our results are in accordance with other studies suggesting that the effect peaks at around 3 days and lasts approximately 1 week [Uchida et al., 2004; Pai et al., 2006]. In fact, the effects observed in pSil_1 T47D-transfected cells may be supported by the rapid decline of survivin levels involving the ubiquitin-proteasome pathway [Zhao et al., 2000].

It may be reasonable to hypothesize that cancer cells survive based on the predominance of antiapoptotic over proapoptotic molecules and that the disturbance of such balance by transducing an siRNA against survivin might lead to cancer cell apoptosis. Moreover, silencing of survivin in lung cancer cells not only increased the levels of apoptosis, but also enhanced the susceptibility of tumor cells to chemotherapeutic agents. These results are consistent with earliest results of Altieri and colleagues, who reported that functional ablation of *Survivin* using a dominant-negative mutant (T34A) was sufficient to trigger apoptosis in cancer cells without the requirement of additional cell death inducers [Mesri et al., 2001].

The molecular mechanisms underlying the anti-apoptotic function of survivin still remain unclear. It has been demonstrated that survivin is able to bind caspase-3 and caspase-7 in vitro [Shin et al., 2001]. Moreover, it has been proposed that survivin may inhibit apoptosis through suppression of caspases activities [Tamm et al., 1998]. However, comparison of the X-ray crystallographic structure of survivin with that of the XIAP (BIR2):caspase-3 complex fails to show the ability of survivin to suppress caspase-3 directly [Riedl et al., 2001]. Although the single BIR domain in the survivin protein has been shown to be closely related to the XIAP BIR3 domain on the basis of three-dimensional structure, a direct evidence of survivin binding to caspase-9 still remains to be demonstrated [Shi, 2000]. Wu and colleagues reported that survivin can bind to the pro-apoptotic mitochondrial protein Smac/DIABLO, both in vitro and in vivo. Furthermore, the authors proposed that binding of survivin to Smac/DIABLO may reduce Smac/DIABLO antagonism to IAPs, such as XIAP, thereby enabling free XIAP to directly block caspases [Song et al., 2003]. Alternatively, the relevance or survivin in the regulation of the cell cycle, and cell viability might be compromised by the occurrence of mitotic catastrophe [Lens et al., 2006].

Another line of evidence suggested a new mechanism by which the primary function of survivin in apoptosis is associated with the inhibition of the apoptosis-inducing factor (AIF) and secondarily with the inhibition of caspase activity [Liu et al., 2004]. AIF is a mitochondrial flavoprotein that induces cytochrome *c* release and subsequent caspase activation; AIF also translocates to the nucleus and causes nuclear fragmentation; this effect is not blocked by caspase inhibitors [Susin et al., 1999; Daugas et al., 2000].

In the present study, we attempted to elucidate the mechanisms of survivin protection, which are critical not only for the understanding of the fundamental role of this protein in cancer cells but also in the development of potential therapeutic strategies. We observed that silencing survivin does not trigger a caspase-dependent apoptotic pathway in T47D human breast cancer cells and that the earliest proapoptotic event in survivin-targeted cells was the nuclear translocation of mitochondrial AIF. It is intriguing to speculate that AIF translocation and caspase-independent apoptosis may represent a backup apoptotic mechanism in normal cells in which caspases fail or become deregulated. In fact, various caspases have been found to be mutated or their activity has been shown to be blocked or interrupted in normal and malignant cells [Reed and Bischoff, 2000]. Hence, expression of survivin may provide tumor cells with the ability to suppress AIF activity and eliminate this backup mechanism, further enhancing the resistance of cancer cells to agonist-induced cell death. Understanding the integration of survivin and AIF in the context of the cellular apoptotic machinery would have important therapeutic implications for promoting apoptosis in cancer cells or preventing exacerbated cell death in degenerative diseases.

In recent years, manipulation of certain genes in cancer have provided clues as to how we can intervene to make tumor cells specifically more sensitive to chemical and physical antitumor agents [Chawla-Sarkar et al., 2004; Plescia et al., 2005; Kappler et al., 2007; Zaffaroni et al., 2007]. Such tumor specificity can only be obtained by exploiting a basic difference between normal and malignant cells. In this context, targeting survivin is particularly attractive because of its high levels of expression in tumor cells and its proven association with disease progression. Overall, the results obtained by different studies aimed at targeting survivin by means of different approaches demonstrated that inhibition of this cytoprotective factor can promote spontaneous apoptosis in tumor cells and enhance the efficacy of several types of conventional treatments including chemotherapy, radiotherapy and immunotherapy. In fact, survivin expression has been reported as a mechanism by which tumor cells evade T cell-mediated cytotoxicity [Croci et al., 2007; Rabinovich et al., 2007]. Moreover, the evidence that survivin plays a crucial role also in tumor angiogenesis [Caldas et al., 2007] would suggest that targeting survivin can increase the overall tumor response to treatment not only through direct interference with the apoptotic pathways in cancer cells, but also by favoring the apoptotic involution of newly formed tumor vasculature.

In conclusion we found, using two different constructs that siRNA approaches can efficiently down-regulate the expression of survivin, suppress tumor growth and promote apoptosis in human breast cancer cells. Silencing survivin gene expression results in the

induction of a caspase-independent apoptotic pathway involving translocation of AIF to the nucleus. Therefore, targeting survivin expression using siRNA strategies in combination with chemotherapy, radiotherapy or immunotherapy may increase the clinical effectiveness of cancer treatments.

ACKNOWLEDGMENTS

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICTO; V.A.R.), the Cancer Research Institute (G.A.R.), Fundación Sales (G.A.R.), Agencia Nacional de Promoción Científica y Tecnológica (PICT 2003-05-13787; G.A.R.), Universidad de Buenos Aires (M091; G.A.R.), Fundación Rene Baron (O.P.), Amigos del Instituto Leloir para la Lucha contra el Cáncer (AFULIC) (O.P), Comisión Honoraria de Lucha Contra el Cáncer (Uruguay; E.O.) and Programa de Cooperación Bilateral Argentina-Uruguay (E.O and G.A.R). D.O.C. thanks the University of Buenos Aires and S.I.C. thanks SECYT UNRC for the fellowships granted.

REFERENCES

Adams JM, Cory S. 1998. The Bcl-2 protein family: Arbiters of cell survival. Science 281:1322–1326.

Altieri DC. 2001. Cytokinesis, apoptosis and survivin: Three for tango? Cell Death Differ 8:4–5.

Altieri DC. 2003. Validating survivin as a cancer therapeutic target. Nat Rev Cancer 3:46–54.

Altieri DC. 2004. Molecular circuits of apoptosis regulation and cell division control: The survivin paradigm. J Cell Biochem 92:656–663.

Altieri DC. 2008. Survivin, cancer networks and pathway-directed drug discovery. Nat Rev Cancer 8:61–70.

Caldas H, Fangusaro JR, Boue DR, Holloway MP, Altura RA. 2007. Dissecting the role of endothelial SURVIVIN DeltaEx3 in angiogenesis. Blood 109:1479–1489.

Canovas PM, Guadagno TM. 2007. Functional analysis of Survivin in spindle assembly in Xenopus egg extracts. J Cell Biochem 100:217–229.

Chawla-Sarkar M, Bae SI, Reu FJ, Jacobs BS, Lindner DJ, Borden EC. 2004. Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. Cell Death Differ 11:915–923.

Croci DO, Zacarias Fluck MF, Rico MJ, Matar P, Rabinovich GA, Scharovsky OG. 2007. Dynamic cross-talk between tumor and immune cells in orchestrating the immunosuppressive network at the tumor microenvironment. Cancer Immunol Immunother 56:1687–1700.

Cryns V, Yuan J. 1998. Proteases to die for. Genes Dev 12:1551–1570.

Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, Prevost MC, Leber B, Andrews D, Penninger J, Kroemer G. 2000. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. FASEB J 14:729–739.

Deveraux QL, Reed JC. 1999. IAP family proteins—Suppressors of apoptosis. Genes Dev 13:239–252.

Duxbury MS, Whang EE. 2004. RNA interference: A practical approach. J Surg Res 117:339–344.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494–498.

Grossman D, Altieri DC. 2001. Drug resistance in melanoma: Mechanisms, apoptosis, and new potential therapeutic targets. Cancer Metastasis Rev 20:3–11.

Grossman D, McNiff JM, Li F, Altieri DC. 1999. Expression of the apoptosis inhibitor, survivin, in nonmelanoma skin cancer and gene targeting in a keratinocyte cell line. Lab Invest 79:1121–1126.

Hannon GJ. 2002. RNA interference. Nature 418:244–251.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407:770–776.

Hinnis AR, Luckett JC, Walker RA. 2007. Survivin is an independent predictor of short-term survival in poor prognostic breast cancer patients. Br J Cancer 96:639–645.

Huynh T, Walchli S, Sioud M. 2006. Transcriptional targeting of small interfering RNAs into cancer cells. Biochem Biophys Res Commun 350: 854–859.

Jiang X, Wilford C, Duensing S, Munger K, Jones G, Jones D. 2001. Participation of Survivin in mitotic and apoptotic activities of normal and tumor-derived cells. J Cell Biochem 83:342–354.

Kappler M, Bache M, Bartel F, Kotzsch M, Panian M, Wurl P, Blumke K, Schmidt H, Meye A, Taubert H. 2004. Knockdown of survivin expression by small interfering RNA reduces the clonogenic survival of human sarcoma cell lines independently of p53. Cancer Gene Ther 11:186–193.

Kappler M, Rot S, Taubert H, Greither T, Bartel F, Dellas K, Hansgen G, Trott KR, Bache M. 2007. The effects of knockdown of wild-type survivin, survivin-2B or survivin-delta3 on the radiosensitization in a soft tissue sarcoma cells in vitro under different oxygen conditions. Cancer Gene Ther 14:994–1001.

Lens SM, Vader G, Medema RH. 2006. The case for Survivin as mitotic regulator. Curr Opin Cell Biol 18:616–622.

Li F, Ling X. 2006. Survivin study: An update of ''what is the next wave''? J Cell Physiol 208:476–486.

Li F, Yang J, Ramnath N, Javle MM, Tan D. 2005. Nuclear or cytoplasmic expression of survivin: What is the significance? Int J Cancer 114:509–512.

Liu T, Brouha B, Grossman D. 2004. Rapid induction of mitochondrial events and caspase-independent apoptosis in Survivin-targeted melanoma cells. Oncogene 23:39–48.

Meng H, Lu C, Mabuchi H, Tanigawa N. 2004. Prognostic significance and different properties of survivin splicing variants in gastric cancer. Cancer Lett 216:147–155.

Mesri M, Wall NR, Li J, Kim RW, Altieri DC. 2001. Cancer gene therapy using a survivin mutant adenovirus. J Clin Invest 108:981–990.

Nomura T, Yamasaki M, Nomura Y, Mimata H. 2005. Expression of the inhibitors of apoptosis proteins in cisplatin-resistant prostate cancer cells. Oncol Rep 14:993–997.

Olie RA, Simoes-Wust AP, Baumann B, Leech SH, Fabbro D, Stahel RA, Zangemeister-Wittke U. 2000. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res 60:2805–2809.

Pai SI, Lin YY, Macaes B, Meneshian A, Hung CF, Wu TC. 2006. Prospects of RNA interference therapy for cancer. Gene Ther 13:464–477.

Pennati M, Folini M, Zaffaroni N. 2007. Targeting survivin in cancer therapy: Fulfilled promises and open questions. Carcinogenesis 28:1133–1139.

Plescia J, Salz W, Xia F, Pennati M, Zaffaroni N, Daidone MG, Meli M, Dohi T, Fortugno P, Nefedova Y, Gabrilovich DI, Colombo G, Altieri DC. 2005. Rational design of shepherdin, a novel anticancer agent. Cancer Cell 7: 457–468.

Rabinovich GA, Gabrilovich D, Sotomayor EM. 2007. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 25:267–296.

Reed JC. 1999. Dysregulation of apoptosis in cancer. J Clin Oncol 17:2941–2953.

Reed JC, Bischoff JR. 2000. BIRinging chromosomes through cell division–and survivin' the experience. Cell 102:545–548.

Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, Liddington RC, Salvesen GS. 2001. Structural basis for the inhibition of caspase-3 by XIAP. Cell 104:791–800.

Salvesen GS, Dixit VM. 1997. Caspases: Intracellular signaling by proteolysis. Cell 91:443–446.

Shi Y. 2000. Survivin structure: Crystal unclear. Nat Struct Biol 7:620–623.

Shin S, Sung BJ, Cho YS, Kim HJ, Ha NC, Hwang JI, Chung CW, Jung YK, Oh BH. 2001. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry 40:1117–1123.

Song Z, Yao X, Wu M. 2003. Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. J Biol Chem 278:23130–23140.

Sperandio S, de Belle I, Bredesen DE. 2000. An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci USA 97:14376–14381.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446.

Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC. 1998. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 58:5315–5320.

Uchida H, Tanaka T, Sasaki K, Kato K, Dehari H, Ito Y, Kobune M, Miyagishi M, Taira K, Tahara H, Hamada H. 2004. Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth in vitro and in vivo. Mol Ther 10:162–171.

Yan H, Thomas J, Liu T, Raj D, London N, Tandeski T, Leachman SA, Lee RM, Grossman D. 2006. Induction of melanoma cell apoptosis and inhibition of tumor growth using a cell-permeable Survivin antagonist. Oncogene 25:6968–6974.

Zaffaroni N, Pennati M, Folini M. 2007. Validation of telomerase and survivin as anticancer therapeutic targets using ribozymes and small-interfering RNAs. Methods Mol Biol 361:239–263.

Zhao J, Tenev T, Martins LM, Downward J, Lemoine NR. 2000. The ubiquitin-proteasome pathway regulates survivin degradation in a cell cycle-dependent manner. J Cell Sci 113(Pt 23):4363–4371.